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Neurogenní plicní edém u potkanů s poraněním míchy

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S disertací je možno se seznámit na děkanátě 2. LF UK v Praze

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Seznam zkratek

ALI	Acute Lung Injury, akutní plicní poškození (selhání)
AMPA	α-amino-3-hydroxyl-5-methyl-4-isoxazolpropionová kyselina
ARDS	(Adult Respiratory Distress Syndrome) - syndrom akutní dechové tísně
ATP	adenozin trifosfát
BBB score	škála hodnocení neurologických funkcí u potkana s poškozenou míchou nazvaná podle prvních písmen příjmení autorů: Basso, D.M., Beattie M.S., and Bresnahan J.C. (Basso et al., 1995)
BDNF	Brain Derived Neurotrophic Factor
BM	Bone Marrow
cAMP	cyklický adenosin trifosfát
CNS	centrální nervový systém
CSPGs	chondroitin sulfát proteoglykany
CT	Computed Tomography, počítačová tomografie
DAD	diffuse alveolar damage, difúzní poškození alveolů
DNA	Deoxyriboneukleová kyselina
ESCs	Embryonic Stem Cells, embryonální kmenové buňky
FLASh	Fast Low Angle Shot, nízkoúhlový rychlý paprsek
FOV	field of view, zorné pole
FSCs	Fetal Stem Cells, fetální kmenové buňky
GFAP	Glial Fibrillary Acidic Protein, marker astroglie
HEMA	2-hydroxyethyl methacrylát
HPMA	N-(2-hydroxypropyl) metacrylamid
IL-1, 2, 3…	Interleukin-1, Interleukin-2 etc.
inh.	inhalační
MAG	Myelin Associated Glycoprotein
MASCIS	Multicenter Animal Spinal Cord Injury Study, oficiální protokol k provádění míšního poranění (Basso et al., 1996).
MR	Magnetická Rezonance
MRI	Magnetic Resonance Imaging, zobrazení magnetickou rezonancí
NASCIS	National Acute Spinal Cord Injury Study, klinické studie míšního poranění (Bracken et al., 1985; Bracken et al., 1990; Bracken et al., 1997)
MSCs	Mesenchymal Stem Cells, mesenchymální kmenové buňky
MSH	melanocyty stimulující hormon
NGF	Nerve Growth Factor, nervový růstový faktor

NMDA	N–metyl-D-aspartát
NSCs	Neural Stem Cells, neurální kmenové buňky
NT-3	neurotrophin-3
NT-4	neurotrophin-4
NYU imp.	New York University impactor, přístroj pro provedení míšního poranění
OECs	Olfactory Ensheating Cells, kmenové buňky čichového epitelu
OSU imp.	Ohio State University impactor, přístroj pro provedení míšního poranění
PBS	Phosphate Buffer Saline, fosfátový pufr, obohacený o NaCl
pCO2	parciální tlak oxidu uhličitého
PEEP	Positive End Expiratory Pressure, přetlaková mechanická ventilace
PFA	paraformaldehyd
P-index	plicní index
PNS	periferní nervový systém
pO2	parciální tlak kyslíku
RARE	Rapid Acquistion with Relaxation Enhancement
RTG	Rentgen, Rentgenové vyšetření
s.c.	subkutánně, podkožně
SCI	Spinal Cord Injury, míšní poranění
S.E.M	Standard Error of Mean, směrodatná ochylka
TE	echo time
TEM	Transverse Electro Magnetic (technický údaj)
Th1, 2, 3…	hrudní obratel 1, 2, 3 etc.
TR	repetition time – čas opakování
TrueFISP	True Fast Imaging with Steady-state free Procession
TSH	thyroideu stimulující hormon
VIP	vasoaktivní intestinální peptid

Obsah

1. Úvod

Plicní komplikace zůstávají stále důležitou příčinou morbidity a mortality u pacientů s náhle vzniklým poškozením míchy (Urdaneta et al., 2003). Jednou z hlavních komplikací poškození míchy je otok plic neuro-humorálně-stresové etiologie – **neurogenní plicní edém**, vznikající v bezprostřední vazbě na náhle vzniklé poškození centrálního nervového systému (Leal Filho et al., 2005a, 2005b; Kandatsu et al., 2005). Etiopatogenetický mechanismus, vedoucí k jeho rozvoji, je stále předmětem diskusí. Jedním z faktorů, podezřívaných z potenciace rozvoje neurogenního plicního edému, je i druh podané anestezie (Leal Filho et al., 2005b). Tato práce se snaží přispět k objasnění vlivu anestezie na rozvoj neurogenního plicního edému, vznikajícího v závislosti na traumatickém poškození míchy.

1.1. Patofyziologie poranění míchy

Páteřní (hřbetní) mícha je součástí centrálního nervového systému (CNS). Jedná se o ventrodorsálně oploštělý pruh nervové tkáně, uložený v *saccus durae matris spinalis*, uvnitř osteo-ligamentózní schránky páteřního kanálu. Z míchy vystupuje druhově specifický počet míšních nervů, které náleží k perifernímu nervovému systému (PNS). Na průřezu míchy můžeme pozorovat šedou hmotu míšní, tvořenou těly neuronů a jejich výběžky, uspořádánou do tvaru motýla okolo centrálního míšního kanálu a bílou hmotu míšní, která šedou hmotu obklopuje. Bílá hmota je tvořena pouze výběžky neuronů.

Poranění míchy (angl. spinal cord injury – SCI) patří mezi jeden z nejvýznamnějších celospolečenských lékařských problémů, zejména s vysokým rozvojem a nárůstem automobilové dopravy a extrémních druhů sportovních aktivit. Průměrný věk pacientů s míšním poraněním je 32 let. Je čtyřikrát častější u mužů. Okolo 40-50% úrazů míchy je spojených s požitím alkoholu. Incidence poranění míchy je ve vyspělých zemích udávána okolo 5 případů na 100.000 obyvatel, v Severní Americe se výskyt odhaduje na 10,000 nových případů ročně. Zanedbatelný není ani dopad ekonomický, například v USA dosahují roční výdaje, spojené s léčbou míšního poranění 10 miliard dolarů (Bracken, 1991; Bracken et al., 1997).

K míšnímu poranění dochází v návaznosti na poškození osteoligamentózního aparátu páteře buď přímo, při prudkém ohnutí, napnutí, nebo rotaci míchy, nebo nepřímo, stlačením kostním úlomkem, fragmentem intervertebrálního disku, nebo cizím tělesem. Míšní poranění rozlišujeme kompletní a částečné (inkompletní). Při částečném míšním poranění není mícha postižena v celém průřezu a symptomatologie závisí od rozsahu a umístění léze. Výsledný neurologický deficit závisí na mechanismu míšního poranění, úrovni a rozsahu léze a následné péči o pacienta.

Snížení motorických funkcí označujeme termínem paréza, jejich vyhasnutí jako plegie, souhrnně pak českým termínem obrna. Poškození bílé a šedé hmoty míšní vyústí ve spastickou obrnu (hypertonie svalstva díky ztrátě inhibičních vlivů vyšších center), poškození předních míšních kořenů v obrnu chabou (přerušení nervového vlákna). Snížení sensitivních funkcí označujeme jako hypestezie, jejich vyhasnutí potom termínem anestezie. K nim mohou být přidruženy i pozitivní sensitivní symptomy, jako jsou bolesti, parestézie, dysestezie, hyperestézie, hyperpatie a allodynie. Z poruch autonomních funkcí je třeba zmínit poruchu vyprazdňování močového měchýře a močovou retenci (detruzor-sfinkterová dyssynergie – snížený tonus detruzoru a současně zvýšený tonus sfinkteru močového měchýře), poruchu udržení moči (hyperreflexie měchýře), paralytický ileus, anální inkontinenci, erektilní dysfunkci, ortostatickou hypotenzi a autonomní dysregulaci.

1.1.1. Primární a sekundární poškození míchy
Koncepce primárního a sekundárního míšního poškození (Taoka a Okajima, 1998) označuje jako primární poškození mechanické zhmoždění a laceraci nervové tkáně, včetně mechanického poškození cév. Termínem sekundární poškození je označován soubor buněčných a molekulárních procesů, které jsou vyvolány a bezprostředně navazují na primární poškození (Tator a Fehlings, 1991; Urdziková, 2006). Mezi hlavní sekundární procesy patří krvácení uvnitř míchy, změny krevního tlaku po poškození, ischemie a reperfúze, poškození endotelových buněk, porušení hemato-encefalické bariéry, uvolnění toxických excitačních aminokyselin, akumulace endogenních opioidů, hydrolýza lipidů, vznik volných kyslíkových radikálů, zánětlivá reakce, apoptotická a nekrotická smrt buněk a iontová nerovnováha (Taoka a Okajima, 1998; Urdziková, 2006). Patomorfologicky se tyto procesy projeví jako hemoragie, edém, axonální a

neuronální nekróza, demyelinizace a později jako tvorba cyst a infarktových ložisek (Kakulas, 1984). Tyto progresivní změny jsou označovány souborným názvem spinální posttraumatický infarkt (Urdziková, 2006).

1.1.2. Mechanismy buněčné smrti

Biologická smrt buňky v míšní tkáni může proběhnout dvěma základními mechanismy:

- **nekrózou** (náhodnou smrtí buňky), charakterizovanou pasivním otokem buňky, ztrátou energetických dějů, silným poškozením mitochondrií a zhroucením vnitřní homeostázy, které vyústí v lýzu buněčné membrány s únikem intracelulárních částic do okolí (Cohen, 1993a, 1993b)

- **apoptózou** (programovanou smrtí buňky), charakterizovanou zvětšením celé buňky, kondenzací chromatinu, svraštěním jádra, fragmentací DNA a později rozpadu buňky na menší části, obklopené membránami (Raff, 1998).

Nekróza je obecně považována za děj patologický, kterým buňky umírají na podkladě patologického inzultu, apoptóza je naproti tomu dějem fyziologickým – jejím prostřednictvím tělo odstraňuje staré a nepotřebné buňky. V případě, kdy způspbuje spuštění apoptotické kaskády patologický impulz, je možné i ji považovat za děj patologický. Porušení buněčné integrity při nekróze buňky je provázeno zánětlivou reakcí, zatímco fagocytózu apoptotických fragmentů buňky žádná zánětlivá reakce organismu neprovází (Cohen, 1993a, 1993b; Raff, 1998).

Při poranění míchy se uplatňují oba druhy buněčné smrti. V celém procesu primárního i sekundárního míšního poranění převažuje nekróza, zatímco apoptóza se uplatňuje méně, zejména u buněk, které ztratily souvislost s okolím a staly se tak nepotřebnými nebo nadbytečnými. Nekróza i apoptóza byla pozorována jak u neuronů, tak u buněk glie a to jak u pokusných zvířat, tak u člověka (Crowe et al., 1997; Li et al., 1996a; Emery et al., 1998).

1.1.3. Změny krevního tlaku a perfúze po poškození míchy

Po poškození míchy dochází k přechodnému vzestupu středního arteriálního tlaku, po němž následuje hluboký pokles (Faden, 1981; Wells a Hansebout, 1978). Prvotní vzestup krevního tlaku je způsoben aktivitou sympatických pre- a paravertebrálních sympatických ganglií, druhá část pak vyplavením katecholaminů dřeně nadledvin (Young, 1988; Faden, 1981). Krevní průtok v bílé hmotě dramaticky klesá během prvních 5 minut po poškození, poté se však opět začíná normalizovat. Tento dramatický pokles je důsledkem vazospasmu místních cév, který souvisí se sympatickou reakcí na poranění.

1.1.4. Poškození cév při poškození míchy

Při poškození míchy se jako zdroj krvácení uplatňují hlavně drobnější hluboké cévy, uložené ve středu léze, zatímco velké povrchové cévy bývají často ušetřeny. K poškození cév v centru míchy přispívá rovněž menší mechanická odolnost šedé hmoty (Tator a Fehlings, 1991). Rozsah krvácení je přitom přímo úměrný rozsahu léze. Poškození drobných cév navíc způsobí distribuční poruchy prokrvení míchy a ztrátu autoregulace prokrvení míšního parenchymu. Jako další faktory zde působí vasospasmus cév, vazogenní edém v důsledku porušení hemato-encefalické bariéry, tlak okolních edematózních tkání, agregace a humorální působení látek, uvolňovaných z endotelu a poškozených krvinek (Yang et al., 1994; Clozel et al., 1993; Urdziková, 2006). Tyto procesy vedou ke vzniku ischemických ložisek. Krvácení do parenchymu vede k pozdějšímu vzniku vřetenovitých pseudocyst, které jsou typické několik týdnů po inzultu v místě léze (Hejčl et al., 2006, 2008). Úloha krve jako etiopatogenetického mechanismu vzniku léze byla prokázána při experimentálním vpravení krve do míšního parenchymu, po kterém se takové pseudocysty vyvinuly (Noble a Wrathall, 1989).

1.1.5. Porušení hemato-encefalické bariéry po poškození míchy

Hemato-encefalická (hemato-spinální) bariéra tvoří přirozenou obrannou bariéru proti průniku škodlivých látek z krve do tkáně centrálního nervového systému (neuropilu). Její morfologický podklad tvoří modifikované endotelové a gliové buňky, schopné regulovat a omezovat transport vybraných molekul do CNS a zachovávat tak optimální prostředí pro nervové a gliové buňky CNS. Porušení hemato-encefalické bariéry způsobí extravazaci plazmatických proteinů, která je nejvíce vyjádřena mezi 4. – 28. dnem po poranění. Porucha hemato-encefalické bariéry není lokalizována pouze na místo poranění, nýbrž ji

lze pozorovat i v jiných úsecích míchy (Noble a Wrathall, 1989). Díky poškození hemato-encefalické bariéry je neuropil míchy navíc vystaven působení zánětlivých elementů a neurotoxickému účinku aminokyselin, zejména glutamátu (Schlosshauer, 1993). Rovněž jsou vyplavovány markery poškození buněk (heat shock protein 32) a markerů oxidativního stresu (heat shock protein 70) (Fukuda, 1996; Gonzales et al., 1989; Ferrante et al., 1997). Mezi látky, způsobující následné poškození buněk hemato-encefalické bariéry patří endotelin-1, endotelin-2, endotelin-3 a vazoaktivní intestinální peptid (VIP) (Salzman et al., 1996).

1.1.6. Imunitní odpověď po poškození míchy

Zánět je přirozenou reakcí organismu na poškození, vyskytuje se proto v parenchymu míchy po jejím poškození. K nastartování imunitní odpovědi dochází v návaznosti na poškození hemato-encefalické bariéry, kdy stoupá koncentrace imunitních látek (plazmatické proteiny, cytokiny, chemokiny, růstové faktory, trofické faktory etc.) a extravasaci imunitních buněk (neutrofilní leukocyty, T-lymfocyty a makrofágy). V časné fázi míšního poranění, tedy od jedné hodiny do 48 hodin po poškození, se v míše hromadí neutrofily. Teprve poté nastupují do místa léze T-lymfocyty a makrofágy. Antigenně specifické T-lymfocyty zde zůstávají přibližně po dobu 7 dní, nespecifické fagocytující makrofágy pak po dobu 2–4 týdnů (Tator a Fehlings, 1991).

Navzdory přirozenosti imunitní reakce a jejímu celkově menšímu rozsahu ve srovnání s okolními tkáněmi, působí imunitní reakce v míše více škody, nežli užitku. Hlavní důvod pro to je ten, že do neuropilu míchy vstupují buňky z intravasálního prostoru, kterým není toto prostředí vlastní. Pokud jsou z krve pokusného zvířete odstraněny neutrofily nebo makrofágy, poškozená tkáň se po poranění míchy lépe regeneruje (Taoka et al., 1997; Popovich et al., 1997). Imunitní odpověď buněk glie se proto jeví jako výhodná, zatímco odpověď buněk, které do neuropilu vstoupí po poškození hematoencefalické bariéry jako nevýhodná. V dnešní době se vede poměrně extenzivní diskuse, zda zánětlivou reakci potlačovat nebo naopak podporovat. Výsledkem diskusí bude zřejmě selektivní ovlivnění určitých složek imunity.

1.1.7. Excitotoxické a iontové poškození buněk

Experimentální poškození míchy vyvolá několikanásobné zvýšení koncentrace extracelulárních excitačních neurotransmiterů, zejména glutamátu a aspartátu. Vysoké extracelulární koncentrace glutamátu a aspartátu jsou pro neurony, gliové buňky i myelinové pochvy axonů toxické (Li et al., 1999). Extracelulární koncentrace glutamátu po poškození míchy konstantně narůstá, a to hlavně díky jeho omezenému vychytávání, exocytóze Ca-dependentních glutamátových synaptických vezikul a uvolňování glutamátu a intracelulárního Ca^{2+} při lýze buňky (Li et al., 1999). Nadbytek extracelulárního glutamátu a Ca^{2+} nadměrně stimuluje ionotropní glutamátové AMPA/kainátové a NMDA receptory, zvyšuje influx Ca^{2+} do intracelulárního prostoru a oběma mechanismy současně spouští kaskádu excitotoxické buněčné smrti (Doble, 1999; Li a Stys, 2000; Li et al., 1999). Naopak, blokáda glutamátových receptorů a/nebo snížení množství extracelulárního Ca^{2+} působí protektivně, a to jak při poškození míchy, tak při anoxii (Li et al., 1999).

1.1.8. Role volných radikálů po poškození míchy

Během snížené perfúze nebo reperfúze ischemického ložiska Vzniká a hromadí se celá řada reaktivních volných radikálů, na jejichž přítomnost je centrální nervový systém citlivý. Za normálních okolností, tj. v přítomnosti minimálního množství volných radikálů je míšní tkáň schopna tyto radikály vychytat pomocí antioxidantů (kyselina askorbová, glutathion, vitamín E) a odstranit enzymy (kataláza, superoxiddismutáza a glutathionperoxidáza) (Halliwell, 1992). Po poškození míchy rychle nárůstá množství volných radikálů, které už nejsou přirozenými mechanismy odstranitelné. To vede k poškozování buněčných lipidů, proteinů a DNA. Bylo prokázáno, že podání antioxidantů a enzymů, odstraňujících volné radikály, působí po poškození míchy neuroprotektivně (Vaziri et al., 2004).

1.1.9. Pohlavní rozdíly při poškození míchy

Klinické studie ukazují, že výsledné poškození míchy u žen je menší, než výsledné poškození míchy u mužů. Tato situace je zřejmě způsobena neuroprotektivním vlivem ženských pohlavních hormonů, estrogenu a progesteronu (Roof a Hall, 2000). Pokud jsou tyto látky exogenně podány pokusnému zvířeti mužského pohlaví, je jeho výsledný neurologický status po poškození míchy lepší, než u kontrolních zvířat (Yune et al., 2004; Roof a Hall, 2000). Estrogeny mají antioxidační efekt, redukují neurotoxicitu a excitotoxicitu

a zvyšují expresi antiapoptotického faktoru bcl-2. Progesteron má zase pozitivní účinek na stabilizaci membrán a redukuje poškození způsobené lipidovou peroxidací. Jeho neuroprotektivní vliv se projevuje i supresí neuronové hyperexcitability (Roof a Hall, 2000).

1.2. Modely poranění míchy

Ke studiu patofyziologických mechanismů traumatického poškození míchy je využívána řada modelů u pokusných zvířat. Mezi požadavky na dobrý experimentální model patří jednoduchost provedení, standardnost, reprodukovatelnost, kvantifikovatelnost, vytvoření nekompletního poškození a co největší anatomická a patofyziologická podobnost s klinickou situací. Dobré reprodukovatelnosti lze však dosáhnout jen s minimalizací změn parametrů vnitřního prostředí, jako je tělesná teplota, krevní tlak, pO_2, pCO_2 a pH krve (Collins, 1983). Ne vždy je možno všem těmto požadavkům vyhovět, někteří autoři dokonce udávají, že díky současnému působení kompresivních, angulačních a distrakčních sil a současně v anatomické variabilitě, dokonce mezi jedinci téhož inbredního kmene, je vytvoření ideálního modelu, reprodukujícího klinickou situaci beze zbytku, prakticky nemožné (Hitchon et al., 1988). V dnešní době se však podařilo vytvořit řadu modelů, které se blíží k vytyčenému cíli. Inkompletní poškození žádáme, abychom mohli posoudit jak pozitivní, tak negativní dopad léčebného zákroku (Kwon et al., 2002). Modely poškození míchy můžeme zhruba rozdělit na „ostré" a „neostré".

1.2.1. Ostré modely poškození míchy
Mechanismus vzniku míšního poranění ostrým způsobem předpokládá částečné nebo úplné přetětí míchy s minimální složkou tupého poškození tlakem nože. Mezi klasické zástupce těchto modelů patří:

- **hemisekce** (přetětí poloviny míchy), kde je možné srovnávat regeneraci na postižené straně míchy se zdravou polovinou a
- **transekce** (přetětí celé míchy), kde se však často objevuje retrakce proximálního i distálního pahýlu od místa transekce.

Tyto modely jsou vhodné zejména pro sledování regenerace axonů, neboť umožňují sledovat regeneraci přesně vymezeného úseku míšní dráhy. Jsou

rovněž vhodné k implantaci různých látek pro přemostění léze, jako jsou např. hydrogely (Hejčl et al., 2006, 2008; Syková et al., 2006a; Woerly et al., 1998, 1999). Problémem těchto modelů je často velmi špatná reprodukovatelnost – hemisekci lze jen velmi obtížně provést dvakrát ve stejném rozsahu. U transekcí je problémem retrakce pahýlů, která významně omezuje jakékoli terapeutické snahy. Ostré modely mají tu nevýhodu, že je porušena *dura mater* a likvor může volně odtékat do okolního prostoru. Tento stav je sice možno řešit suturou tvrdé pleny velmi jemným stehem (Hejčl et al., 2006), jedná se však o další zásah, který může ovlivnit sekundární poškození a tvorbou jizvy. Uzavření *dura mater* vnímá naše skupina jako klíčové díky uzavření subarachnoidálního prostoru, zamezení přístupu infekce a hlavně zamezení prorůstání okolních pojivových tkání do místa léze (Hejčl et al., 2006, 2008; Syková et al., 2006a).

1.2.2. Neostré modely poškození míchy

Neostré modely poškození míchy byly a jsou využívány daleko více než ostré. Hlavní důvod je ten, že více tupé stlačení míšní tkáně daleko přesněji odráží klinickou příčinu postižení míchy (stlačení vpáčeným kostním úlomkem, krvácení, tumor, aneurysma apod.). Tyto modely jsou více vhodné pro sledování patofyziologických procesů v míšní tkáni, pro sledování biomechaniky traumatického míšního poranění a na farmakologické studie (Kwon et al., 2002). S výjimkou modelu balónkové kompresní míšní léze je nutné u těchto modelů provést laminektomii.

Mezi zástupce neostrých modelů patří:
- *Weight drop* **model** (Allen, 1911). Princip tohoto modelu, použitého na primátech, kočce, ovci a laboratorním potkanovi, spočívá v dopadu závaží s definovanou váhou z definované výšky na míšní parenchym. Navzdory tomu, že se jedná o jeden z nejstarších modelů, je dodnes používán, zejména díky jeho principiální jednoduchosti a technickému zdokonalení (Behrmann et al., 1992; Gale et al., 1985; Gruner, 1992; Wrathall et al., 1985).
- **Injektáž parafinu** (Ayer, 1919)
- **Stlačení prstem** (McVeigh, 1923)
- **Stlačení skalpelem** (Thompson, 1923)
- **Stlačení kostním voskem** (Craig, 1932)
- **Stlačení Kocherovou svorkou** (Fontaine, 1954)

- **Stlačení lisovacími kleštěmi** (Harvey a Srebnik, 1967)
- **Stlačení aneurysmální svorkou** (Rivlin a Tator, 1978). Mezi přednosti tohoto modelu patří ovlivnitelnost doby, po jakou svorka působí. Naopak, nevýhodná je složitá manipulace s míchou při nasazování svorky.
- **Elektromagnetický model** (Behrmann et al., 1992; Hiruma et al., 1999; Stokes a Jakeman, 2002; Stokes et al., 1992)
- *Blocking weight* **model** (Holtz et al., 1990; Li et al., 1996a; Nystrom a Berglund, 1988)
- **Fotochemická léze míchy** (Verdu et al., 2003)
- **NYU (New York University) impactor** (Gruner, 1992; Scheff et al., 2003). V podstatě se jedná o modifikaci Allenova *weight drop* modelu. V tomto modelu padá závaží o hmotnosti 10g z výšky 6,25, 12,5 a 25 mm na dorsální plochu míchy v úrovni Th9-10. Tento protokol je používaný v osmi laboratořích v USA, sdružených do Multicenter Animal Spinal Cord Injury Study ve zkratce MASCIS (Basso et al., 1996).
- **OSU (Ohio State University) Impactor** (Bresnahan et al., 1987; Stokes, 1992; Stokes et al., 1992). Jedná se o modifikaci NYU impactoru, při které je sonda, způsobující kompresivní lézi míchy pomalu spouštěna na povrch míchy a pak provedena léze. Nedojde zde k odrazu nebo uhnutí míchy od sondy.
- **Model balónkové míšní léze** (viz následující kapitola)

1.2.3. Model balónkové míšní léze

Tento model je probírán v samostatné kapitole, jelikož dle našich výzkumů nejlépe odpovídá klinické situaci. Dokladem je jeho přijetí členy konsorcia mezinárodního projektu Evropské Unie RESCUE. Poškození míchy v tomto modelu vzniká nafouknutím malého balónku (dnes nejčastěji jako součást Fogartyho katétru) v páteřním kanále, které způsobí kompresi míchy, bez poškození okolních vazivově-kostních struktur (tj. okolo místa léze). Balónková kompresní léze byla použita na psech (Tarlov et al., 1953), opicích (Tator a Deecke, 1973), kočkách (Martin a Bloedel, 1973), fretkách (Eidelberg et al., 1976) a u potkanů (Khan a Griebel, 1983, Vanický et al., 2001; Urdziková et al., 2005; Syková et al., 2005). Použití tohoto modelu u malých zvířat ulehčila komerční výroba unifikovaných katétrů, které mají konstantní tvar a velikost a jsou ze stejného materiálu, což zaručuje vysoký stupeň reprodukovatelnosti provedené

míšní léze (Urdziková, 2006). Jednotlivé studie postupně odhalily, že u potkana (váha 300-330g) objem balónku 10 µl způsobí minimální poškození, které je regenerováno *ad integrum*, 15 µl způsobí submaximální poškození a více než 20 µl způsobí již klinický obraz transekce (Vanický et al., 2001; Urdziková, 2006; Martin et al., 1992). Jednou z hlavních předností modelu epidurální kompresní balónkové léze je fakt, že není nutné provádět laminektomii – katétr je zasunut do epidurálního prostoru malým navrtaným otvorem, umístěným dva segmenty kaudálně od místa léze, který jednak nijak neohrozí stabilitu páteře, jednak není v místě budoucí léze, neovlivňuje tedy nijak vlastní lézi.

Obr. 1.1. Balónková kompresní míšní léze **A.** Schéma pracovního postupu. Místo zavedení balónku je vyznačeno dvěma šipkami napravo. Část míchy, využívaná k morfometrické analýze je vyznačena šipkou vlevo. **B.** Magnetická Rezonance - sagitální řez míchou 1 den po provedené balónkové kompresní míšní lézi (hlavička šipky). Šipka – místo zavedení katétru. **C.** Balónková kompresní míšní léze na řezu míchou. Barvení - hematoxylin-eosin.

1.2.4. Srovnání jednotlivých modelů

Srovnávací studie ukazují, že sekundární poškození míchy nejlépe reprodukují model balónkové léze a model stlačení míchy pomocí aneurysmatické svorky. *Weight drop* model má nevýhodu, že probíhá velmi krátce – nedojde tedy k žádnému stlačení cév, vývoj sekundárního míšního poškození je zkreslen a neodpovídá proto klinické situaci. Vyskytuje se například krvácení v centru míchy (hematomyelie), které je v klinických podmínkách spíše ojedinělé (Ducker a Hamit, 1969). Naopak u tohoto modelu nedojde k působení trakčních sil, které je u míšních lézí časté (Maiman, 1988; Ducker a Hamit, 1969). Model s použitím aneurysmatické svorky má zase nevýhodu, že je zde nutná laminektomie v místě poškození, spojená s posunem míchy kvůli nasazení svorky, což se v klinické praxi nestává (Khan a Griebel, 1983). Jako nejvýhodnější se proto zdá být model balónkové kompresní míšní léze, u kterého je laminektomie provedena v jiné části, než ve které bude provedena léze a katétr je na místo určení zasouván epi- nebo subdurálně. Naše pracovní skupina preferuje epidurální cestu katétru, neboť se tak minimalizuje pravděpodobnost poškození míchy již při zavádění katétru (Syková et al., 2005; Vanický et al., 2001; Urdziková, 2006). Navíc je více imitována klinická situace, kde je poškozena dura mater jen výjimečně, při penetrujících poraněních.

Nevýhodou přípravy modelů na zvířatech je fakt, že poškození míchy je prováděno v anestezii, což značně ovlivňuje procesy v míše (Urdziková, 2006). Na druhé straně, provádění míšní léze bez anestezie je nejen neetické (§ 17 zák. odst.1 č. 246/1992 Sb. na ochranu zvířat proti týrání v platném znění), ale zřejmě i technicky velmi špatně proveditelné. Další nevýhodou je fakt, že testované neuroprotektivní látky je možné v experimentu podat, až na výjimky, okamžitě, zatímco v klinické praxi je třeba nejdříve zajistit stabilizaci pacienta (Kwon et al., 2002).

1.2.5. Potkan jako modelové pokusné zvíře

Využití laboratorního potkana ke studiu traumatického míšního poranění má řadu výhod. Není finančně, prostorově ani jinak náročný na chov, dobře se s ním manipuluje a je méně náchylný na infekci. S využitím potkanů je možné experimentovat s větším množstvím zvířat najednou. Nespornou výhodou je i dostupnost řady modelů poškození míchy (viz kap. 1.2.) a behaviorálních testů

(viz kap. 11.2.), vytvořených právě pro laboratorního potkana (Basso et al., 1995; Gale et al., 1985).

1.3. Anestezie zvířat s traumatickým poškozením míchy

Anestezie a s ní související analgezie pokusného zvířete je klíčová ke správnému a bezbolestnému provedení chirurgického zákroku v experimentu na pokusném zvířeti. K tomuto účelu se používají celková anestetika, která kromě reverzibilního navození bezvědomí a utlumení bolesti, tlumí i nežádoucí vegetativní i somatické reflexní reakce, navozují různý stupeň svalové relaxace a způsobují amnézii na období, kdy byl prováděn chirurgický zákrok. Mezi hlavní požadavky na celková anestetika patří nízká toxicita, metabolická inertnost, rychlá eliminace z organismu a dobrá ovlivnitelnost (řiditelnost) hloubky anestezie. Anestetikum musí rovněž dobře přestupovat přes hematoencefalickou bariéru, aby bylo v mozku, zejména v oblasti retikulární formace mozkového kmene a v mozkové kůře, rychle dosaženo jeho koncentrace, potřebné pro navození celkové anestezie. Rychlost probuzení z anestezie je přímo úměrná rychlosti odstranění anestetika z mozku. Celková anestetika rozeznáváme injekční a inhalační, v závislosti na cestě aplikace. Lokální anestetika se v experimentálním poranění míchy používají pouze výjimečně. Někteří autoři aplikují při experimentálních zákrocích na míše lokální anestetikum přímo do operačního pole, aby tak snížili dávku nutného celkového anestetika.

1.3.1. Stádia celkové anestezie

Mezi plným vědomím a hlubokým stupněm celkové anestezie rozeznáváme čtyři stádia, jejichž navození je podmíněno různou citlivostí jednotlivých částí centrálního nervového systému k účinku celkových anestetik (Hynie, 2000) :

1. *Preanestetické stádium.* Je charakterizováno sníženým vnímáním bolesti při zachování vědomí.
2. *Excitační stádium.* Projevuje se zvýšením somatických i vegetativních reflexů při ztrátě vědomí.
3. *Chirurgické stádium.* V tomto stádiu je přítomno bezvědomí, analgezie, areflexie a různý stupeň myorelaxace.

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4. *Paralytické stádium*. Projevuje se poškozením oběhové a dýchací soustavy, které může být ireverzibilní, nebo dokonce končit smrtí.

Jednotlivá stádia jsou nejvíce vyznačena po podání éteru, při použití moderních anestetik už nejsou hranice mezi jednotlivými stádii tak ostré. Pro provádění chirurgických výkonů je nejvíce vhodné stádium chirurgické. Navozování anestezie probíhá od prvního ke čtvrtému stádiu, probouzení z anestezie směrem opačným. Hloubku anestezie lze rovněž posuzovat s použitím hodnot krevního tlaku a dechové frekvence (Hynie, 2000).

1.3.2. Celková anestetika aplikovaná injekčně

Injekčně aplikovaná anestetika jsou pokusnému zvířeti nejčastěji podávána intramuskulárně (i.m.), intravenózně (i.v.), intraperitoneálně (i.p.) a subkutánně (s.c.). Funkci injekčně aplikovaných anestetik popisují buď rozdělovací koeficienty tkáň-krev a krev-mozek (i.m., s.c. a i.p. anestetika), nebo pouze rozdělovací koeficient krev-mozek (i.v. anestetika) (Hynie, 2000). Hlavními zástupci injekčně aplikovaných anestetik jsou pentobarbital, xylazin, ketamin, thiopental a midazolam (Pandey et al., 2000; Mesquita et al., 2002).

1.3.3. Celková anestetika inhalační

Celková anestetika inhalační jsou v experimentu podávána pokusnému zvířeti buď do uzavřeného prostoru (např. plastiková nádoba), nebo přímo do obličejové masky, v obou případech s použitím odpařovače příslušného anestetika. Funkci inhalačních celkových anestetik popisují rozdělovací koeficienty intraalveolární prostor-krev a krev-mozek. Mezi hlavní inhalační anestetika patří éter, halothan, oxid dusný, isofluran, sevofluran, desfluran, enfluran a methoxyfluran (Hynie, 2000).

Isofluran

Isofluran (1-chloro-2,2,2-trifluoroethyl difluoromethyl ether, *Isofluranum*, FORANE, inh., ISOFLURANE, inh.) je kapalné prchavé celkové inhalační anestetikum s nízkým stupněm metabolizování. Nezpůsobuje nahromadění fluoridu a má jen velmi málo nežádoucích vedlejších účinků, o čemž svědčí i jeho časté použití v humánní medicíně, zejména při neurochirurgických výkonech a u pacientů v těžkém zdravotním stavu (Hynie, 2000). Rozpustnost isofluranu v krvi je malá (koeficient rozpustnosti krev/plyn 1,43), proto nástup i

odeznění anestezie jsou rychlé. Anestezie je provázena nižším stupněm analgezie a myorelaxace, mírným zvýšením intrakraniálního tlaku a sníženou spotřebou kyslíku myokardem. Malá část isofluranu je degradována játry, většina je uvolňována plícemi (Hynie, 2000). Předávkování isofluranem způsobuje hypotenzi, periferní vazodilataci a útlum dýchání.

Minimální alveolární koncentrace

Na účinku celkových inhalačních anestetik se podílejí tři hlavní parametry – rozdělovací koeficient mezi krví a plynem, rozdělovací koeficient mezi mozkem a krví a minimální alveolární koncentrace. Posledně jmenovaný parametr je nejsnazší stanovit a pro praxi má nejlepší vypovídací hodnotu (Orliaguet et al., 2001). Stanovení minimální alveolární koncentrace je proto dnes považováno za základní parametr ke srovnání účinků inhalačních anestetik (Eger et al., 2003; Kandatsu et al., 2005; Orliaguet et al., 2001; Xing et al., 2004; Stabernack et al., 2003). Minimální alveolární koncentrace je definována jako koncentrace anestetika ve vdechované směsi plynů, která má u 50 % jedinců centrálně anestetické účinky (Hynie, 2000; Eger et al., 2003).

1.3.4. Nežádoucí účinky celkových anestetik

Nežádoucí účinky celkových anestetik jsou přes intenzivní výzkum a zdokonalování anestetik stále poměrně běžné. Drtivá většina těchto vedlejších účinků je však mírného stupně, jako např. mimovolní svalové pohyby, škytavka a kašel. Ostatní, jako např. neurogenní plicní edém, bronchospasmus, laryngospasmus, hypotenze, srdeční arytmie, respirační deprese, pooperační nauzea a zvracení, poruchy funkce ledvin, střevní atonie, poruchy jater a změny EEG aktivity, již mohou chirurgický zákrok zkomplikovat více. Za nejtěžší komplikaci anestezie je považována maligní hypertermie (Hynie, 2000).

1.4. Testování zvířat po poškození míchy

Testování motorických, sensitivních, reflexních a ev. i autonomních funkcí je velmi důležitou součástí studií na laboratorních zvířatech s poškozením míchy. Testování sensitivních a autonomních funkcí má jistě svůj význam, nejvýznamnější je však testování motorických funkcí, jejichž zlepšení je mimo jiné hlavním cílem všech terapeutických snah v této oblasti výzkumu (Urdziková, 2006; Basso, 2004). O problematice behaviorálního testování

laboratorního potkana jsme podrobněji pojednali v našem přehledovém článku (viz kap. 11.2.).

Motorické schopnosti

Obr. 1.2. Motorické schopnosti potkanů po provedení balónkové kompresní míšní léze, kdy byl balónek nafouknut na finální objem 10 μl, 15 μl nebo 20 μl. Jako nejvýhodnější se jeví využití objemu 15 μl, jelikož potkan po 4 týdnech dosáhne poloviny BBB škály, což může odrážet jak pozitivní, tak negativní dopad terapeutických snah.

1.5. Terapie traumatického poškození míchy v experimentu

Kauzální způsob léčby míšního poranění, navzdory úsilí mnoha tisíc vědců a lékařů, v současné době neexistuje. V podstatě jedinou „léčbou" míšního poranění je dnes aktivní prevence sekundárního poškození správně poskytnutou první pomocí a transportem, neurochirurgicko-traumatologicko-ortopedickou stabilizací poškozené páteře a aplikací kortikoidu metylprednizonu, jak vyplývá z *National Acute Spinal Cord Injury Study*, ve zkratce NASCIS I, II a III (Bracken et al., 1985; Bracken et al., 1990; Bracken et al., 1997) (více viz kap. 1.6.). Ostatní terapeutické snahy jsou zatím v širším měřítku nedostupné, nebo jen velmi málo účinné a omezují se na užší klinické studie nebo výzkum.

Výzkum této oblasti je zaměřen zejména na ovlivnění:

1. **Neuroprotekce** - ovlivnění sekundárních procesů v poraněné míše s cílem zmenšit rozsah léze,
2. **Regenerace** - eliminace inhibičních faktorů, zabraňujících regeneraci poškozených nervových vláken v centrálním nervovém systému a podpora axonální regenerace pomocí trofických faktorů, transplantace kmenových buněk, implantace biomateriálů přemosťujících lézi, ovlivnění genů atd.
3. **Rehabilitace** - aktivace automatických pohybových vzorců v úseku míchy pod místem poranění, užití ortéz apod.

1.5.1. Neuroprotekce

Snahy o ovlivnění sekundárních procesů po poranění míchy, nejčastěji farmakologickou cestou, jsou cílené na snížení nebo eliminaci škodlivého vlivu jednotlivých sekundárních procesů (viz kap. 1.1.).

Farmakologická léčba

V experimentu i klinické praxi byly použity barbituráty (thiopental) (Hitchon et al., 1982), antagonisté opioidních receptorů (naloxon) (Benzel et al., 1990), antagonisté kalciových kanálů (nimodipin) (Ransom et al., 1990; Tator a Fehlings, 1991), adrenergní agonisté (phenylephrin) (Dyste et al., 1989), neuropeptidy (TSH, MSH), protizánětlivá léčba (chlorochin, kolchicin, cyclosporin A, metylprednizon, minocyklin) (Buki et al., 1999; Vanický et al., 2002), léčba podporující zánětlivý proces (IL-1, IL-2, IL-6) (Klusman a Schwab,

1997) a antagonisté AMPA glutamátových receptorů. Některé farmakologické preparáty jsou zaměřené proti několika sekundárním procesům najednou, např. kortikoid methylprednizon má zároveň protizánětlivý a anti-lipoperoxidační efekt, nebo antibiotikum minocyklin působí proti apoptóze v míšní lézi a zabraňuje aktivaci mikroglie v místě poškození (Kwon et al., 2005).

Léčba zaměřená na zastavení sekundárního poškození musí začít ve velmi časné fázi po poškození míchy. Nutnost použití v časné fázi po poranění vylučuje bohužel předem celou řadu léčiv z této indikační skupiny (Urdziková, 2006).

Hypotermie
Mezi lékaři i vědci panuje názor, že hypotermie má jednoznačně neuroprotektivní účinky. Do jaké míry je tento účinek skutečný je předmětem diskusí. Objevují se práce, které označují neuroprotektivní efekt hypotermie za výrazný (Cambria et al., 1997; Stys et al., 1992), jiné jej popírají (Wells a Hansebout, 1978). V jednom však mají vědečtí pracovníci i lékaři jasno, hypertermie má při poškození míchy jednoznačně negativní vliv (Urdziková a Vanický, 2005; Urdziková, 2006).

Udává se, že principem účinku hypotermie je snížení metabolického obratu v poškozené tkáni (Cambria et al., 1997; Urdziková, 2006). Je popsáno, že poškození míchy nad úrovní Th6 vyřadí z funkce termoregulační centrum, což způsobuje zvýšení tělesné teploty, rychlejší projevy nedostatku ATP, zvýšení akumulace laktátu a excitačních aminokyselin v místě poškození, zvýšení metabolismu kalcia v místě poškození, zvýšení tvorby volných radikálů a zvýšení exprese adhezivních molekul a s tím související podporu vstupu leukocytů do místa léze (Dietrich et al., 1991, 1996; Chopp et al., 1989; Busto et al., 1989; Castillo et al., 1999; Globus et al., 1995; Kawai et al., 2000; Urdziková a Vanický, 2005; Urdziková, 2006). Řada studií ukazuje, že tyto procesy jsou u poškozené míchy za hypotermických podmínek utlumené, což podporuje obecný názor protektivního vlivu hypotermie (Dietrich et al., 1991, 1996; Chopp et al., 1989; Busto et al., 1989; Castillo et al., 1999; Globus et al., 1995; Kawai et al., 2000; Urdziková a Vanický, 2005; Urdziková, 2006). Zůstává tedy vyřešit, jakým způsobem lze hypotermii využít v klinické praxi.

1.5.2. Regenerace

Regenerace poškozených axonů v periferním nervovém systému probíhá při správné a včasné terapii velmi dobře. Naproti tomu, regenerace v centrálním nervovém systému byla donedávna považována za nemožnou, nebo jen velmi mizivou. Zatímco v periferním nervovém systému proximální pahýl aktivně regeneruje, v centrálním nervovém systému se axonální konec pahýlu ztlušťuje v „end bulb", které, v souvislosti s řadou dalších faktorů, znemožňuje regeneraci (Cajal, 1928; Schwab a Bartholdi, 1996). V míše se fyziologicky vyskytuje řada faktorů, inhibujících růst a regeneraci axonů. Tyto inhibiční vlivy jsou spojeny s činností neuroglie.

Gliová jizva

Po několika týdnech od traumatického poškození míchy se v neuropilu míchy vytvoří gliová jizva, tvořená hlavně GFAP pozitivními astrocyty a proteoglykany, včetně CSPGs. Tato jizva tvoří jednak bariéru mechanickou, přestavovanou hustou impermeabilní matrix, jednak bariéru molekulární, přestavovanou růst-inhibujícími molekulami. Díky svému uspořádání neumožňuje prorůstání axonů (Hatten et al., 1991). Davies et al. ukázali, že inhibiční potenciál gliové jizvy koreluje s množstvím proteoglykanů gliové jizvy (Davies et al., 1997). Nové výzkumy ukazují, že na vytvoření a zachování gliové jizvy se rovněž podílejí látky ze skupiny semaforinů a efrinů (Niclou et al., 2006). Inhibice těchto látek může být jednou z experimentálních strategií k potlačení rozvoje gliové jizvy a regenerace axonů (Niclou et al., 2006).

Látky, používané k přemostění léze

Existuje a je testováno několik možností, jak přemostit místo léze (angl. scaffold) a vytvořit tak mechanickou podporu pro vrůstající buňky a axony. K tomuto účelu je využívána řada materiálů ve formě nejrůznějších gelů, pěn i nanovláken. Mezi nejvíce slibné patří implantace hydrogelů (Woerly et al., 1998, 1999). Hydrogely jsou netoxické, chemicky inertní, uměle vytvořené polymery s vysokým obsahem vody a velkým povrchem, například na bázi N-(2-hydroxypropyl) metacrylamidu (HPMA) nebo 2-hydroxyethyl methacrylátu (HEMA). V dnešní době je možné vytvořit také biodegradabilní hydrogely. Díky chemické inertnosti reaguje na jejich přítomnost míšní tkáň jen minimálně. Pro růst axonů je výhodná jejich poréznost, která zvyšuje pravděpodobnost jejich vrůstání do gelu. Vlastnosti hydrogelů lze libovolně upravovat, například

jejich póréznost, mechanické a chemické vlastnosti, nebo je možné je kombinovat s nejrůznějšími látkami, jako např. s růstovými faktory a kmenovými buňkami.

Studie v naší laboratoři ukazují, že s implantací hydrogelů je lépe několik týdnů vyčkat; implantace hydrogelů bezprostředně po poranění má mnohem horší prognózu, pravděpodobně díky vysoké aktivitě procesů, souhrnně označovaných jako sekundární míšní poranění a díky tomu, že ještě není vytvořena finální kavita (viz kap. 1.1.) (Syková et al., 2006a; Přádný et al., 2005; Hejčl et al., 2006, 2008; Lesný et al., 2002, 2006).

Mezi další látky, kterými je možné přemostit vzniklou lézi, patří např. přípravky na bazi fibrinu, kolagenu, fibronektinu, alginátu, agarózy, hyaluronové kyseliny, chitinu, poly-β-hydroxybutyrátu, polyglykolové kyseliny, polylaktátu, polykarbonátu a polyethylenglykolu (Nomura et al., 2006).

Chondroitinasa ABC
Nejsilnější inhibiční složkou gliové jizvy je skupina chondroitinsulfát proteoglykanů (CSPGs). Mezi ně patří například versican, fosfocan, nebo neurocan. Po přidání chondroitinasy ABC, enzymu schopného rozrušení sacharidové složky struktury chondroitinsulfát proteoglykanů, dojde *in vitro* i *in vivo* k prorůstání axonů přes materiál gliové jizvy a u *in vivo* pokusů se zlepší i motorické funkce pokusného zvířete (Smith-Thomas et al., 1994; Davies et al., 1999). Chondroitinasu ABC za normálních okolností produkuje bakterie *Proteus vulgaris* s cílem lepšího průniku do okolních tkání, což naznačuje i potenciální riziko použití tohoto druhu terapie – totiž destrukce chondroitinsulfátů extracelulárního prostoru zdravé tkáně.

Nogo
Ve tkáni míšního parenchymu se i za normálních okolností nacházejí proteiny, které inhibují prorůstání axonů. V roce 2000 se objevil v časopisu Nature článek Martina E. Schwaba a kolegů z Brain Research Institute při Univerzitě v Zürichu (Chen et al., 2000), který otřásl s vědeckou veřejností a do té doby neměnným názorem, že tkáň centrálního nervového systému není schopna regenerace. Jeho skupina objevila myelinový protein, který nazvali Nogo, po

jehož inhibici specifickou protilátkou IN-1 dojde k okamžité aktivaci axonální regenerace a kompenzační plasticity v centrálním nervovém systému (Chen et al., 2000; Schwab et al., 2005). Posléze byl objeven i receptor, prostřednictvím kterého Nogo působí (Strittmatter et al., 2002; Fournier et al., 2001). Zklamáním je absence regenerace axonů u myší s vyřazeným genem pro Nogo (Zheng et al., 2003). Kapitola Nogo je však stále předmětem extensivního výzkumu mnoha laboratoří (Schwab et al., 2005).

Myelin associated glycoprotein

Další molekulou, která byla objevena v souvislosti s inhibicí axonální regenerace je myelin associated glycoprotein (MAG). Bylo prokázáno, že inhibiční aktivita MAG a Nogo je přibližně stejně velká (GrandPre et al., 2000). Bohužel, *in vivo* se účinek MAG neukázal tak významný jako *in vitro* (Bartsch et al., 1995; Li et al., 1996b).

cAMP

V roce 1999 bylo objeveno, že cyklický adenosin-monofosfát (cAMP) blokuje aktivitu inhibitorů růstu jako je myelin nebo Myelin associated glycoprotein (Cai et al., 1999). Jeho role při ovlivnění míšního poranění je nyní testována v experimentu.

4-amidopyridin

4-amidopyridin (Fampridine-SR®) je blokátor draslíkových kanálů, schopný zlepšit vedení vzruchu v poškozených axonech. Jeho podání v experimentu mírně zlepšilo neurologické funkce, snížilo spasticitu a tremor končetin pokusných zvířat (McBride et al., 2007; Hayes, 2007). Navíc se zlepšila retence moči a sexuální funkce (Segal et al., 1999). V současnosti probíhající rozsáhlá klinická studie ukáže do budoucna více.

GM-1 gangliosid

Experimentálně bylo prokázáno, že GM-1 gangliosid (Sygen®) potencuje růst a regeneraci axonů u koček s poraněním míchy. Rovněž měl účinky při potlačování některých sekundárních procesů v poraněné míše. Výsledky klinické studie na lidech však ukázaly jen mizivý efekt v klinické praxi (Chinnock a Roberts, 2005).

Kmenové buňky

Novým trendem výzkumu je léčba míšního poranění s použitím kmenových buněk (Syková et al., 2005; 2006a, 2006b; Jendelová et al., 2003, 2005). Jako kmenové buňky označujeme buňky s neomezenou schopností sebeobnovy, které jsou zdrojem buněčných populací ve vyvíjejícím se, nebo již vyvinutém organismu. Kmenová buňka se obecně dělí na diferencující se buňku progenitorovou, která směřuje za svým osudem a na buňku kmenovou, která zaujímá místo původní kmenové buňky. Tento proces se nazývá asymetrické dělení. Progenitorové a kmenové buňky se označují souborným termínem prekurzorové buňky. Kmenové buňky takto představují prakticky nevyčerpatelný zdroj buněk pro organismus. Naopak, progenitorová buňka podléhá ireverzibilnímu procesu diferenciace a nemůže se už nikdy stát multipotentní buňkou kmenovou (Glogarová, 2006).

Kmenové buňky rozdělujeme na primordiální zárodečné buňky (angl. primordial germ cells), embryonální kmenové buňky (angl. embryonic stem cells, ESCs), fetální kmenové buňky (angl. fetal stem cells, FSCs) a kmenové buňky z dospělého organismu (angl. adult stem cells). Podle zdrojového místa uložení pak rozdělujeme kmenové buňky např. na neurální kmenové buňky (angl. neural stem cells, NSCs), mesenchymální kmenové buňky z kostní dřeně (angl. mesenchymal stem cells, MSCs), kmenové buňky čichového epitelu (angl. olfactory ensheating glia, OEGs), mesenchymální kmenové buňky tukové tkáně (angl. adipose tissue derived stem cells) apod.

Cílem terapeutických snah s použitím kmenových buněk je jednak nahradit ztracenou buněčnou populaci, jednak vyplnit vzniklé kavity a troficky podpořit regeneraci axonů (Syková et al., 2005; Urdziková, 2006). Zároveň je možné žádaným způsobem předem upravovat genetickou výbavu buněk ve smyslu produkce růstových faktorů a jim podobných látek.

V současné době je známo, že mesenchymální kmenové buňky jsou při exogenním podání schopné selektivně vyhledávat místo míšní nebo mozkové léze (Akiyama et al., 2002). Některé z těchto buněk se následně transformují na buňky nervového systému (Kopen et al., 1999; Jendelová et al., 2003). Kromě toho jsou schopny sekrece cytokinů a trofických faktorů, které dále podporují regeneraci poraněné nervové tkáně (Bjorklund et al., 2002). V naší laboratoři bylo prokázáno, že současná exogenní aplikace mesenchymálních kmenových

buněk a stimulace uvolňování mesenchymálních kmenových buněk kostní dřeně vyústí v morfologicky i behaviorálně pozorovatelný pozitivní efekt (Urdziková et al., 2006).

Implantace gliových buněk
Kromě kmenových buněk je k podpoře regenerace možné použít i gliové buněčné typy, jako například Schwannovy buňky (Franklin a Barnett, 2000), oligodendrocyty (Duncan a Milward, 1995), olfaktorické kmenové buňky nebo astrocyty (Franklin et al., 1991). Je testována přímá implantace gliových elementů do místa léze. Idea této strategie spočívá v neuroprotekci, tvorbě růstových faktorů a výživě poškozených neuronů pro účely jejich maximálního zachování. Podle experimentálních údajů mají být rovněž tyto buňky schopny přemostit lézi a umožnit tak axonům růst.

Neurotrofiny
Dalším z faktorů, které jsou studovány v souvislosti se zmírněním následků míšního poranění jsou nervové růstové faktory, zvané rovněž neurotrofiny, mezi které patří u savců nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrofin-3 (NT-3) a neurotrofin-4 (NT-4) (Tessarollo, 1997). V rámci podpory regenerace axonů v centrálním nervovém systému se uplatňují zejména NGF a BDNF (Behar et al., 2000). Význam BDNF tkví v podpoře regenerace a větvení cholinergních vláken v místě léze (Ankeny et al., 2001; Xu et al., 1995). Jako nejdůležitější faktor při terapeutickém podání neurotrofinů se ukazuje doba podání po míšním poranění, která má být co nejkratší, nejlépe bezprostředně po poranění, zatímco po 4–6 týdnech po poranění už léčba neurotrofiny nemá žádný efekt (Shumsky et al., 2003). Ve stadiu výzkumu je implantace buněčné linie fibroblastů, schopných produkce NT-3 (Grill et al., 1997). Největší význam mají však neurotrofiny ve vývoji a regeneraci periferního nervového systému, jak bylo prokázáno ve vývojových studiích na mechanoreceptorech (Li et al., 2006; Zelená, 1994), nebo klinických studiích v návaznosti na poranění periferního nervu (Roehm a Hansen, 2005).

1.5.4. Rehabilitace
Pokusy na zvířatech i klinické studie prokázaly, že terapeutické snahy v podobě intenzivní rehabilitace, včetně elektrické stimulace a chirurgických plastických

výkonů významným způsobem ovlivňují objektivní i subjektivní stránky pacientova poškození.

1.5.5. Akutní a chronické poškození

Jako zásadní se jeví rozdíl mezi terapeutickou snahou v akutním nebo chronickém stadiu po poškození míchy. Hranice mezi těmito dvěma stavy je udávána mezi 3-5 týdny. Akutnímu stadiu, tedy období od poškození do 3-5 týdnů po poranění, vévodí sekundární procesy, které jsou přístupné aplikaci nejrůznějších látek s cílem terapeutického potlačení těchto procesů. Jsou také vhodné pro buněčnou terapii. Chronické stadium poškození míchy se zdá být výhodnější pro aplikaci hydrogelů.

1.6. Terapie traumatického poškození míchy v klinické praxi

V klinické praxi jsou v současné době velmi omezené možnosti terapie. V první řadě směřují terapeutické snahy ke stabilizaci pacientových životních funkcí a fixaci páteře. Na neurochirurgickém oddělení je posléze provedena, diferenciálně diagnostická rozvaha, podpořená několika zobrazovacími metodami (RTG, CT, MRI) a následně chirurgická dekomprese poraněné míchy a stabilizace páteře. V předoperačním, peroperačním i pooperačním období je nutná monitorace krevního tlaku, srdeční a dechové frekvence a saturace krve kyslíkem (Bracken et al., 1990; Bracken et al., 1997; Trivedi, 2002).

V dnešní době se jako nejdůležitější jeví pooperační fáze, která zahrnuje rehabilitaci motorických, sensitivních a autonomních funkcí, edukace pacienta, chirurgické zákroky plastického charakteru na odstranění kontraktur, elektrická stimulace ochrnutých svalů, prevence dekubitů, lázeňskou léčbu, psychofarmakoterapii etc. (Trivedi, 2002).

1.6.1. Metylprednizon

V současné době je standardním postupem při traumatickém poškození míchy, dle multicentrické klinické studie National Acute Spinal Cord Injury Study II (NASCIS II), provedené na 487 pacientech, podání syntetického glukokortikoidu metylprednizonu (Bracken et al., 1990, 1992). Podle tohoto protokolu je podáváno 30 mg metylprednizonu na 1 kg tělesné hmotnosti pacienta v bolusové dávce, co nejdříve po úrazu, avšak nejpozději do 8 hodin. Následuje 23-hodinová infúze s dávkou 5,4 mg metylprednizonu na 1 kg

hmotnosti pacienta (Bracken et al., 1990). Některé světové společnosti, jako např. Kanadská neurochirurgická společnost však nepodání metylprednizonu v akutním stadiu po poškození míchy nepovažují za non-lege artis postup, nýbrž pouze jako metodu volby (Hugenholtz et al., 2002).

Mechanismus účinku metylprednizonu předpokládá úlohu při snížení zánětlivé reakce, hydrolýzy a peroxidace lipidů, redukce tvorby míšního edému a zlepšování průtoku v cévách poškozené míchy (Xu et al., 1992, 1998; Young a Flamm, 1982; Urdziková, 2006; Vanický et al., 2002). O skutečném účinku metylprednizonu při traumatickém poranění míchy se však dodnes vedou spory, které neusnadnilo ani provedení tří studií NASCIS (Bracken et al., 1985; Bracken et al., 1990; Bracken et al., 1997). Objevují se totiž práce, které prokazují jak pozitivní účinek methylprednizonu v experimentu (Green et al., 1980) nebo v klinické praxi (Bracken et al., 1990; Otani et al., 1996), tak práce prokazující nulový efekt metylprednizonu v experimentu (Bartholdi a Schwab, 1995) nebo klinické praxi (Hurlbert, 2000; Nesathurai, 1998; Short et al., 2000; Pointillart et al., 2000). Někteří autoři jej dokonce považují za kontraindikovaný pro pacienty s poškozením míchy (Hurlbert, 2000; Hugenholtz et al., 2002).

Léčba methylprednizonem může mít několik nežádoucích účinků, zejména při dlouhodobém podávání, jako je například snížená imunitní odpověď díky imunosupresivnímu účinku kortikoidů. U pacientů s traumatickým poškozením míchy v akutním stadiu se pak častěji výskytuje pneumonie a sepse (Hugenholtz et al., 2002; Bracken et al., 1990). Mezi další komplikace patří hyperglykémie, akutní myopatie a gastrointestinální potíže (Galandiuk et al., 1993; Gerndt et al., 1997; Qian et al., 2000). Tyto nežádoucí účinky lze do jisté míry odstranit lokálním intrathekálním podáním (Koszdin et al., 2000). Jeho účinek však zůstává nadále sporný (Vanický et al., 2002; Hurlbert, 2000; Hugenholtz et al., 2002). Klinické studie NASCIS nesporně naznačily jednu z cest možné časné terapie míšního poranění tím, že posunuly terapeutické okno do ranných stadií po míšním poranění (Schwartz a Fehlings, 2002).

1.6.2. Ostatní látky, testované v klinických studiích
Existují další látky, které se v experimentu jevily jako slibné, avšak klinické studie prokázaly opak. Příkladem takové látky je GM1 gangliosid, kde první část klinických studií prokázala jeho pozitivní efekt (Geisler et al., 1992, 1993),

zatímco v další fázi se ukázalo, že GM1 gangliosid nemá žádné protektivní účinky na poraněnou míchu (Geisler et al., 2001a, 2001b). Jiným příkladem je podávání vitamínu E, který sice zlepšuje sledované neurologické parametry po poškození míchy, musí však být podáván už před poškozením míchy, což je v klinické praxi neproveditelné (Anderson et al., 1988).

V současnosti probíhají tyto klinické studie:

- Implantace progenitorů oligodendrocytů – fáze I (Myckatyn, 2004)

- Implantace lidských embryonálních kmenových buněk – studie v přípravě (Vogel, 2005)

- Léčba gradientem elektrického pole – fáze I. Zlepšuje motorické i sensitivní funkce po poškození míchy

- Minocycline – fáze II.

- Cethrin- Rho inhibitor – fáze I. Subdurální aplikace

- Aktivované makrofágy – probíhá fáze II. (Schwarz et al., Israel)

- Mononukleární kmenové buňky kostní dřeně – fáze I (Syková et al., 2006, ČR)

- Implantace olfaktorické glie (McKay-Sin, Brisbane)

1.6.3. Klinická studie v České Republice

V rámci Centra buněčné terapie a tkáňových náhrad při 2. Lékařské Fakultě Univerzity Karlovy v Praze probíhá v pražské Fakultní Nemocnici Motol klinická studie s použitím autologních mononukleárních buněk kostní dřeně. Vedoucím projektu je prof. MUDr. Eva Syková, DrSc. Kmenové buňky jsou aplikovány jednak intravenózně, jednak intraarteriálně a to co nejdříve po poranění míchy. Jako nejslibnější se jeví aplikace kmenových buněk intraarteriální cestou do blízkosti místa léze, s použitím katetrizace arteria vertebralis v prvních 4 týdnech po poranění (Syková et al., 2006b).

1.6.4. Budoucnost

Souhrnem lze tedy uvést, že terapeutické úspěchy řešení traumatického poranění míchy, nejsou, navzdory intenzivnímu výzkumu zdaleka uspokojivé. Jediným lékem zůstává methylprednizon, ale jeho účinek je sporný. Hlavním problémem je to, že problematika traumatického poškození míchy je velmi obsáhlá a velké množství procesů, probíhajících v poškození míše, není ještě dostatečně prozkoumáno. Navíc, člověk zde platí i daň za fylogenetickou vyspělost centrálního nervového systému. Proto jinak úspěšné studie *in vitro*, nebo i studie *in vivo* na nižších obratlovcích při aplikaci u člověka selhávají.

1.7. Neurogenní plicní edém

Neurogenní plicní edém je akutní komplikace náhle vzniklého poškození centrálního nervového systému, charakterizovaná zvýšeným množstvím intersticiální a intraalveolární plicní tekutiny, spojeným s výskytem plicních hemoragií. Jako první popsal neurogenní plicní edém Shanahan již v roce 1908 jako komplikaci opakovaných epileptických záchvatů u 11 pacientů a byl to právě on, kdo spojil těžké poškození centrálního nervového systému se vznikem plicního edému a zároveň první, kdo použil pro tento edém název „neurogenní"(Shanahan, 1908). Ve světové (Leal Filho et al., 2005a, 2005b) i české odborné literatuře (Vinš, 2003) byl NPE již vymezen jako samostatná klinická jednotka, řazená do skupiny nekardiogenních (extrakardiálních) plicních edémů. Na rozdíl od kardiogenních (při levostranné srdeční insuficienci) nebo ostatních nekardiogenních (toxického, obstrukčního, renálního, vysokohorského, lymfatického, eklamptického a iatrogenního) plicních edémů vzniká neurogenní plicní edém v bezprostřední vazbě na náhle vzniklé poškození centrálního nervového systému (Leal Filho et al., 2005a, 2005b; Kandatsu et al., 2005; Vinš, 2003).

Obr. 1.3. Histologický obraz závažného akutně vzniklého neurogenního plicního edému. Na obrázku je patrné masivní prokrvácení plicní tkáně, typické pro neurogenní plicní edém. Barvení – hematoxylin-eosin.

1.7.1. Výskyt neurogenního plicního edému

Epidemiologická data, vázající se k celosvětovému výskytu neurogenního plicního edému buď chybí úplně, nebo nejsou z hlediska uspořádání studie nebo počtu pacientů uspokojivá. Extrémní rozdílnost epidemiologických dat ukazuje práce Weira, který nalezl neurogenní plicní edém u 71% pacientů se subarachnoideálním krvácením (Weir, 1978) a práce Grafa a Rossiho, kteří nalezli neurogenní plicní edém pouze u 2 ze 3940 pacientů, tedy pouze 0,05 %, zemřelých na neurologické onemocnění (Graf a Rossi, 1975). Je pravděpodobné, že posledně jmenované číslo je zkreslené díky zahrnutí všech neurologických onemocnění do vybrané skupiny. Nejobjektivnější a nejvíce citovaná je epidemiologická práce Fontese a kolegů, kteří zjistili, že 62% pacientů s neurogenním plicním edémem jsou ženy, průměrného věku 31,6 let, nejčastější příčinou edému je subarachnoideální krvácení a mortalita pacientů v přímé souvislosti s neurogenním plicním edémem je 9,5 % (Fontes et al.,

2003). Většina ostatních autorů usuzuje, že neurogenní plicní edém se vyskytuje u více než poloviny pacientů s těžkým poškozením centrálního nervového systému – u pacientů s tupým nebo penetrujícím poraněním mozku nebo míchy, subarachnoidálním nebo intracerebrálním krvácením, u epileptického záchvatu typu grand mal, cerebrovaskulárních příhod, hyponatremické encefalopatie a dalších podobných případů (Vinš, 2003; Stocker a Burgi, 1998; Brito et al., 1995; Dragosavac et al., 1997; Antoniuk et al., 2001; Urban et al., 2001). Výskyt neurogenního plicního edému však není v řadě případů řazen do statistik (Leal Filho et al., 2005a, 2005b; Fontes et al., 2003; Macleod, 2002). Existující epidemiologická data navíc ukazují, že neurogenní plicní edém vzniká nejčastěji na základě poškození centrálního nervového systému, které doprovází zvýšený intrakraniální tlak.

1.7.2. Patofyziologie neurogenního plicního edému

Neurogenní plicní edém je charakterizován významným stupněm dilatace plicních kapilár, kongescí kapilár krvinkami, intraalveolárním krvácením a intersticiálním (perivaskulárním) a intraalveolárním edémem, který je tvořen na proteiny-bohatým tkáňovým mokem – **exsudátem**, jež obsahuje více než 70 % plazmatické hladiny proteinů (Kondo et al., 2004; Leal Filho et al., 2005a). Stejně jako ostatní druhy plicního edému, je i neurogenní plicní edém charakterizován třemi fázemi (Vinš, 2003):

1. **preedémovou fází**, kdy probíhá zvýšený pohyb tekutiny z kapilár do intersticia. Přebytek přívodu tekutiny je však kompenzován zvýšenou lymfatickou drenáží,
2. **intersticiální edém**, kdy je překročena drenážní kapacita intersticia až o 30 % a tekutina se začíná hromadit v poddajném vazivu mezi bronchioly, arteriolami a venulami. Klesá poddajnost plicní tkáně a zvyšuje se plicní cévní odpor,
3. **alveolární edém**, kdy jsou překročeny objemové možnosti intersticiálního prostoru, a tlak tekutiny způsobuje rozrušení spojů alveolárních membrán.

V případě neurogenního plicního edému však na sebe všechny fáze navazují velmi rychle a navíc jsou doplněny o hemoragie. Patofyziologicky představuje vznik neurogenního plicního edému nerovnováhu Starlingových sil, odvislých

od permeability kapilárního endotelu, cévního povrchu a hydrostatického a osmotického tlaku (Malik, 1985; Colice, 1984).

Roli Starlingových sil při vzniku neurogenního plicního edému naznačuje Starlingova rovnice (Starling, 1918):

$$Q = K_{fk} \cdot (P_k - P_i) - K_d (\pi_k - \pi_i)$$

kde Q označuje eflux tekutiny, P_k - hydrostatický tlak v kapiláře, P_i - hydrostatický tlak v intersticiu, K_{fk} - kapilární filtrační koeficient, K_d – koeficient odrazu (nabývá hodnot od 0, kdy je stěna kapiláry extrémně propustná pro proteiny do 1, kdy je stěna pro proteiny nepropustná), π_k – onkotický tlak v kapiláře a π_k – onkotický tlak v intersticiu. Míra efluxu tekutiny je tedy přímo úměrná hydrostatickému tlaku v kapiláře a nepřímo úměrná onkotickému tlaku v kapiláře.

Předchozí výzkum naznačil skupinu možných etiopatogenetických mechanismů, které by mohly vést k rozvoji neurogenního plicního edému (Toka and Okajima, 1998; Urdaneta et al., 2003), ten skutečný mechanismus však zůstává stále zahalen rouškou tajemství a je předmětem intenzivních spekulací. Na vzniku neurogenního plicního edému se nejpravděpodobněji podílí náhle vzniklá systémová aktivace sympatiku a uvolnění velkého množství katecholaminů a dalších substancí, označovaná jako **katecholaminová bouře** (angl. catecholamine storm) nebo méně často Cushingova odpověď (angl. Cushing response) (Toka and Okajima, 1998; Urdaneta et al., 2003; Fontes et al., 2003; Pender a Pollack, 1992; Walder et al., 2002; Cushing, 1901). Výsledkem je generalizovaná alfa-adrenergní vasokonstrikce a nárůst systémového a plicního tlaku, způsobující centralizaci oběhu. Následkem těchto změn je zvýšení plicního hydrostatického tlaku, poškození cévní a alveolární stěny, nárůst permeability alveolární stěny a uvolňování tekutiny do intraalveolárního prostoru (Fontes et al., 2003; Vinš, 2003).
Epitel alveolární stěny hraje kritickou úlohu při minimalizaci vlhkosti intraalvelárního prostoru vzdušných plicních sklípků, která je nezbytná pro správnou výměnu plynů (Rezaiguia-Delclaux et al., 1998). Mnohé studie ukazují klíčovou roli epitelu alveolární stěny při absorpci a reabsorpci

isotonické tekutiny z intraalveolárního prostoru plicních sklípků proti vzrůstajícímu osmotickému gradientu do lumina plicních kapilár (Rezaiguia-Delclaux et al., 1998; Matthay a Wiener-Kronisch, 1990). Je známo, že hlavní podíl na tomto procesu má transport Na+ a K+ iontů, což lze nepřímo prokázat inhibicí procesu vstřebávání tekutiny z intraalveolárního prostoru amiloridem a ouabainem (Rezaiguia-Delclaux et al., 1998). Porušení této iontové rovnováhy může potencovat vznik neurogenního plicního edému.

1.7.3. Klinický obraz neurogenního plicního edému

Klinický obraz neurogenního plicního edému je poměrně nespecifický. Subjektivně se vyskytuje náhle vzniklá dušnost, bolesti na hrudi, zhoršené odkašlávání, nauzea, zvracení, bolesti hlavy, pocity slabosti, schvácení a obavy o vlastní život. Objektivně lze nalézt vazbu na poranění centrálního nervového systému, tachypnoe, tachykardii, vrzoty a chrůpky při bazích plic, narůžovělé sputum až hemoptýzu, hypoxemii, případně zvýšený intrakraniální tlak, poruchy vědomí a spíše mírnější zvýšení teploty. Někteří autoři popisují u svých pacientů **smrtelný chropot** (angl. death rattle) (Macleod, 2002; Keegan a Lanier, 1999). Známky zánětu jsou minimální nebo žádné. Na RTG lze v 90 % případů pozorovat různý stupeň difúzního zastření obou plicních polí, zejména v hilové oblasti, nástřik plicních cév a normální velikost srdečního stínu (Vinš, 2003; Wasowska-Krolikowska et al., 2000; Fontes et al., 2003).

1.7.4. Terapie neurogenního plicního edému

Základem terapie je potlačení hypoxie přímým podíváním kyslíku nebo přetlakovou mechanickou ventilací pomocí PEEP (Positive End Expiratory Pressure), monitorování pacienta a úprava hemodynamických parametrů, včetně zvýšení diurézy (furosemid, mannitol), zvýšené polohy hlavy a analgetické léčby (Vinš, 2003; Leal Filho et al., 2003; Macleod, 2002). V časném stadiu mohou být užitečné alfa-blokátory (Vinš, 2003) a kortikoidy (Chang et al., 2005), farmakologická terapie však obvykle mnoho nevyřeší. Neurogenní plicní edém se u většiny pacientů podaří zvládnout do 48-72 hodin.

1.7.5. Diferenciální diagnostika neurogenního plicního edém

Neurogenní plicní edém může imitovat celá řada stavů, nejpravděpodobnější jsou však následující:

ALI a ARDS

Od neurogenního plicního edému je třeba odlišovat akutní plicní selhání (ALI – acute lung injury) a syndrom akutní dechové tísně (ARDS – adult respiratory distress syndrome). Tyto syndromy totiž vznikají na zánětlivém podkladě, vedoucímu k difúznímu poškození alveolů (DAD – diffuse alveolar damage) a současně bez akutní vazby na poškození centrálního nervového systému (Vinš, 2003). Mortalita ARDS a ALI je vyšší než mortalita neurogenního plicního edému.

Aspirační pneumonie

Klinický obraz aspirační pneumonie je podobný neurogennímu plicnímu edému (pacient v těžkém stavu, dechová nedostatečnost, restriktivní porucha dýchání, tachykardie, tachypnoe). Aspirační pneumonie však nevzniká tak dramaticky rychle jako neurogenní plicní edém a je u ní přítomna horečka. Je třeba dát pozor na stavy, kdy je horečka způsobena poškozením vlastního centrálního nervového systému (zejména oblasti ventrálního hypothalamu) – to však není na vrub neurogenního plicního edému, tento nemá zánětlivou složku. Odeznění příznaků aspirační pneumonie navíc trvá déle, okolo 2 týdnů.

1.8. Vliv anestezie na rozvoj neurogenního plicního edému

Různí autoři popsali jak potenciaci (Pandey et al., 2000) tak inhibici (Mesquita et al., 2002) vzniku neurogenního plicního edému při podání různých druhů anestetik. Mezi nejvýznamnější anestetika, uplatňující se při vzniku neurogenního plicního edému, patří lidokain, ketamin, xylazin, pentobarbital, halothan, isofluran a sevofluran (Laffon et al., 2002; Leal Filho et al., 2005a, 2005b; Pandey et al., 2000; Kandatsu et al., 2005; Molliex et al., 1998).

Je známo, že halothan a isofluran u potkanů indukují reversibilní snížení alveolární epiteliální tekutinové clearance a snižují tak práh pro vznik plicního edému (Rezaiguia-Delclaux et al., 1998; Laffon et al., 2002). Při pokusech bylo zjištěno, že halothan navíc snižuje aktivitu amilorid-sensitivních sodíkových kanálů a Na^+/K^+ ATPázy v pneumocytech II. typu v alveolární stěně, které společnými silami odstraňují Na^+ (a s ním i vodu) z intraalveolárního prostoru (Molliex et al., 1998), což potvrzuje významnou roli Na^+ iontů v procesu vzniku plicního edému. Bylo totiž popsáno, že dobře fungující iontový transport Na^+ iontů a vody zvyšuje alveolární epiteliální clearance, která je klíčová jak pro

prevenci vývoje plicního edému, tak pro rozpouštění a vstřebávání edematózní tekutiny u pacientů s vyvinutým plicním edémem (Matthay a Wiener-Kronisch, 1990; Ware a Matthay, 2001).

2. Cíle práce

1. Stanovit koncentrace isofluranu, při kterých se rozvíjí neurogenní plicní edém u potkanů s traumatickým poškozením míchy na modelu epidurální balónkové kompresní léze.

2. Stanovit rozsah poškození plic u různých koncentrací isofluranu pomocí stanovení stupně subpleurálního krvácení, relativní hmotnosti plic, histologie a *in vivo* zobrazovacích metod.

3. Posoudit význam sympatického nervového systému při rozvoji neurogenního plicního edému sledováním krevního tlaku a tepové frekvence před a po blokádě sympatiku pentoliniem.

4. Stanovit funkční neurologický dopad neurogenního plicního edému u zvířat s těžkým plicním edémem, navozeným epidurální balónkovou míšní lézí v mírné isofluranové anestezii.

5. Optimalizovat protokol vyvolání míšního poškození u laboratorního potkana.

6. Posoudit možnost využití získaných poznatků při vytvoření modelu těžkého neurogenního plicního edému u potkana s poraněnou míchou.

7. Stanovit dopad postupné gradace expanze balónku v míšním kanálu na změny krevního tlaku, tepové frekvence a stupeň rozvoje neurogenního plicního edému.

8. Přispět k diskuzi o neurogenním plicním edému formulací vlastní hypotézy o etiopatogenetickém vlivu nízkého stupně anestezie na rozvoj neurogenního plicního edému.

3. Materiál a metodika

Vzhledem k tomu, že je metodika podrobně zpracována v přiložených publikacích, je zde uveden pouze stručný přehled metod, použitých v práci.

3.1. Pokusná zvířata

V našich studiích, zabývajících se patofyziologií a terapií míšního poranění, jsme použili celkem 264 potkanů kmene Wistar (Velaz, Česká Republika). Potkani byli umístěni v akreditovaném zvěřinci na Ústavu experimentální medicíny, v.v.i. nebo Fyziologickém ústavu AVČR, v.v.i. s 12ti hodinovým cyklem den/noc a krmeni standardní dietou a vodou *ad libitum*. Všichni potkani byli mužského pohlaví, vzhledem k jejich hormonální stabilitě (Roof a Hall, 2000). Abychom minimalizovali variabilitu průměru míšního kanálu, používali jsme pouze potkany o váze 300-330 g. Výběr do jednotlivých skupin byl prováděn náhodně. Se zvířaty bylo nakládáno dle etických a právních norem podle § 17 zák.odst.1 č. 246/1992 Sb. na ochranu zvířat proti týrání v platném znění.

3.2. Anestezie

K anestezii zvířat byl použit isofluran (Forane, Abbot Labaratories, Ltd., Queenborough, Velká Británie). K jeho aplikaci byla použita nosní maska domácí výroby, připojená na přístroj Isoflurane Vapor 19.3 (Drägerverk AG Lűbeck, Německo). Zvířata byla zvážena a uložena do uzavíratelné plastikové nádoby o průměru 16,5 cm a výšce 13 cm, do které byl zaveden přívod z odpařovače isofluranu a zahájena anestezie 5% isofluranem ve vzduchu při proudu 300 ml inhalační směsi za minutu. Po navození narkózy byli potkani vyjmuti a připojeni na nosní masku s definovanou koncentrací isofluranu ve vzduchu 1,5%, 2%, 2,5%, 3%, 4% nebo 5%, při proudu 300 ml inhalační směsi za minutu. V indikovaných případech byla zvířata narkotizována pentobarbitalem (30 mg/kg).

3.3. Stanovení bezpečné koncentrace isofluranu

Před započetím experimentu jsme stanovili minimální a maximální hranici isofluranu, ve které mohou být zvířata bezpečně narkotizována. Při použití

nižší anestezie než 1,5% isofluranu ve vzduchu byly pozitivní reakce na bolest při kompresi prstů na pánevních končetinách a konce ocasu (digital a tail pinch reflex) a současně korneální reflex, nebylo tedy etické podrobovat zvířata nižší anestezii. Při použití více než 4% isofluranu ve vzduchu umírala zvířata na předávkování anestetikem.

3.4. Tělesná teplota

Všechny zákroky na míše byly prováděny při tělesné teplotě 37° C, neboť hypo- nebo hypertermie může mít vliv na rozsah léze (Cambria et al., 1997; Urdziková a Vanický, 2005). Tělesná teplota 37° C byla dosažena s použitím vyhřívací dečky (Heizkissen, Typ HK3, Německo) a poté udržována na 37° C, měřeno rektálním teploměrem (Roth, Česká Republika).

3.5. Balónková kompresní míšní léze

K navození míšního poškození jsme použili dříve popsaný model epidurální balónkové kompresní míšní léze (Vanický et al., 2001; Urdziková et al., 2006). Operace byla provedena v aseptických podmínkách. V anestezii byla pokusnému zvířeti oholena záda a provedena povrchová desinfekce kůže 70 % ethanolem. Byl proveden sagitální řez v délce 3 cm kraniálně od místa, kde se poslední pár žeber připojuje na páteř. Bylo protnuto podkoží a povrchové fascie. Hluboké zádové svalstvo bylo skalpelem oboustranně odříznuto od sloupce *processus spinosi* a odsunuto laterálně. S použitím Luerových kleští jsme odstranili *processus spinosi* Th10 a Th11. Spinální výběžek obratlů Th8-9 byl zachycen do zahnutého peánu, uchyceného do stojanu a zvíře nadzvednuto. Tímto manévrem jsme dosáhli dorsálního ohnutí páteře a zvětšení epidurálního prostoru pro následné zasunutí katétru. Pomocí stereomikroskopu (Leica S6, Švýcarsko) a zubní vrtačky (W&H, MF Perfecta, Rakousko) s kuličkovým vrtáčkem, byl do středu středu *arcus vertebrae* vyvrtán otvor o průměru 1,5 mm. Dvěma ostrými pinzetami byl šetrně odstraněn periost a zkontrolováno neporušení *dura mater*. Na oblouku obratle Th11 jsme zubní vrtačkou vyvrtali žlábek, který posloužil ke správnému zavedení Fogartyho katétru (2-French Fogarty catheter, Baxter Healthcare Corporation, Irvine, CA, USA) do epidurálního prostoru. Střed balónku byl zasunut do hloubky 1 cm, čímž bylo dosaženo míšního segmentu Th8-9. Fogartyho katétr byl naplněn sterilní destilovanou vodou a napojen na 50 µl plynotěsnou Hamiltonovu stříkačku (typ 1705, TLL - TEFLON® Luer Lock). Stříkačka byla uchycena v

mikromanipulátoru, který umožňoval přesné dávkování 15 µl tekutiny, potřebných pro naplnění balónku. Z celého systému byly ještě před započetím experimentu odstraněny vzduchové bubliny. Poté byla stabilizována tělesná teplota. Poškození míchy bylo vyvoláno okamžitým nebo postupným nafouknutím balónku na dobu 5 minut. Po uplynutí této doby byl balónek vyfouknut a odstraněn z epidurálního prostoru. Operační rána byla šita ve vrstvách.

3.6. Transversální přerušení míchy
V anestezii byl za aseptických podmínek proveden přístup jako v předchozím případě. V oblasti Th7 byla provedena laminektomie. Následně bylo skalpelem provedeno úplné přetětí míchy v oblasti míšní Th8.

3.7. Pooperační péče
Zvířatům určeným k behaviorálním studiím byla provedena sutura rány a po operaci byla umístěna do klecí po dvou pro minimalizaci sociálního stresu a pro lepší rehabilitaci po poškození míchy. Všichni potkani byli krmeni standardní dietou *ad libitum*. Jednou z hlavních komplikací po poškození míchy je porucha vyprazdňování močového měchýře (dyssynergie detruzoru-sfinkteru), jež vede jednak k mechanickému poškození jeho stěny, jednak k infekčním komplikacím (Urdziková, 2006). Jelikož po poškození míchy jsou přerušeny dráhy, které kontrolují jeho vyprazdňování, bylo nutno potkanům močový měchýř manuálně vyprazdňovat. Vyprazdňování bylo prováděno nejprve 2x denně, posléze 1x denně, dokud se zvířatům neustavil měchýř automatický.

3.8. Eutanázie zvířete, vyjmutí plic, autopsie
Eutanázie byla provedena u zvířat, určených k studiu akutní faze neurogenního plicního edému. Pokud zvíře přežilo 15 minut od započetí míšního poranění (nafouknutí balónku), tedy 10 minut po vyfouknutí balónku, byla provedena jeho eutanazie gilotinou. Následně byly plíce potkana vyjmuty, zváženy a dále zpracovány. Pro minimalizaci posmrtných změn na plicích a vyvarování se jejich poškození byl celý proces vyjímání plic ještě před započetím pokusů nacvičen tak, aby netrval déle než 30 sekund. Při vyjímání plic byl brán zřetel na zachování celistvosti plicního parenchymu a odstřižení cév plicního hilu v úrovni jejich výstupu z plic za účelem minimalizace změny jejich hmotnosti pro následné stanovení plicního indexu. Na závěr byla provedena pitva zvířete a

vyhledáváno makroskopické krvácení do jiného orgánu (mozek, srdce, thymus, játra, střevo, ledviny, slezina a močový měchýř).

3.9. Reprodukovatelnost a hodnocení poškození míchy

Reprodukovatelnost modelu epidurální balónkové míšní léze byla v našich experimentálních podmínkách mnohokrát prokázána (Syková et al., 2005; Jendelová et al., 2004; Syková a Jendelová, 2005; Urdziková a Vanický, 2006; Urdziková, 2006; Vanický et al., 2002). Uspořádání části naší studie bohužel nedovolovalo behaviorální testování pokusných zvířat ihned po zákroku. Ve všech případech však byl před výkonem zkontrolován balónek Fogartyho katétru pro přítomnost vzduchových bublin. Jeho správné a rovnoměrné nafouknutí bylo ověřeno s použitím stereomikroskopu vždy před a po provedení balónkové léze. Na správné provedení balónkové léze jsme usuzovali i podle průvodných známek míšního poranění - svalových tonicko-klonických křečí zádového a končetinového svalstva a velmi často rovněž krátkodé zástavy dechu.

3.10. Vyhodnocení stupně subpleurálního krvácení

Stupeň subpleurálního krvácení do plic byl hodnocen makroskopicky, bezprostředně po vyjmutí plic z hrudníku potkana a jejich zvážení. Každá plíce byla posuzována zvlášť a zařazena do jednoho z následujících stupňů:

- *zdravá plíce* (žádné krvácení na povrchu plic),
- *Grade I* (maximálně 10 % povrchu plic je prokrváceno),
- *Grade II* (25-50 % povrchu plic prokrváceno) a
- *Grade III* (více než 50 % povrchu plic je prokrváceno).

Mírný stupeň krvácení v oblasti plicního hilu, cca. 2 mm v průměru, byl standardním nálezem, souvisejícím s odříznutím plicních cév, přítomný u všech skupin.

3.11. Plicní index

Ke zjišťování stupně plicního edému byla použita jednoduchá, avšak velmi citlivá technika stanovení plicního indexu (Leal Filho et al., 2005a, 2005b), který v podstatě odráží relativní hmotnost plic. Tento index je možné spočítat jako podíl mokré hmotnosti plic a tělesné hmotnosti zvířete v gramech. Plicní index

do 0,55 ukazuje na zdravou plicní tkáň, bez přítomnosti plicního otoku. Hodnoty nad 0,55 ukazují rozvinutý plicní edém, hodnoty nad 0,70 již těžký plicní edém (Leal Filho et al., 2005b).

3.12. Fixace a histologické zpracování plic

Zvážené a posouzené plíce byly fixovány 4% paraformaldehydem ve fosfátovém pufru imerzí po dobu 1-2 dní. Tkáně byly odvodněny vzestupnou řadou etanolů, prosyceny benzenem a dále parafinem a nakonec zality do parafinu. Parafinové řezy, silné 5 μm byly připevněny na sklíčko směsí bílku s glycerinem na tepelné destičce. Dále byly řezy odparafinovány xylenem, rehydratovány pomocí sestupné řady alkoholů a barveny hematoxylinem-eosinem.

3.13. Měření tloušťky alveolární stěny

Pro zjištění stupně edému alveolární stěny bylo využito vybraných parafinových řezů, barvených hematoxylinem-eosinem. Vyhodnocení řezů bylo prováděno mimo hilovou oblast. U každého reprezentativního řezu byla změřena tloušťka všech alveolárních stěn v daném poli s použitím programu Neurolucida (MicroBrightField, Inc., USA), dokud nebylo dosaženo 100 měření. Z jednotlivých měření byl vypočítán průměr a směrodatná odchylka. Data z jednotlivých skupin byla porovnávána Studentovým t-testem.

3.14. Zobrazení RTG

Ke stanovení stupně plicního edému *in vivo* jsme využili RTG zobrazení s použitím přístroje Image Station In-Vivo FX System (Kodak, Německo). Potkani byli, 1 hodinu po skončení zákroku, narkotizováni pentobarbitalem (50 mg/kg) a uloženi do přístroje, kde byl zhotoven RTG snímek.

3.15. Sledování nástupu neurogenního plicního edému

Ke stanovení času, ve kterém se vzniká neurogenní plicní edém, jsme nejdříve odhalili *pleura parietalis* a *in vivo* tak sledovali rozvoj neurogenního plicního edému na plicním parenchymu během následné balónkové léze. Zvířata byla narkotizována 1.5% isofluranem a umístěna do polohy na břiše. Dorsolaterální kožní řez byl proveden v rozsahu Th7-9, povrchové svaly hrudní stěny byly proťaty kolmo na svalové snopce a odsunuty do stran. Následně byly odstraněny *musculi intercostales* a žebra v příslušných segmentech a *pleura*

parietalis očištěna. Krvácení z mezižeberních tepének a žil bylo staveno jemným zhmožděním stěny cévy s následnou tamponádou jemnými kousky buničiny. Elektrokauterizace nebyla využita v důsledku snahy o zachování maximální translucence pleury. Speciální péče byla věnována prevenci vytvoření pneumothoraxu. Následně byla provedena balónková léze a v jejím průběhu a po něm byl sledován plicní parenchym. Po celou dobu byl zaznamenáván čas. Čas, kdy začínal plicní parenchym měnit barvu a zejména čas, kdy se objevilo subpleurální krvácení byl zaznamenán.

3.16. Behaviorální testování – BBB test

 Pro otestování hybnosti pánevních končetin zvířat po míšním poranění jsme použili BBB test (BBB score), jež je dnes považován za zlatý standard behaviorálního testování laboratorních potkanů s poškozením míchy (Basso et al., 1995). BBB test je udáván od 0 (žádný pohyb pánevních končetin) do 21 (plná pohyblivost pánevních končetin), pro každou končetinu zvlášť (tab. 4.3.). Výsledná hodnota je aritmetickým průměrem hodnot pro pravou a levou pánevní končetinu. BBB test lze hrubě rozdělit na tři fáze, podle stupně zotavení (angl. recovery) - **rannou fázi** (0-7), **střední fázi** (8-13) a **pozdní fázi** (14-21).

 Potkani s traumatickým poškozením míchy byli testováni před zákrokem, 24 hodin po zákroku a dále po 7, 14, 21, 28, 35, 42 a 49 dnech. Zvířata byla testována na rovném povrchu ve vymezeném kruhovém prostoru s průměrem 90 cm. Testování provádějí vždy dva testující, kteří stojí proti sobě, aby mohli pozorovat pohyby končetin ze dvou stran. Hodnotí se jen volní pohyby, nikoli pohyby reflexní.

Tab. 4.3. BBB test (Basso et al., 1995)

Číslo	Charakteristika
0	Žádný viditelný pohyb pánevní končetiny
1	Nepatrný pohyb jednoho nebo dvou kloubů, obvykle kyčle a/nebo kolene
2	Výrazný pohyb jednoho kloubu
	Výrazný pohyb jednoho kloubu a nepatrný pohyb dalšího kloubu
3	Výrazný pohyb dvou kloubů
4	Nepatrný pohyb všech tří kloubů
5	Nepatrný pohyb dvou kloubů a současně výrazný pohyb třetího
6	Výrazný pohyb dvou kloubů a současně nepatrný pohyb třetího
7	Výrazný pohyb všech tří kloubů
8	Zametání bez váhové podpory
	Plantární umístění packy bez váhové podpory
9	Plantární umístění packy s váhovou podporou pouze ve stoji
	Občasná, častá nebo konsistentní chůze po dorsum pedis s váhovou podporou a absence plantárního umístění packy
10	Občasné plantární umístění packy s váhovou podporou a žádná předozadní koordinace
11	Časté až konzistentní plantární umístění packy s váhovou podporou a žádná předozadní koordinace
12	Časté až konzistentní plantární umístění packy s váhovou podporou a občasná předozadní koordinace
13	Časté až konzistentní plantární umístění packy s váhovou podporou a častá předozadní koordinace
14	Konzistentní plantární umístění packy s váhovou podporou a konzistentní předozadní koordinace. Packa je rotována zevně nebo navnitř při zdvihnutí packy a při prvním kontaktu.
	Části plantární umístění packy s váhovou podporou, konzistentní předozadní koordinace a občasná chůze po dorsum pedis s váhovou podporou
15	Konzistentní plantární umístění packy s váhovou podporou a konzistentní předozadní koordinace. Žádné nebo občasné zvedání prstů. Packa je paralelně k tělu při prvním kontaktu.
16	Konzistentní plantární umístění packy s váhovou podporou a konzistentní předozadní koordinace. Časté zvedání prstů. Packa je paralelně k tělu při prvním kontaktu a rotována ve fázi zdvihnutí.
17	Konzistentní plantární umístění packy s váhovou podporou a konzistentní předozadní koordinace. Časté zvedání prstů. Packa je

	paralelně k tělu při prvním kontaktu i ve fázi zdvihnutí.
18	Konzistentní plantární umístění packy s váhovou podporou a konzistentní předozadní koordinace. Konzistentní zvedání prstů. Packa je paralelně k tělu při prvním kontaktu a rotována ve fázi zdvihnutí.
19	Konzistentní plantární umístění packy s váhovou podporou a konzistentní předozadní koordinace. Konzistentní zvedání prstů. Packa je paralelně k tělu při prvním kontaktu i ve fázi zdvihnutí. Ocas je při zemi část nebo celou dobu.
20	Konzistentní plantární umístění packy s váhovou podporou a konzistentní předozadní koordinace. Konzistentní zvedání prstů. Packa je paralelně k tělu při prvním kontaktu i ve fázi zdvihnutí. Nestabilita trupu. Konzistentní zvedání ocasu.
21	Konzistentní plantární umístění packy s váhovou podporou a konzistentní předozadní koordinace. Konzistentní zvedání prstů. Stabilita trupu. Konzistentní zvedání ocasu.

3.17. Behaviorální testování – plantární test

K testování sensitivity potkanů s poškozenou míchou jsme využili Plantární test (*Plantar test* – Ugo Basile, Comerio, USA). Tento test umožňuje kvantifikovat stupeň citlivosti pánevní končetiny na podráždění tepelným podnětem v podobě času, které potkan potřebuje k odtažení packy. Potkani s traumatickým poškozením míchy byli testováni před zákrokem, 24 hodin po zákroku a dále po 7, 14, 21, 28, 35, 42 a 49 dnech.

3.18. Zobrazení míchy magnetickou rezonancí

K ověření postupu vytvoření léze bylo u všech potkanů v této části provedeno MR vyšetření v místě léze den po poranění. Mícha byla vyšetřena *in vivo* experimentálním MR tomografem (Bruker Biospec 47/20, síla pole 4,7 T, vnitřní průměr magnetu 20 cm) vybaveným gradientním systémem o síle 200 mT/m a páteřní cívkou vlastní výroby (Burian a Hájek, 2004). Sagitální řezy (obrazová matice 512 x 160 bodů, zorné pole o velikosti 10 x 3 cm, tloušťka řezu 0,5 mm s mezerou mezi řezy 0,5 mm a echo-časem 70 ms a repetičním časem 2800 ms) byly snímány běžnými RARE sekvencemi s turbo faktorem 8.

3.19. Perfúze zvířat

V hluboké anestezii jsme otevřeli hrudník potkana, zpřístupnili srdce, odstřihli ouško pravé předsíně a zavedli kanylu do levé komory srdeční. Přes kanylu byl peristaltickou pumpou aplikovaný fyziologický roztok po dobu 2 minut a následně 4% paraformaldehyd ve fosfátovém pufru. Na perfúzi bylo použito nejméně 1 litr fixativa na 1 kg tělesné hmotnosti zvířete. Po perfúzi byla vyjmuta páteř v rozsahu minimálně 1,5 cm nad a pod místem léze a uložena do 5% paraformaldehydu ve fosfátovém pufru na dobu 24 hodin při teplotě 4°C. Poté byla mícha vyjmuta a uložena do stejného fixačním roztoku pro další zpracování.

3.20. Histologické zpracování

Z míchy byl excidován 2 cm dlouhý úsek s poškozeným místem uprostřed. Před odvodněním a prosycením parafinem byla excidovaná mícha zafixována na pevné podložce, aby nedošlo k jejímu ohnutí. Celý 2 cm dlouhý segment byl zalit do paraplastu s použitím speciální formy na odlévání 2 cm vysokého bloku. Příčné 5 μm silné řezy byly připraveny na sáňkovém mikrotomu a natahovány na podložní želatinou-potažená sklíčka. Následné barvení probíhalo na těchto sklíčkách.

Histochemické barvení Luxol Fast Blue a Cresyl Violet
Pro následnou morfometrickou analýzu bylo použito barvení Luxol Fast Blue a Cresyl Violet. Toto barvení zobrazuje kontrastním způsobem šedou a bílou hmotu míchy i při malém zvětšení, což je výhodné při zakreslování plochy zachovalé tkáně a následné morfometrické měření (Urdziková, 2006).

Morfometrické měření zachování bílé a šedé hmoty
Rozsah léze byl kvantifikován jako objem zachované bílé a šedé hmoty v poškozeném míšním segmentu. Použili jsme sérii řezů s odstupem 1 mm. Z odebraného segmentu jsme získali sérii cca 20 řezů. V každém z těchto řezů byla obkreslena část zbývající šedé a bílé hmoty, jejichž plochu jsme odměřili pomocí programu pro analýzu obrazu *Image Tool for Windows 2.00*. Sebraná data tvořily v diagramu U křivku, ve které jsme definovali střed léze jako řez, který rozděloval tuto U křivku na co nejsymetričtější poloviny. Pro účely analýzy jsme brali do úvahy jen oblast míchy, ve které byla hlavní oblast léze. Objem

zachovalé tkáně v analyzovaném segmentu byl vypočítán jako součet ploch z jednotlivých řezů, vynásobených vzdáleností mezi řezy.

3.21. Měření krevního tlaku a tepové frekvence

V isofluranové anestezii bylo z laterálního krčního přístupu proniknuto k *arteria carotis communis sinistra*, jejíž 1 cm dlouhý úsek byl mobilizován. Kraniálně od operovaného úseku byla naložena sutura, kaudálně svorka. Do lumina cévy byl zaveden katétr PE 50, který byl vyveden podkožím na povrch v místě dorsální strany krku. V místě svorky byla okolo katétru naložena sutura a svorka byla odstraněna. Katétr byl připojen k monitorovacímu zařízení PowerLab system (AD Instruments, Colorado Springs, USA). Rána byla zacelena tkáňovým lepidlem. Celý měřící systém byl naplněn fyziologickým roztokem s heparinem.

V pokusech, kde bylo třeba podat gangliový blokátor pentolinium (5 mg/kg i.v., Sigma-Aldrich), byla kromě katétru do *arteria carotis* současně zavedena i kanyla PE 10 do *vena jugularis interna sinistra*, která byla vyvedena na povrch stejným způsobem. Do této kanyly pak bylo v průběhu pokusu, tj. před nafouknutím balónku, aplikováno pentolinium. V průběhu experimentu jsme zaznamenávali hodnoty krevního tlaku (v mm Hg) a tepové frekvence (v tepech za minutu - bpm) v různých časových úsecích v závislosti na uspořádání experimentu.

3.22. Statistická analýza

Všechna data byla analyzována s použitím aritmetického průměru ± směrodatné odchylky (S.E.M.). Porovnávání dvou početních celků bylo prováděno Studentovým t-testem na hladině významnosti $p < 0.05$ (označené *). Kromě toho byly použity neparametrický Kruskall-Wallisův test a Mann-Whitney test.

BBB test jsme hodnotili tak, že z naměřených hodnot pravé a levé končetiny jsme vypočítali aritmetický průměr. Jednotlivé skupiny jsme porovnávali pomocí neparametrického Kruskall-Wallis a Mann-Whitney testu na hladině významnosti $p < 0.05$ (označené *). Při morfometrických měřeních rozsahu poškození jsme analyzovali rozdíly mezi skupinami dvěma způsoby:

1) V diagramu jsme porovnávali jednotlivé naměřené hodnoty v různých vzdálenostech od centra léze.

2) Po vypočítání objemu zachovalé šedé a bílé hmoty jsme analyzovali rozdíly mezi skupinami nepárovým Studentovým T testem. Hmotnost potkanů jsme porovnávali též Studentovým t-testem.

Statistické zpracování bylo provedeno s použitím programu Microsoft Excel, InStat 3 a Sigmaplot.

4. Výsledky

Nízké koncentrace isofluranu umožňují rozvoj neurogenního plicního edému u potkanů s poraněnou míchou

Studovali jsme vliv různých koncentrací isofluranu na rozvoj neurogenního plicního edému u potkanů s poraněnou míchou. Nejdříve jsme stanovili bezpečnou minimální koncentraci isofluranu jako 1,5% isofluran ve vzduchu při proudu anestetické směsi 300 ml/min. Zvířata jsme rozdělili do následujících skupin:
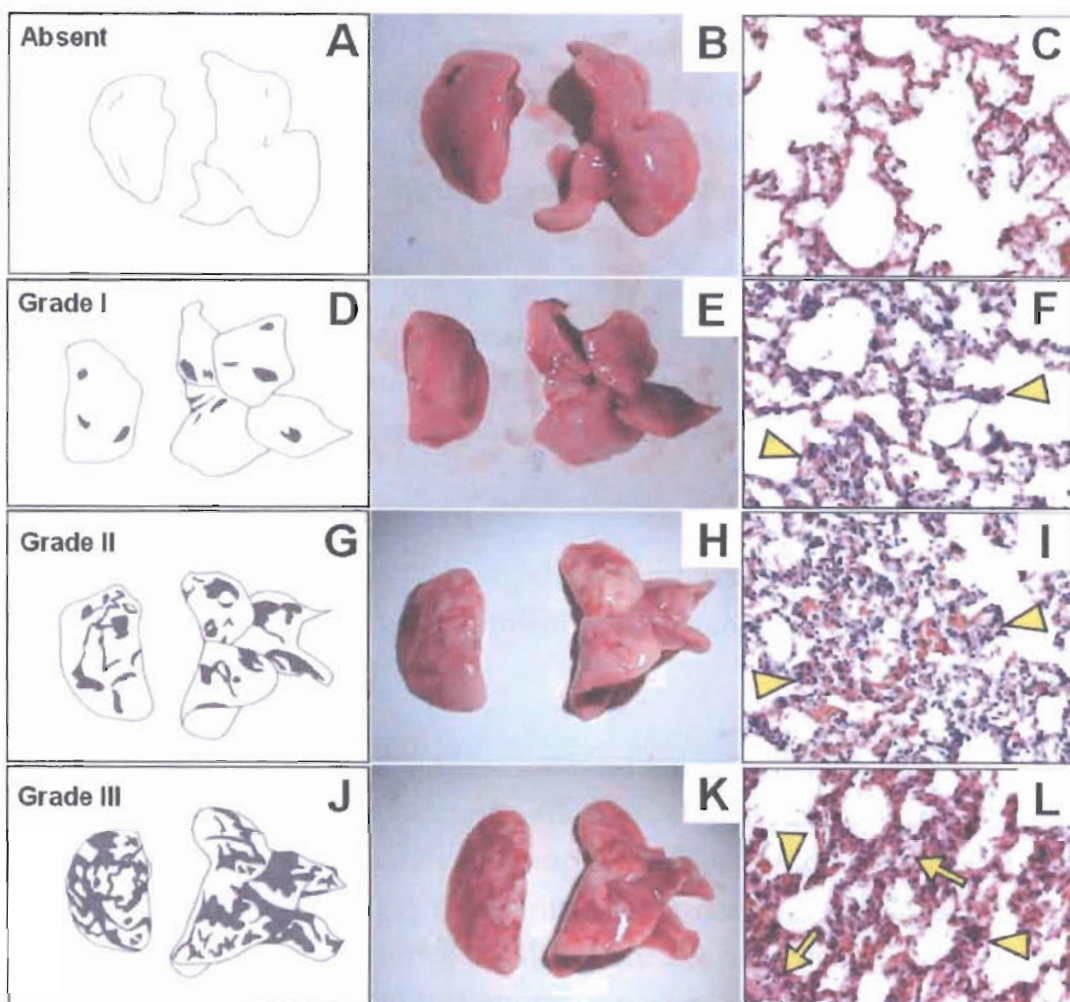
1. Zvířata narkotizovaná nízkou koncentrací anestetika (1,5-2% isofluran)
2. Zvířata narkotizovaná střední koncentrací anestetika (2,5-3% isofluran)
3. Zvířata narkotizovaná vysokou koncentrací anestetika (4-5% isofluran)

A. Vliv isofluranu na rozvoj neurogenního plicního edému

Zvířata jsme narkotizovali pomocí příslušné koncentrace isofluranu a provedli balónkovou kompresní míšní lézi o objemu 15 µl po dobu 5 minut. Po následujících 10 minutách jsme zvíře usmrtili, okamžitě vyjmuli plíce a zvážili je a poté uložili do fixačního roztoku. U každého zvířete jsme detailně monitorovali peroperační průběh, stanovili plicní index, stupeň subpleurálního krvácení a na histologických řezech i tloušťku alveolární stěny. U 10 zvířat byl proveden RTG plic.

Zvířata narkotizovaná nízkou koncentrací isofluranu

U všech těchto zvířat se rozvinul neurogenní plicní edém. U všech zvířat byl přítomen signifikantně vyšší stupeň subpleurálního krvácení, zvýšený plicní index i zvětšená tloušťka alveolární stěny. RTG obraz odpovídal plicnímu edému. Téměř 42% zvířat zemřelo na následky edému. Ve srovnání se zvířaty narkotizovanými 3% isofluranem bylo v návaznosti na provedení balónkové kompresní míšní léze pozorováno signifikantní zvýšení středního arteriálního tlaku a signifikantní snížení srdeční frekvence. Tato reakce vznikala na podkladě aktivace sympatiku, jak bylo prokázáno pomocí aplikace pentolinia.

Obr. 4.1. Stupně subpleurálního krvácení (chybějící - absent, stupeň I.-III. - grade I.-III.) a histologické vyšetření plicní tkáně. **A,B,C.** Chybění subpleurálního krvácení. Histologický obraz v (C) je normální plicní tkáň. **D,E,F.** Stupeň I subpleurálního krvácení kde je postiženo více než 10% povrchu plic krvácením. Tloušťka alveolárních stěn odráží počínající intersticiální edém (hlavičky šipek v F). **G,H, I.** Stupeň II subpleurálního krvácení, kde je postiženo 11-50% povrchu plic. Histologické vyšetření (I) ukazuje na ztluštění alveolárních stěn (hlavičky šipek v I) a krvácení. **J,K,L.** Stupeň III subpleurálního krvácení, kde je více než 51% povrchu plic postiženo krvácením. Masivní ztluštění alveolárních stěn (hlavičky šipek v L), intersticiální a intraalveolární edém (šipky v L) a masivní extravazace erytrocytů.

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Zvířata narkotizovaná střední koncentrací isofluranu

U žádného z těchto zvířat se nerozvinul těžký neurogenní plicní edém. Plicní index, tloušťka alveolární stěny a RTG vyšetření neprokázalo přítomnost edému. Pouze 28% zvířat mělo přítomno Grade I nebo II subpleurální krvácení. Žádné zvíře nezemřelo.

Zvířata narkotizovaná vysokou koncentrací isofluranu

Všechna tato zvířata zemřela na následky předávkování anestetikem. U žádného z nich se nerozvinul neurogenní plicní edém.
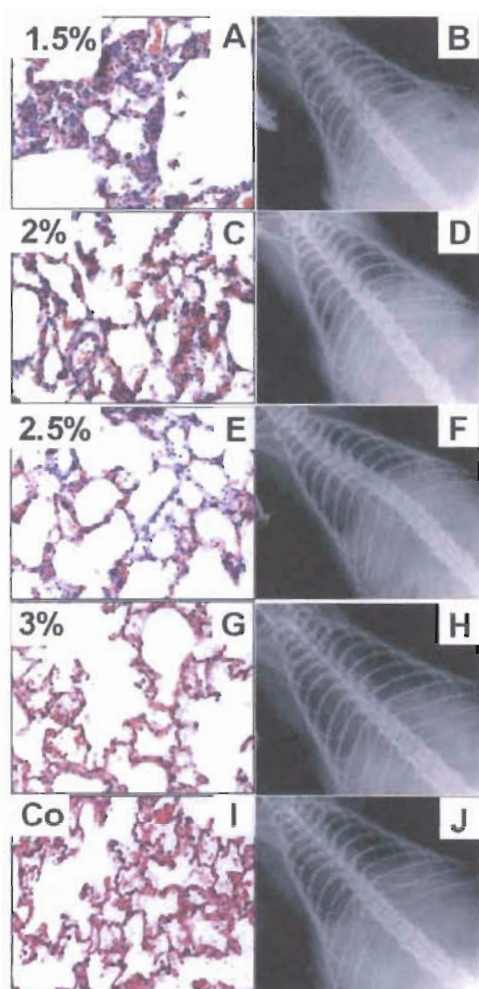
Kontroly

U žádného ze zvířat narkotizovaných různými koncentracemi isofluranu (1,5-3%), u kterých nebyla provedena míšní léze, se nerozvinul neurogenní plicní edém. Žádné zvíře nezemřelo.

B. Vliv isofluranu na velikost léze a návrat neurologických funkcí

Zvířata z 1,5% a 3% skupiny jsme narkotizovali pomocí příslušné koncentrace isofluranu a provedli balónkovou kompresní míšní lézi o objemu 15 μl po dobu 5 minut. Po dobu následujících 7 týdnů byl monitorován návrat neurologických funkcí s použitím lokomotorického BBB testu a sensitivního plantar testu. Po uplynutí této doby byla zvířata usmrcena, perfundována fixačním roztokem a provedena morfometrická analýza stávající šedé a bílé hmoty míšní.

Vliv přítomnosti neurogenního plicního edému se odrazil v signifikantně horším návratu lokomočních i sensitivních neurologických funkcí v 2-3. týdnu po provedené lézi. Následně se stav normalizoval.

Morfometricky nebyly nalezeny rozdíly mezi oběma skupinami. Vyšetření magnetickou rezonancí *in vivo* rovněž neprokázalo žádné signifikantní rozdíly.

Obr. 4.2. Neurogenní plicní edém u skupin narkotizovaných 1.5%, 2%, 2.5% nebo 3% isofluranem. A. Histologické vyšetření ukazující masivní neurogenní plicní edém – ztluštění alveolárních stěn, intersticiální edém a masivní krvácení u potkanů narkotizovaných 1.5% isofluranem. B. RTG ukazující difúzní zastínění plicního parenchymu, zejména v perihilární oblasti, ukazující neurogenní plicní edém u potkanů narkotizovaných 1.5% isofluranem. C. Histologické vyšetření ukazující rozvoj neurogenního plicního edému se ztluštěním alveolárních stěn a občasnými okrsky krvácení u potkanů narkotizovaných 2% isofluranem. D. RTG ukazující difúzní zastínění plicního parenchymu, zejména v perihilární oblasti, ukazující neurogenní plicní edém u potkanů narkotizovaných 2% isofluranem. E. Občasná místa prokrvácení plicního parenchymu bez přítomnosti plicního edému u potkanů narkotizovaných 2.5% isofluranem. F. Normální RTG obraz plic u potkanů narkotizovaných 2.5% isofluranem. G. Normální histologický obraz plic u potkanů narkotizovaných 3% isofluranem. H. RTG normálních plic u potkanů narkotizovaných 3% isofluranem. I. Histologie plicní tkáně kontrolního zvířete J. RTG normálních plic u kontrolního zvířete.

Posuzovali jsme možnost využití balónkové kompresní míšní léze v 1,5% a 2% isofluranové anestezii jako modelu těžkého neurogenního plicního edému. Zvířata jsme narkotizovali pomocí příslušné koncentrace isofluranu a provedli balónkovou kompresní míšní lézi o objemu 15 µl po dobu 5 minut. Po následujících 10 minutách jsme zvíře usmrtili, okamžitě vyjmuli plíce a zvážili je a poté uložili do fixačního roztoku. U každého zvířete jsme detailně monitorovali peroperační průběh, stanovili plicní index, stupeň subpleurálního krvácení a na histologických řezech i tloušťku alveolární stěny. Následně jsme porovnali stupeň subpleurálního krvácení a histologické analýzy. U 3 zvířat z každé skupiny byl proveden *in vivo* RTG plic. U 3 zvířat z 1,5% skupiny jsme před provedením míšní léze chirurgicky odstranili všechny vrstvy boční hrudní stěny s výjimkou průsvitné pohrudnice a peroperačně jsme makroskopicky sledovali nástup neurogenního plicního edému.

Neurogenní plicní edém

U všech zvířat narkotizovaných 1,5% nebo 2% isofluranem ve vzduchu se rozvinul neurogenní plicní edém. Stanovení stupně subpleurálního krvácení, plicního indexu, tloušťky alveolárních stěn a *in vivo* RTG vyšetření dokumentovalo, že u 1,5% skupiny byl edém rozsáhlejší než u 2% skupiny, v obou případech však těžkého stupně. Více než 33% zvířat z 1,5% skupiny na jeho následky zemřelo v průměrné době 8,45 minuty po provedení léze. U 2% skupiny přežila 15-minutový interval po začátku léze všechna zvířata. První známky ztmavnutí povrchu plic se u 1,5% skupiny objevily v průměrné době 6,67 minut, zatímco první subpleurální hemorrhagie až v průměrné době 8,00 minut po nafouknutí balónku v epidurálním prostoru.

Využití modelu

Model neurogenního plicního edému v 1,5% isofluranové anestezii lze využít u menších nebo předběžných studií, jelikož má 33% mortalitu. Naopak model neurogenního plicního edému v 2% isofluranové anestezii je vhodný pro rozsáhlejší studie.

Pomalá míšní komprese zabrání rozvoji neurogenního plicního edému u potkanů
s poraněnou míchou

Stanovili jsme význam rychlosti nástupu a objemu míšní léze na rozvoj neurogenního plicního edému. Zvířata jsme narkotizovali pomocí 1,5% isofluranu ve vzduchu, zavedli kanylu pro monitoraci krevního tlaku a tepové frekvence a provedli míšní transekci nebo balónkovou kompresní míšní lézi několika různými způsoby:

1. Okamžité nafouknutí na 15 µl.
2. Rychlé postupné nafukování (5 µl - 5 µl – 5 µl)
3. Pomalé postupné nafukování (3 µl – 2 µl – 2 µl – 2 µl – 2 µl – 2 µl – 2 µl)
4. Okamžité nafouknutí na 5 µl
5. Okamžité nafouknutí na 10 µl

Ve všech případech jsme balónek ponechali nafouknutý *in situ* na dobu 5 minut po poslední změně. Po následujících 10 minutách jsme zvíře usmrtili, okamžitě vyjmuli plíce, zvážili je a poté uložili do fixačního roztoku. U každého zvířete jsme detailně monitorovali peroperační průběh, stanovili plicní index, stupeň subpleurálního krvácení a na histologických řezech i tloušťku alveolární stěny. Následně jsme porovnali stupeň subpleurálního krvácení a histologické analýzy.

Rychlost nafouknutí balónku
U skupiny s okamžitým nebo rychle nafouklým balónkem došlo k rozvoji typického těžkého neurogenního plicního edému. Naopak u skupiny, kde byl balónek nafukován pomalu, k těžkému edému nedošlo; pouze u 36% zvířat byl přítomen lehký stupeň subpleurálního krvácení. Rozdíl ve středním arteriálním tlaku mezi skupinou s rychle a s pomalu nafukovaným balónkem nebyl výrazný – mnohem větší byl rozdíl ve změnách tepové frekvence, která výrazně klesala u zvířat s NPE, což odráží větší účinnost barereflexu u této skupiny.

Neúplné nafouknutí balónku a transekce
Neúplné nafouknutí balónku ani míšní transekce nebyly schopny vyvolat rozvoj neurogenního plicního edému.

Hluboká anestezie zabrání rozvoji neurogenního plicního edému

Formulovali jsme hypotézu o protektivním vlivu hluboké anestezie na rozvoj neurogenního plicního edému. Při hluboké anestezii jsou více utlumena hypothalamická, kmenová a míšní vasoaktivní sympatická centra, což zabrání jejich hyperaktivaci na podkladě náhle vzniklého poranění centrálního nervového systému. Díky tomu se neuplatní ani prvotní šok nervové tkáně ani zvýšení intrakraniálního tlaku. Neurony vasoaktivních center pak nespouštějí rozsáhlou hyperaktivaci sympatiku, neboli katecholaminovou bouři, takže nedochází ke zvýšení krevního tlaku, generalizované periferní vasokonstrikci, zvýšení systémové cévní rezistence, centralizaci oběhu a snížení poddajnosti levé komory srdeční. Neuplatní se ani plicní vasokonstrikce, zvýšení plicního kapilárního hydrostatického tlaku, poškození alveolární stěny a uvolnění tekutiny do intersticia – tedy nedochází k rozvoji neurogenního plicního edému.

Mechanismy rozvoje neurogenního plicního edému

V této práci je podán přehled teorií etiopatogeneze neurogenního plicního edému a jsou diskutovány možnosti jeho vzniku na podkladě nově získaných dat. Diskutována je zejména role intrakraniálního tlaku, centrálních vasoaktivních sympatických center, typů poranění centrálního nervového systému, sympatické hyperaktivace, zánětu a anestezie. Kromě toho jsou shrnuta dostupná epidemiologická a klinická data o neurogenním plicním edému. Navíc je prezentován přehledný výčet experimentálních modelů neurogenního plicního edému.

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pulmonary edema in spinal cord injured rats. J Neurotrauma

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Low Concentration of Isoflurane Promotes the Development of Neurogenic Pulmonary Edema in Spinal Cord Injured Rats

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ABSTRACT

Anesthetics can either promote or inhibit the development of neurogenic pulmonary edema (NPE) after central nervous system (CNS) injury. The influence of isoflurane was examined in male Wistar rats using 1.5%, 2%, 2.5%, 3%, 4%, or 5% isoflurane in air. Epidural balloon compression of the thoracic spinal cord was performed. The development of NPE was examined *in vivo* and on histologic sections of lung tissue. Animals anesthetized with 1.5% or 3% isoflurane were behaviorally monitored using the BBB and plantar tests for 7 weeks post-injury. The spinal cord was examined using MRI and morphometry of the spared white and gray matter. All animals from the 1.5% and 2% groups developed NPE. Almost 42% of the animals in the 1.5% group died of severe pulmonary hemorrhage and suffocation; x-rays, the pulmonary index, and the histological picture revealed a massive NPE. More than 71% of the animals from the 2.5% and 3% groups did not develop any signs of NPE. Blood pressure after spinal cord compression rose more in the 1.5% group than in the 3% one. In the 1.5% group, the sympathetic ganglionic blockade prevented the neurogenic pulmonary edema development. Animals from the 3% group recovered behaviorally more rapidly than did the animals from the 1.5% group; morphometry and MRI of the lesions showed no differences. Thus, low levels of isoflurane anesthesia promote NPE in rats with a compressed spinal cord and significantly complicates their recovery. The optimal concentration of anesthesia for performing a spinal cord compression lesion is 2.5–3% isoflurane in air.

Key words: blood pressure; isoflurane; lesion; neurogenic pulmonary edema; rat; spinal cord injury

INTRODUCTION

RESPIRATORY COMPLICATIONS are still an important cofactor of morbidity and mortality in patients with spinal cord or brain injuries (Urdaneta and Layon, 2003). Neurogenic pulmonary edema is an acute life-threatening complication following spinal cord or brain injury. It is characterized by marked pulmonary vascular conges-

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tion, extravasation of protein-rich edema fluid and intraalveolar hemorrhage (Kandatsu et al., 2005; Kondo et al., 2004; Leal Filho et al., 2005a,b).

Epidemiological data of neurogenic pulmonary edema are scarce; its morbidity in patients with severe central nervous system (CNS) injury has been reported to be 40–50% and its mortality around 9% (Fontes et al., 2003; Dragosavac et al., 1997; Antoniuk et al., 2001). To date, several data on the neurogenic pulmonary edema in spinal cord injured patients has been reported (Karlsson, 2006; Stocker and Burgi, 1998; Troll and Dohrmann, 1975; Tsao et al., 1999). However, a comprehensive epidemiological study, systematically examining the occurence of neurogenic pulmonary edema in spinal cord injured patients is still lacking.

Many pathophysiological mechanisms have been implicated in the development of neurogenic pulmonary edema, but the exact cascade leading to its development is still unclear (Leah Filho et al., 2005a,b). Both the release of vasoactive substances and a severe transient sympathetic discharge are thought to participate in this process (Taoka and Okajima, 1998; Urdaneta et al., 2003). These processes lead to the constriction of the pulmonary veins, an increase in pulmonary capillary hydrostatic pressure, damage to the alveolar wall, and the leakage of fluid into the intraalveolar space (Fontes et al., 2003).

Several authors have reported that different anesthetic drugs either promote or inhibit the development of neurogenic pulmonary edema in spinal cord or brain injured rats (Leah Filho, 2005a,b; Mesquita et al., 2002; Pandey et al., 2000). The influence of ketamine, xylazine and pentobarbital on the development of neurogenic pulmonary edema has been shown previously (Leah Filho, 2005a,b; Mesquita et al., 2002; Pandey et al., 2000). However, the possible role of isoflurane, one of the most prevalent anesthetics used in experimental spinal cord injury has not yet been examined in detail.

In preliminary experiments, we observed that some animals were dying due to bleeding from the airways and suffocation during balloon-induced spinal cord compression, when using lower levels of isoflurane anesthesia. To evaluate whether the anesthesia levels or other factors are responsible for such reactions, we undertook a comprehensive study of different doses of anesthesia using the balloon compression lesion model (Vanický et al., 2001). In addition, we monitored blood pressure, heart rate, the level of functional recovery and spinal cord tissue sparing in animals with or without neurogenic pulmonary edema. To demonstrate the role of the sympathetic nervous system, we also studied blood pressure and heart rate in animals anesthetized by 1.5% isoflurane that were subjected to ganglionic blockade by pentolinium prior to balloon inflation.

METHODS

Animals

We used 148 male Wistar rats (Velaz, Prague, Czech Republic) with body weights of 300–330 g. This study was performed in accordance with the European Communities Council Directive of 24th of November 1986 (86/609/EEC) regarding the use of animals in research and was approved by the Ethics Committee of the Institute of Experimental Medicine ASCR, Prague, Czech Republic.

Design of the Study

In preliminary experiments, we determined the minimum safe concentration of isoflurane anesthesia, under which no corneal, tail pinch or interdigital toe reflexes occur, to be 1.5% isoflurane in air (flow of anesthetic mixture was 300 mL/min).

In the first part of our study, animals were anesthetized with 1.5% ($n = 12$), 2% ($n = 12$), 2.5% ($n = 12$), 3% ($n = 16$), 4% ($n = 3$), or 5% ($n = 3$) isoflurane in air, and a spinal cord balloon compression lesion was made (Fig. 1). Animals were sacrificed 10 min after lesioning, and the grade of neurogenic pulmonary edema was evaluated using macroscopic visual examination of subpleural bleeding, the p-index (lung weight/body weight), and histological examination of lung tissue sections. Routine paraffin embedding and hematoxylin-eosin staining of the spinal cord lesion sites were performed. In an additional 10 animals (two from each of the 1.5%, 2%, 2.5%, 3%, and control groups), in vivo x-ray examination was performed. Moreover, blood pressure and heart rate were monitored in animals from the 3% ($n = 6$) and 1.5% ($n = 6$) groups. Controls were healthy noninjured animals, sacrificed immediately after the induction of anesthesia. To make sure that the observed pulmonary edema was "neurogenic," we anesthetized 3 animals from each group with isoflurane (1.5%, 2%, 2.5%, or 3%) for 40 min, while no surgery was performed, and then examined their lungs.

In the second part of the study, we evaluated the level of functional recovery and spinal cord tissue sparing in animals with (1.5% group) or without (3% group) neurogenic pulmonary edema for 7 weeks following a balloon compression lesion. On the second day after the injury, in vivo MR images of the injured spinal cord were taken to verify the lesion procedure (Fig. 1). Animals
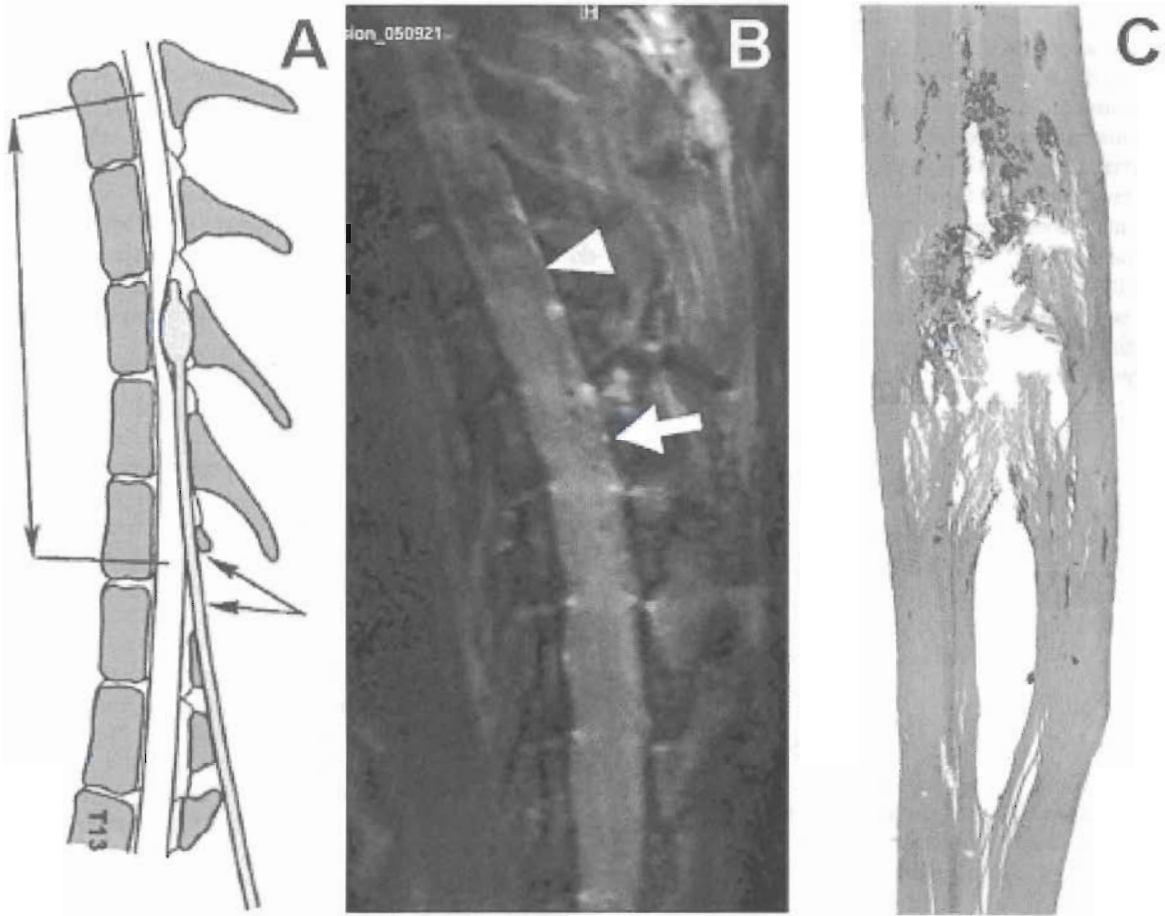
FIG. 1. Balloon compression lesion. (**A**) Schematic drawing of the injury procedure. The insertion site of the balloon is marked by two arrows on the right; the part of the spinal cord used for morphometric analysis is marked by the left arrow. (**B**) Sagittal magnetic resonance image of a spinal cord in which a balloon compression lesion (arrowhead) was performed one day earlier. Arrow, site of insertion of the catheter. (**C**) Hematoxylin-eosin-stained tissue section of a balloon compression spinal cord lesion.

were behaviorally tested using the Basso-Beattie-Bresnahan (BBB) locomotor test and the plantar test 24 h post-injury and then once per week. In addition, post mortem morphometric analysis of the volume of the spared white and gray matter was performed.

Balloon-Induced Spinal Cord Injury

After the induction of anesthesia with 5% isoflurane in room air (flow 300 mL/min), animals were maintained in 1.5%, 2%, 2.5%, 3%, 4%, or 5% isoflurane anesthesia (flow 300 mL/min) via a face mask throughout the operation. All animals were heated to 37°C, and their body temperature was measured by a rectal thermometer to standardize the procedure and to exclude the influence

of hypo- or hyperthermia (Cambria et al., 1997; Urdzíková and Vanický, 2006). For spinal cord injury, we used the model of an epidural balloon compression lesion, as described in detail previously (Vanický et al., 2001). Briefly, under aseptic conditions, a 2-cm median skin incision at the Th10-L1 level was made. The dorsal muscles were shifted laterally, and the Th10 and Th11 spinous processes were removed. A hole was drilled into the Th10 lamina with a dental drill. Then, a 2-F French Fogarthy catheter (Baxter Healthcare Corporation, Irvine, CA) was filled with distilled water and connected to a 50-μL Hamilton syringe and inserted into the dorsal epidural space 10 mm rostrally, to reach the Th8-Th9 spinal level (Fig. 1). The balloon was rapidly inflated with 15 μL of distilled water for 5 min, using a micro-

manipulator. Subsequently, the balloon was deflated and removed. Soft tissues and the skin were sutured.

To verify the injury procedure, the balloon was inflated before and immediately after the injury procedure to confirm the inflation of the balloon in the spinal channel. The inflation of a balloon to 15 μL in the spinal channel produces an incomplete lesion, so after 7 weeks, the hindlimbs of the animals are able to support body weight and occasionally forelimb-hindlimb coordination is observed. This state corresponds to a BBB score of 9–11 at 7 weeks post-injury.

Evaluation of Neurogenic Pulmonary Edema

In the first part of the study, animals were sacrificed 10 min after the removal of the catheter; the lungs were immediately removed and weighed. Subsequently, selected organs (brain, heart, thymus, liver, intestine, kidney, spleen, and urinary bladder) were dissected to detect other possible sites of hemorrhage or other pathologic changes. In all cases, a mild hematoma, maximally 1 mm in diameter, was found in the hilus area due to the manipulation of the pulmonary vessels during lung removal (not taken into further account). The level of pulmonary subpleural bleeding was evaluated macroscopically as "Absent" (no bleeding on the lung surface), "Grade I" (small bleeding areas, occupying not more than 10% of the lung surface), "Grade II" (medium-sized bleeding areas, occupying 11–50% of the lung surface), and "Grade III" (massive bleeding areas, occupying more than 50% of the lung surface; Fig. 2). Each lung was evaluated separately. To estimate the liquid gain of the lungs, both lungs were weighed, and the relative pulmonary weight was calculated as the pulmonary index (lung weight/body weight × 100), which has been previously considered to be very sensitive to the degree of pulmonary edema (Leal Filho et al., 2005a,b; Mesquita et al., 2002; Minnear and Connel, 1982). The lungs were immediately fixed in 4% paraformaldehyde in phosphate buffer (pH 7.4) for 2 days, embedded in paraffin and stained with hematoxylin and eosin. Five-micron sections were cut, and the thickness of the alveolar walls measured using Neurolucida software (MicroBrightField, Inc.). From each lung, three sections (from the inferior, middle, and superior parts of the lung) were taken, an d all alveolar wall thicknesses in one representative field from each section were measured. A representative field was defined as a site in the non-subpleural lung parenchyma, without any large vessel or bronchus, outside of the hilus region.

Measurement of Blood Pressure and Heart Rate Changes

Systolic, diastolic and mean arterial blood pressure, together with heart rate, were monitored in animals from the 3% and 1.5% groups using a PowerLab system (AD Instruments, Colorado Springs). Under isoflurane anesthesia, a catheter was inserted into the left carotid artery, exteriorized in the interscapular region, the animal put into a prone position and a balloon compression lesion performed. The systolic, diastolic, and mean arterial pressure (mm Hg), together with heart rate (bpm), were monitored for 5 min before the procedure, throughout the entire procedure and for 5 min after the procedure. The values obtained were (1) the baseline value, (2) the value during the skin incision (minimum), (3) the value during the muscle incision (minimum), (4) the value during the inflation of the balloon (maximum), (5) the value of inflated balloon (2-min interval from the beginning of inflation), and (6) the value after 5 min of recovery.

Ganglionic Blockade

To eliminate the influence of the sympathetic nervous system, we administered the ganglionic blocker pentolinium (5 mg/kg i.v., Sigma) to five animals anesthetized with 1.5% isoflurane at 3 min before the balloon inflation. Blood pressure and heart rate were monitored as described above.

X-Ray Imaging

To analyze the extent of neurogenic pulmonary edema *in vivo*, we used x-ray imaging employing the Image Station In-Vivo FX System (Eastman Kodak Company). Animals were anesthetized with 1.5%, 2%, 2.5%, or 3% isoflurane and a balloon compression lesion was made. Before awakening from the anesthesia, pentobarbital (30 mg/kg) was injected, the animals were placed in the Image Station, and routine x-ray images were taken (35 kVP, exposure time 5 min).

Postoperative Care

After the lesion procedure, the animals developed a complete paraplegia for 2–3 days post-injury, followed by a gradual recovery during 5 weeks after the injury. The animals were housed in pairs, to reduce stress from isolation, on a 12-h light-dark cycle with standard rat chow and water *ad libitum*. After lesioning, manual bladder expression was performed. Generally, the expression of the bladder was performed twice a day during the early postoperative period. With the improvement of the animal's condition, it was performed once a day until the end of the second week, by which time a reflex bladder was usually established.

Behavioral Testing of Animals

All animals included in the second part of the study were allowed to survive for 7 weeks post-injury.
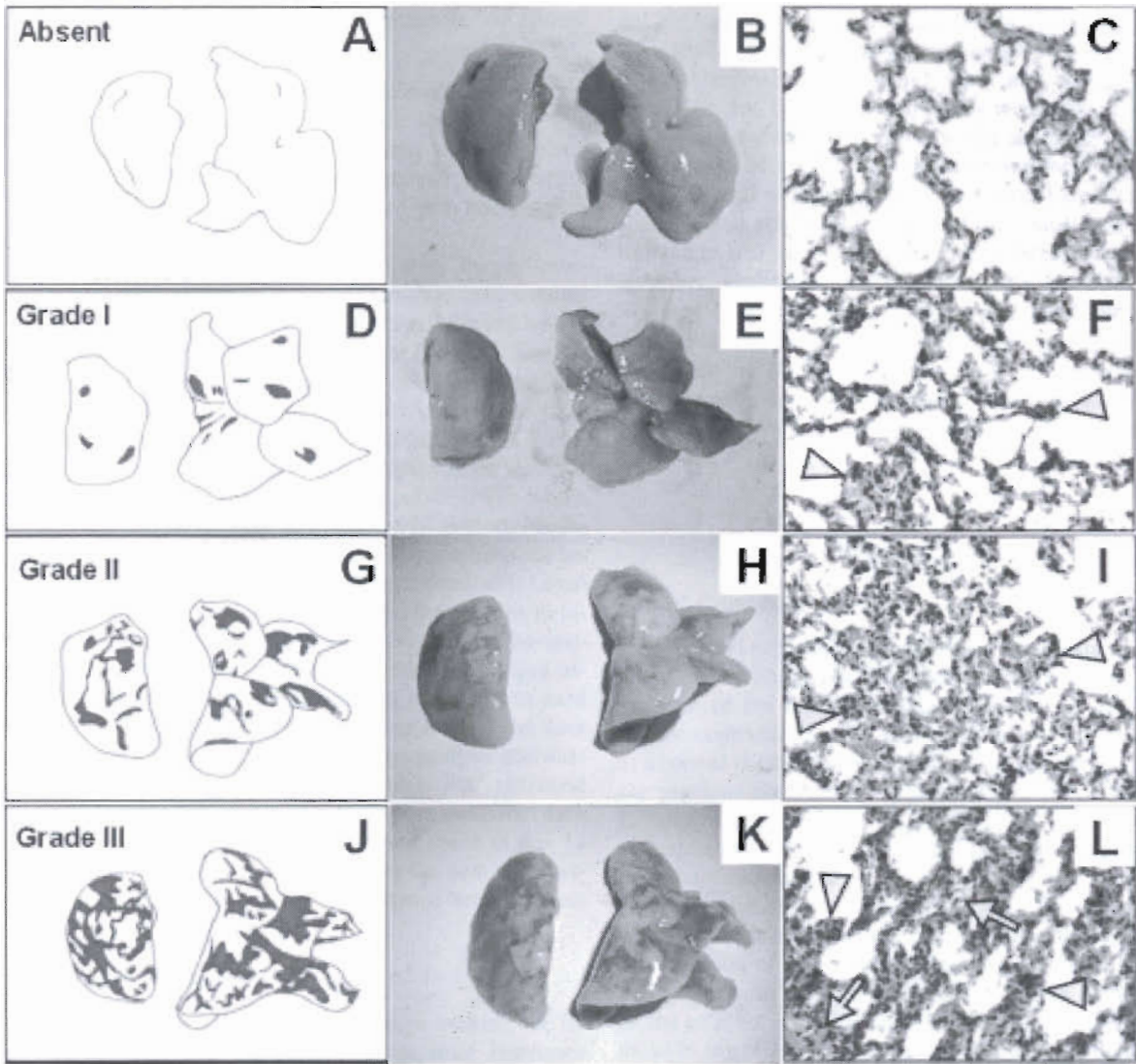
FIG. 2. The grading of subpleural hematoma (absent, grade I, II, III) and histological examination of lung tissue. (**A–C**) The absence of subpleural bleeding. The histology (C) corresponds to that of normal lungs. (**D–F**) Grade I subpleural bleeding in which no more than 10% of the lung surface is affected, with small hemorrhagic areas. The thickness of the alveolar wall indicates the beginning of interstitial edema (arrowheads in F), and occasional erythrocyte extravasation can be seen. (**G–I**) Grade II subpleural bleeding in which 11–50% of the lung surface is affected. Histology (I) reveals a thickening of the alveolar walls (arrowheads in I), and bleeding is apparent. (**J–L**) Grade III subpleural bleeding in which more than 51% of the lung surface is affected. A massive thickening of the alveolar wall (arrowheads in L), interstitial and intraalveolar edema (arrows in L), and a massive extravasation of erythrocytes are visible.

Hindlimb performance was evaluated using the BBB open field locomotor test developed by Basso et al. (1995). Sensation in the hindlimbs was determined according to the latency (in seconds) of hindlimb withdrawal from thermal stimulation using the plantar test (Ugo Basil, Comerio, Italy), as described previously (Syková et al., 2005; Urdziková et al., 2006). Two observers performed the BBB and plantar tests before injury, at 24 h after injury, and then once a week throughout the survival period.

Histological Procedures and Assessment of Spinal Tissue Sparing

For routine hematoxylin-eosin staining, a 3-cm-long segment of spinal cord containing the lesioned site was dissected, put into 4% paraformaldehyde in phosphate buffer (pH 7.4) for at least 2 days, embedded in paraffin, cut into 30-μm-thick sections, stained with hematoxylin and eosin and mounted.

At 7 weeks post-injury, animals were deeply anesthetized with chloral hydrate (400 mg/kg), and animals were transcardially perfused with saline, followed by 4% paraformaldehyde in phosphate buffer (pH 7.4). The entire spinal cord was left in the spinal canal overnight, and then carefully removed and postfixed in the same fixative and stored at 4°C until further processing. A 2-cm-long segment of the spinal cord containing the lesioned site was dissected and embedded in paraffin. The whole segment was serially cut, and series of 20 sections (thickness 5 μm) were collected (2 mm distance between individual sections). The sections were stained with Luxol Fast Blue and Cresyl Violet. We used these stains to facilitate the discrimination between gray and white matter at low magnifications. Every section was imaged using a digital camera; high resolution images were used to delineate the spared white and gray matter, and their areas were measured using the image analysis software Neurolucida (MicroBrightField, Inc.) For statistical analysis, 13 lesion-centered sections were used from each spinal cord. The volume of the spared tissue in this 11 mm long segment was calculated as the sum of cross-sectional areas multiplied by the distance between them (Vanický et al., 2001).

Magnetic Resonance Imaging

To verify the injury procedure and to evaluate the lesion development, we performed magnetic resonance (MR) imaging of the lesion site 1 day after injury (Fig. 1). Spinal cords were scanned *in vivo* on an MR spectrometer (Bruker Biospec 47/20, 4.7 Tesla, 20 cm room temperature bore) equipped with 200 mT/m gradient system and a home-made quasi TEM mode operating microstrip surface coil for spinal cord imaging (Burian and Hájek, 2004). Sagittal images (matrix 512 × 160, FOV 10 × 3 cm, slice thickness 0.5 mm, contiguous slices, TE/TR 70/2500 msec) and axial images (matrix 256 × 128, FOV 4 × 2 cm, slice thickness 0.5 mm, slice gap 0.5 mm, TE/TR = 70/2800 msec) were acquired using an ordinary RARE sequence with the RARE factor equal to 8.

Statistical Analysis

The mean p-index of each group, the thickness of the alveolar wall as well as blood pressure and heart rate val-

ues are reported as mean ± SEM. Intergroup differences were analyzed using a non-paired Student's t-test. In individual animals, BBB scores were averaged across hindlimbs, and intergroup differences were analysed using the non-parametric Kruskall-Wallis and Mann-Whitney U-tests. Morphometric measurements were used to construct plots of consecutive cross-sectional areas of the spared tissue at individual levels of the spinal cord rostral and caudal to the epicenter. The differences at each level were analyzed using the Kruskall-Wallis and Mann-Whitney U-tests. Body weights at individual survival time points and the calculated volumes of the spared tissue within the 11-mm-long segments were compared by a non-paired Student's t-test. Statistically significant differences ($p < 0.05$) are marked in figures and tables by asterisks.

RESULTS

Spinal Cord Injury

The spinal cord lesioning procedure was performed in all animals without any unexpected complications. The inflation of the balloon was accompanied by skeletal muscle contractions in all cases, which was considered as a normal reaction to injury and was in accordance with our previous observations (Syková et al., 2005, 2006a; Urdzíková et al., 2006).

Respiratory failure, accompanying spinal cord compression, followed the onset of the injury procedure in 30% of cases (37 of 124 operated animals), independently of the concentration of isoflurane anesthesia used. The duration of respiratory arrest ranged from 5 to 40 sec, with a mean duration of 21.56 ± 9.11 sec. Although all the animals were subjected to an autopsy, no hemorrhage in any other examined organ (brain, heart, thymus, liver, intestine, kidney, spleen, and urinary bladder) was found.

According to our results, we divided the animals into a "low isoflurane group" (1.5–2% isoflurane), in which all animals developed neurogenic pulmonary edema, a "medium isoflurane group" (2.5–3% isoflurane), with a very low occurrence of neurogenic pulmonary edema, and a "high isoflurane group" (more than 4% isoflurane), in which all animals died due to anesthesia overdose (Table 1).

Neurogenic Pulmonary Edema in Groups Anesthetized with a Low Concentration of Isoflurane

All animals anesthetized with a low concentration of isoflurane (1.5% and 2% groups) developed neurogenic

TABLE 1. IMPAIRMENT OF LUNG FUNCTION AFTER SPINAL CORD INJURY IN ANIMALS ANESTHETIZED WITH 1.5%, 2%, 2.5%, 3%, 4%, OR 5% ISOFLURANE AND CONTROL GROUPS

Isoflurane	N	Absent (% of 2N)	Grade I (% of 2N)	Grade II (% of 2N)	Grade III (% of 2N)	p-index	Died (% of 2N)
1.5%	12	—	—	1 (4.17%)	23 (95.83%)	0.92 ± 0.18*	5 (41.67%)
2%	12	—	3 (12.50%)	7 (29.17%)	14 (58.33%)	0.74 ± 0.11*	—
2.5%	12	15 (62.50%)	6 (25.00%)	3 (12.50%)	—	0.51 ± 0.06	—
3%	16	26 (81.25%)	4 (12.50%)	2 (6.25%)	—	0.50 ± 0.06	—
4%	3	6 (100.00%)	—	—	—	0.48 ± 0.01	3 (100.00%)
5%	3	6 (100.00%)	—	—	—	0.47 ± 0.02	3 (100.00%)
Control	12	24 (100.00%)	—	—	—	0.45 ± 0.02	—

The absence or presence of subpleural bleeding (evaluated as Grade I–III) in different groups (the total number of lungs in each group; the right and left lung were considered separately—and the percentage of all lungs in the corresponding group).

*A significant elevation ($p \pm 0.05$) of the p-index (mean values ± SEM [standard error of the mean]).

The occurrence of death is shown as the total number of deaths in each group and as the percentage of all animals in the group. Control animals are animals without spinal cord injury, sacrificed immediately after the onset of anesthesia. N, number of rats; 2N, number of lungs.

pulmonary edema. Pulmonary subpleural bleeding developed in all animals anesthetized with 1.5% or 2% isoflurane. In 77% of lungs, Grade III subpleural bleeding was present. In the remaining lungs, either Grade II (17%) or Grade I (6%) subleural bleeding occurred (Table 1, Figs. 2 and 3). In these animals, the pulmonary index differed significantly from controls: in the 2% isoflurane group, it was 64% higher in comparison with animals from the control group ($p = 0.0000005$), while in the 1.5% group it was even higher, at 101% ($p = 0.0000008$).

Five of 12 animals (42%) in the 1.5% group died in 7.50 ± 3.15 min (range from 5 to 12 minutes) after the beginning of balloon inflation (Table 1). A few minutes before death, their breathing frequency started to increase slowly, and they began to develop a so-called "death rattle." Subsequently, their ventilation stopped and after several seconds, gaseous blood came out of their noses, followed by the cessation of their heart beat. In contrast, no animal from the 2% group died (Table 1).

Microscopic examination of the lungs showed that lower concentrations of isoflurane caused edema of the alveolar membrane, perforation of thin capillary walls, massive bleeding and the leakage of intravascular fluid into the alveoli. The combination of interstitial and intraalveolar leakage of transsudate with intraparenchymal hemorrhage, consequent to spinal cord injury, was considered as the picture of neurogenic pulmonary edema (Figs. 2 and 3). In the 1.5% group, the thickness of the alveolar membrane was 264% larger in comparison with controls, in the 2% group, 199% larger than in controls ($p = 0.0004$ and $p = 0.006$, respectively; Table 2). In addition, in vivo x-ray imaging showed diffuse hyperintensive infiltrates in both lungs, mainly around the hilus regions (Fig. 3). Thus, lower concentrations of isoflurane are causative for the development of massive neurogenic pulmonary edema in spinal cord injured rats.

Neurogenic Pulmonary Edema in Groups Anesthetized with a Medium Concentration of Isoflurane

More than 28% of animals anesthetized with a medium concentration of isoflurane in air (2.5% and 3% groups) developed lung hemorrhage, but none of these cases were Grade II or III, and in 72% of cases no subpleural hematoma was present (Table 1, Figs. 2 and 3). In addition, the pulmonary index values were slightly higher in both the 2.5% and 3% groups in comparison with controls (Table 1), but these differences did not reach statistical significance ($p = 0.07$ and $p = 0.06$, respectively).

Macroscopic evaluation of the occurrence of subpleural bleeding showed that animals anesthetized with
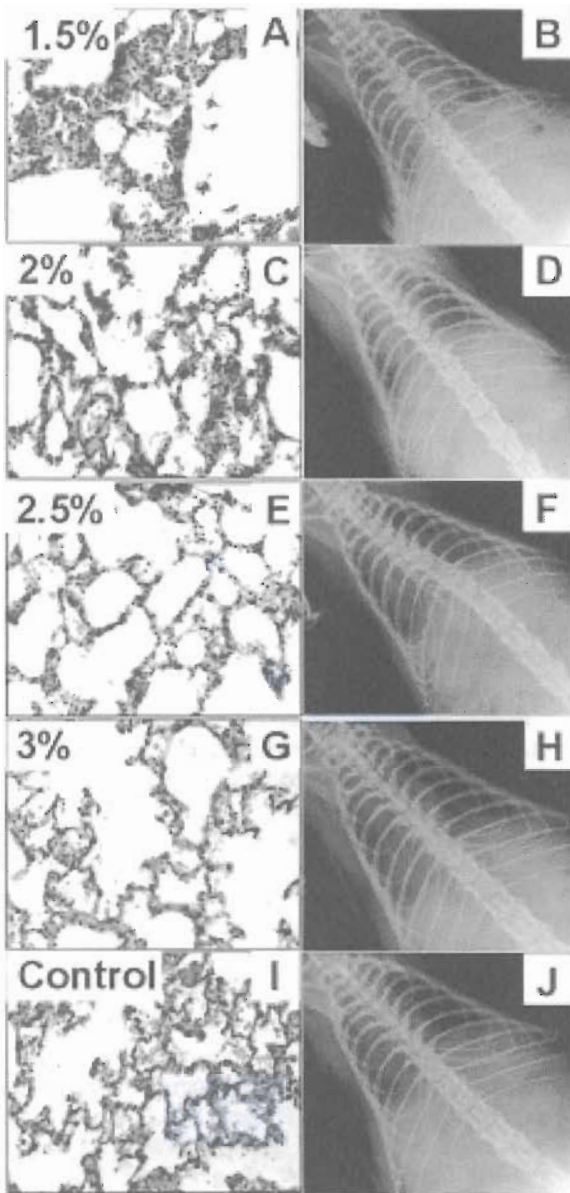
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mal lung tissue, with a slightly increased thickness of the alveolar walls and limited extravasation of blood elements (Figs. 2 and 3). In the 2.5% group, the thickness was 30% larger and in the 3% group only 18% larger in comparison with controls (Table 2). Thus, only in the 2.5% group did the difference in alveolar wall thickness reach statistical significance ($p = 0.03$). *In vivo* x-ray examination showed lungs that were comparable to controls (Fig. 3). Thus, lesioned animals anesthetized with medium concentrations of isoflurane developed only a very low level of neurogenic pulmonary edema. In addition, 3% isoflurane can be considered as the safest concentration to use for balloon compression spinal cord lesioning.

Neurogenic Pulmonary Edema in Groups Anesthetized with a High Concentration of Isoflurane

All animals from the high anesthesia groups (4% or 5% isoflurane) died due to an overdose of anesthesia (Table 1). Animals from the 4% isoflurane group died in 15.33 ± 2.81 min and animals from the 5% group in 6.33 ± 2.52 min after the onset of anesthesia. There were no rattle or seizures present in these animals. Their breathing rate slowly decreased until it stopped. Post mortem examination of their lungs revealed no macroscopic or microscopic signs of neurogenic pulmonary edema (Tables 1 and 2, Fig. 2). In all cases, subpleural bleeding did not occur, and the mean pulmonary indexes were comparable with control animals (Table 1).

3% isoflurane developed neurogenic pulmonary edema less frequently than did animals from the 2.5% group: the difference in the occurrence of subpleural bleeding was almost 20% (19% in the 3% group vs. 38% in the 2.5% group; Table 1). However, the pulmonary index of both groups was comparable (0.50 vs. 0.51), and the difference between both groups and controls did not reach statistical significance ($p = 0.06$ and $p = 0.07$, respectively; Table 1).

Histological examination of animals anesthetized with a medium concentration of isoflurane showed almost nor-

TABLE 2. THICKNESS OF THE ALVEOLAR WALL (μM) IN THE LUNGS OF ANIMALS WITH SPINAL CORD INJURY ANESTHETIZED WITH 1.5%, 2%, 2.5%, 3%, 4%, OR 5% ISOFLURANE AND CONTROL GROUPS

Group	Thickness of alveolar wall (μm)
1.5%	119.78 ± 32.30*
2%	98.30 ± 39.24*
2.5%	42.69 ± 21.00*
3%	38.67 ± 18.54
4%	38.00 ± 12.06
5%	28.57 ± 11.02
Control	32.89 ± 12.50

Statistical significance is denoted by an asterisk ($p < 0.05$).
Control animals are animals without spinal cord injury, sacrified immediately after the onset of anesthesia.

No Pulmonary Edema in Rats without Spinal Cord Injury

We observed no signs of subpleural bleeding or pulmonary edema in animals without injury anesthetized for 40 min with 1.5%, 2%, 2.5%, or 3% isoflurane. No animal from any of these group showed the presence of subpleural bleeding (in every animal it was graded as Absent) or a significantly elevated p-index (0.45 ± 0.01 in the 1.5% group; 0.44 ± 0.02 in the 2% group; 0.49 ± 0.06 in the 2.5% group and 0.44 ± 0.02 in the 3% group). X-ray images, histological examination of lung tissue and the mean thickness of the alveolar wall were also normal (data not shown). No animal died.

The Grade of Subpleural Bleeding Corresponds to Edema Level

When we measured the thicknesses of the alveolar walls in different grades of subpleural bleeding (Absent, Grade I, Grade II, and Grade III), we found a correlation between subpleural bleeding level and alveolar wall thickness (Table 3, Fig. 2). When subpleural bleeding was absent, the average thickness of the alveolar wall was comparable with that of controls. In Grade I, the thickness was 81% greater than in controls ($p = 0.003$), in Grade II it was 105% greater ($p = 0.0007$) and in Grade III, 271% greater ($p = 0.00002$; Table 3). In addition, we observed an increase in blood cell numbers and the amount of interalveolar edema fluid with increasing grade of subpleural bleeding (Fig. 2).

Major Increase of Blood Pressure and Decrease of Heart Rate Associated with Pulmonary Edema

The baseline values of mean arterial pressure and heart rate differed significantly between animals with and with-

out neurogenic pulmonary edema (Table 4). The spinal cord injury procedure increased systolic and diastolic pressure in all animals, both with and without NPE. After an initial decrease of heart rate and blood pressure at the beginning of the surgical approach (during the muscle incision, the removal of spinous processes and the drilling of a hole into the vertebra), a rapid increase in both systolic and diastolic pressure followed the inflation of the balloon (Fig. 6, Table 4).

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The mean arterial pressure decreased during the skin and muscle incisions, but rose over the baseline values during the spinal cord injury. After SCI, the mean arterial pressure decreased again under the baseline values (Table 4). Rats from the 1.5% isoflurane group exhibiting the presence of severe neurogenic pulmonary edema (p-index = 0.72 ± 0.13; subpleural bleeding absent) had significantly higher values of mean arterial pressure before, during, and after the procedure (Table 4) than rats from the 3% group without neurogenic pulmonary edema (p-index = 0.41 ± 0.05; subpleural bleeding grade II or III). The difference in mean arterial pressure between the 1.5% and 3% groups ranged from 5% (during the muscle incision) to 30% (during the 2-min period after balloon inflation). The maximal values of mean arterial pressure were observed after the inflation of the balloon. During the entire surgical procedure, the differences of the blood pressure readings from baseline values were greater in animals from the 1.5% group, indicating their greater sensitivity to all the procedures. Thus, higher values of mean arterial pressure predispose an animal to develop neurogenic pulmonary edema. Heart rate was higher in the 1.5% than in the 3% group unless the balloon was inflated in the epidural space. The major blood pressure rise in the 1.5% group was accompanied by a considerable fall in heart rate, but this was not observed in the 3% group (Table 4).

During blood pressure and heart rate monitoring, one animal from the 1.5% group died of neurogenic pul-

TABLE 3. THICKNESS OF THE ALVEOLAR WALL (μM) IN THE ABSENCE OR PRESENCE OF SUBPLEURAL BLEEDING, EVALUATED AS GRADE I–III IN ANIMALS WITH BALLOON COMPRESSION LESION

Hemorrhage	Thickness of alveolar wall (μm)
Absent	33.24 ± 12.53
Grade I	59.66 ± 21.70*
Grade II	67.34 ± 21.60*
Grade III	122.06 ± 30.87*
Control	32.89 ± 12.50

Statistical significance is denoted by an asterisk ($p < 0.05$).
Control animals are animals without spinal cord injury, sacrified immediately after the onset of anesthesia.

TABLE 4. BASELINE MEAN ARTERIAL PRESSURE AND HEART RATE VALUES AS WELL AS THE
VALUES FOUND AFTER PARTICULAR SURGICAL PROCEDURES IN THE 1.5% AND
3% GROUPS DURING THE PERFORMANCE OF A BALLOON COMPRESSION LESION

	3% group	1.5% group	1.5% group—pentolinium
Mean arterial pressure (mm Hg)			
Baseline values	78 ± 9	93 ± 13[†]	97 ± 3[†]
Skin incision	73 ± 8*	85 ± 11[†]	77 ± 9*
	(−6%)	(−9%)	(−21%)
Muscle incision	55 ± 3*	58 ± 3*[†]	66 ± 11*[†]
	(−29%)	(−38%)	(−32%)
Pentolinium injection	—	—	55 ± 3*
			(−43%)
Balloon inflation, maximum	127 ± 20*	155 ± 21*[†]	75 ± 10*[†]
	(+63%)	(+67%)	(−3%)
Balloon inflation, 2 min	97 ± 15*	126 ± 21*[†]	67 ± 8*[†]
	(+24%)	(+35%)	(−31%)
Recovery	60 ± 6*	77 ± 18*[†]	55 ± 4*
	(−23%)	(−17%)	(−43%)
Heart rate (bpm)			
Baseline values	380 ± 27	433 ± 39[†]	402 ± 26
Skin incision	383 ± 24	430 ± 31[†]	438 ± 23*[†]
	(+1%)	(−1%)	(+9%)
Muscle incision	355 ± 19*	396 ± 30*[†]	425 ± 34[†]
	(7%)	(9%)	(6%)
Pentolinium injection	—	—	313 ± 40*
			(−22%)
Balloon inflation, maximum	359 ± 32	283 ± 73*	386 ± 48
	(−6%)	(−35%)	(−4%)
Balloon inflation, 2 min	378 ± 16	327 ± 48[†]	370 ± 47
	(−1%)	(−25%)	(−8%)
Recovery	341 ± 30*	357 ± 38*	317 ± 47*[†]
	(−10%)	(−18%)	(−21%)

*Statistically significant (paired Student's t-test, $p < 0.05$) in-group differences in comparison to baseline values are marked with an asterisk.

[†]Significant (non-paired Student's t-test, $p < 0.05$) differences from the 3% group are marked with a dagger.

Relative changes from baseline values are shown in parentheses.

monary edema. Although its baseline mean arterial pressure (88 mm Hg) was essentially average for our study (the range in the 1.5% group was 67–105 mm Hg), its heart rate (472 bpm) was the highest observed in our study (the range of the other animals in the 1.5% group was 377–463 bpm). During the entire procedure, the animal's blood pressure and heart rate were within the range of the other animals: even the maximum value of mean arterial pressure observed while the balloon was inflated in the spinal channel (167 mm Hg) was not the highest in the 1.5% group (two animals reached 170 mm Hg). The animal's heart rate and blood pressure started to decrease towards the typical values seen in the 1.5% group approximately 4 min after the inflation of the balloon. The animal died during the second minute after the deflation of the balloon.

Ganglionic Blockade Prevents Blood Pressure Rise and Pulmonary Edema Development

The acute inhibition of the sympathetic nervous system abolished the blood pressure rise induced by the balloon compression procedure in rats from the 1.5% isoflurane group. The heart rate response of these animals were comparable to the animals from the 3% group (Fig. 6, Table 4). The p-index (0.42 ± 0.01) indicted the absence of pulmonary edema development, and no subpleural bleeding was observed in animals from the 1.5% group following ganglion blockade. Thus, the inhibition of the sympathetic system, induced by an injection of pentolinium prior to the inflation of the balloon, prevented the development of neurogenic pulmonary edema.
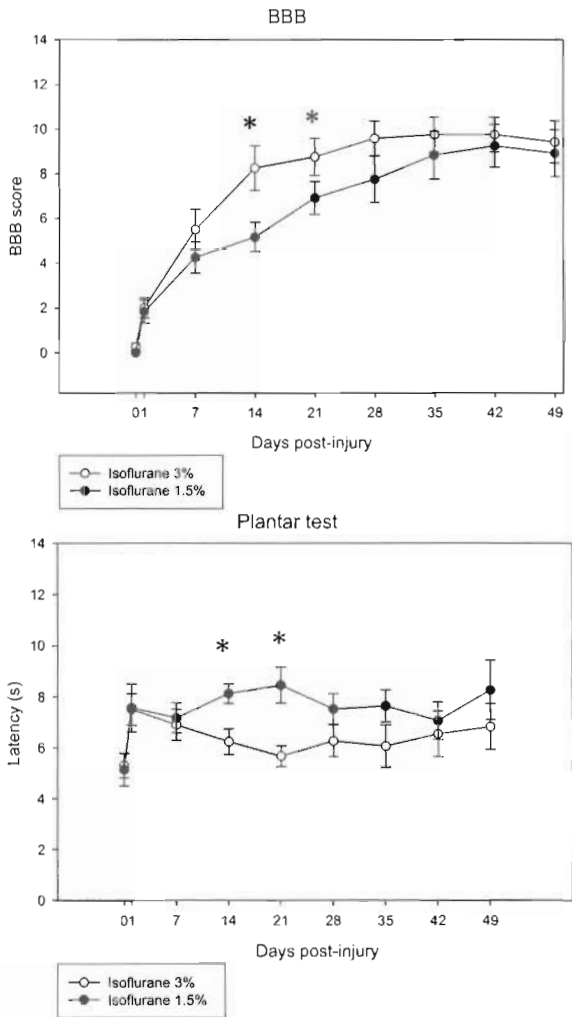
FIG. 4. Behavioral testing of rats with spinal cord injury anesthetized with 1.5% or 3% isoflurane. (A) Basso, Beattie, and Bresnahan (BBB) scores are significantly higher in rats anesthetized with 3% (without neurogenic pulmonary edema) than in rats anesthetized with 1.5% isoflurane. (B) The sensitivity of the hindlimbs is expressed by the latency of hindlimb withdrawal, which is significantly shorter in rats anesthetized with 3% isoflurane.

Behavioral Outcome, Morphometric Analysis, and Magnetic Resonance Imaging

All 24 animals (12 from the 1.5% group and 12 from the 3% group) developed complete paraplegia after the injury procedure, corresponding to a BBB score of 0–1. Throughout the entire recovery period, animals from the 3% group recovered locomotor functions, as shown by their BBB scores, faster than did the animals from the 1.5% group (Fig. 4). This difference reached statistical

significance two and three weeks after the injury ($p = 0.04$ at both time points; Fig. 4). It can also be noted that, for example, the same BBB score achieved by animals from the 3% group 14 days post-injury was reached by animals from the 1.5% group on the 31st day post-injury.

The recovery of sensory functions, estimated by the plantar test, had a similar course (Fig. 4). After the second week post-injury, the sensory functions of animals from the 3% group recovered more rapidly than did the sensory functions of animals from the 1.5% group. In the
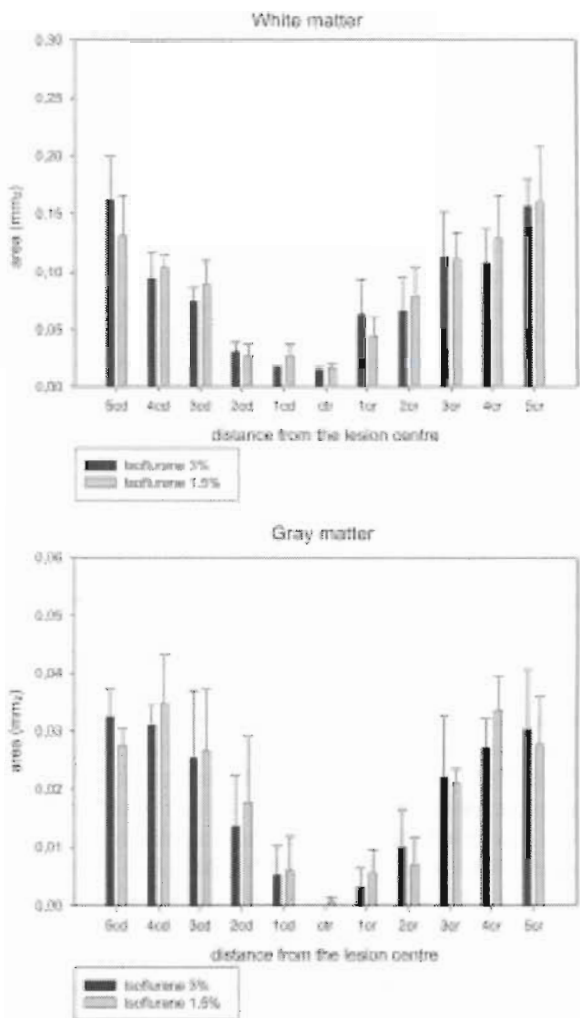

FIG. 5. Area (mm^2) of the preserved gray and white matter in the lesion site of animals with spinal cord injury anesthetized with 1.5% or 3% isoflurane. Note that there are no significant differences in the areas of preserved white or gray matter between the 1.5% and 3% groups.
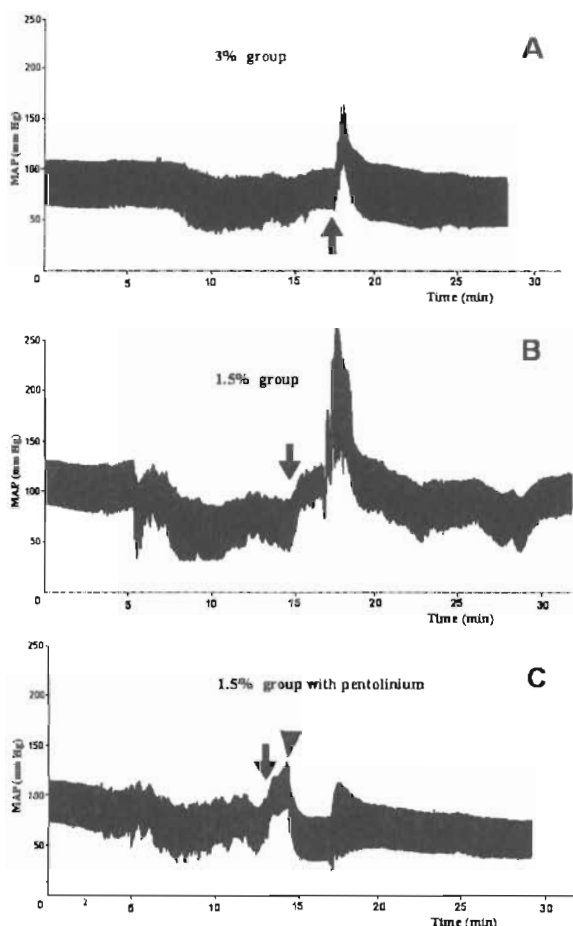
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FIG. 6. The time course of blood pressure during the entire surgical procedure, balloon compression, and recovery period in animals from the 3% group (**A**), the 1.5% group (**B**), and the 1.5% group with ganglionic blockade (**C**). Arrow, inflation of the balloon; arrowhead, pentolinium injection.

2nd and 3rd weeks post-injury, the differences reached statistical significance ($p = 0.04$ and $p = 0.02$, respectively). Thus, animals from the 3% isoflurane group recovered more rapidly than did animals from the 1.5% isoflurane group. Interestingly, the difference in recovery course was accentuated mainly during the second and third weeks after injury, most likely due to the more impaired general health status of rats anesthetized with 1.5% isoflurane.

Morphometric analysis of the volume of spared white and gray matter in the lesion site revealed no significant differences between the 1.5% and 3% groups (Fig. 5). The lesions in both groups showed a hyperintense signal on T2W images, indicating the lesion site and pseudo-

cystic cavities (Fig. 1). *In vivo* assessment of the lesioned tissue revealed no major differences between the two groups (data not shown). Thus, in this case the functional effect of the injury did not correspond to its morphological effect.

DISCUSSION

Our results showed that lower concentrations of isoflurane anesthesia promote the development of neurogenic pulmonary edema in spinal cord injured rats. In addition, rats with neurogenic pulmonary edema had a worse neurological outcome after the injury than did the rats without edema, mainly during the second and third week after injury. However, a morphological analysis of the volume of spared white and gray matter revealed no differences between groups. For experiments involving spinal cord injury, a concentration of 2.5–3% isoflurane in air (flow 300 mL/min) would be optimal. The observed differences between experimental groups indicate the necessity to keep the concentration of isoflurane constant in all animals of all experimental groups throughout a study. The presence or absence of subpleural bleeding according to our criteria (Fig. 2) might be useful for evaluating the presence/absence of neurogenic pulmonary edema.

Neurogenic pulmonary edema has been characterized as interstitial and intraalveolar edema together with intraalveolar hemorrhage, developed as a result of severe central nervous system injury (Fontes et al., 2003). These conditions include spinal cord injury, subarachnoid hemorrhage, primary spinal cord hemorrhage, brain trauma, intracerebral bleeding, severe epileptic grand mal seizure, intracranial tumor, or subdural hematoma (Fontes et al., 2003; Dragosavac et al., 1997).

There are several theories regarding the pathogenesis of neurogenic pulmonary edema (Dragosavac et al., 1997; Fontes et al., 2003; Leal Filho et al., 2005a,b). The most likely explanation is the severe systemic sympathetic discharge, called the "catecholamine storm" (Fontes et al., 2003; Taoka and Okajima, 1998; Urdaneta et al., 2003). This theory proposes the activation of the sympathetic centers in the medulla oblongata, leading to generalized vasoconstriction, an increase in systemic pressure and the augmentation of central blood volume. A rapid increase in blood volume in the pulmonary vascular bed leads to an increase in pulmonary capillary pressure and an imbalance in the Starling forces. Finally, the extravasation of intravascular fluid and microruptures of the capillary wall cause pulmonary edema and intraalveolar bleeding (Fontes et al., 2003; Leal Filho et al.,

2005a,b; Taoka and Okajima, 1998; Urdaneta et al., 2003). The greater increase in blood pressure and decrease in heart rate as a result of spinal cord injury observed in the animals anesthetized with lower concentrations of isoflurane, accompanied by the development of neurogenic pulmonary edema, support this assumption. To demonstrate the importance of the sympathetic nervous system, we inhibited its function by acute ganglionic blockade (pentolinium). Our data clearly demonstrate that neurogenic pulmonary edema does not develop in the absence of sympathetic vasoconstriction.

The role of a sudden increase of intracranial pressure in the development of neurogenic pulmonary edema should also not be underestimated. In the majority of clinical situations, in which neurogenic pulmonary edema develops, a rapid increase of intracranial pressure is the dominant pathophysiological mechanism (Fontes et al., 2003; Urdaneta et al., 2003). One of the locations most sensitive to the rapid elevation of intracranial pressure is the bottom of the fourth ventricle, where the centers of respiratory and vasomotor control are located. Thus, several authors have speculated that increased intracranial pressure plays a similar role in the altered function of these centers (Fontes et al., 2003; Taoka and Okajima, 1998; Urdaneta et al., 2003; Walder et al., 2002). Some clinical papers have provided support for such theories (Macmillan et al., 2002; Ochiai et al., 2001).

For spinal cord injury, we used an epidural balloon compression lesion model in which the lesion is caused by the rapid inflation of a balloon inside the spinal column (Fig. 1), without destabilization of a spine by the laminectomy, as in other models of spinal cord injury. This procedure must lead to a concomitant increase in intracranial pressure above the lesion site. It can thus activate the neurons in the centers on the bottom of the fourth ventricle. Our previous studies demonstrated the reproducibility of the epidural balloon compression model (Syková and Jendelová, 2005; Syková et al., 2005, 2006a; Urdzíková et al., 2006;), so we believe that the amount of pressure increase elicited in the current series of experiments was comparable in all the tested groups.

Experimentally, neurogenic pulmonary edema can be developed by the injection of neuropeptide Y, veratrine, or fibrin (fibrinogen + thrombin) into the cisterna magna (Hirabayashi et al., 1996; Ishikawa et al., 1988; Lane et al., 1998; Maron, 1985). Similarly, an injection of excitotoxic glutamate into the fourth ventricle leads to the development of neurogenic pulmonary edema in experimental animals (Kondo et al., 2004). These experiments show the crucial role of medulla oblongata nuclei in the development of neurogenic pulmonary edema.

It has been shown that halogenated inhalation anesthetics, such as halothane or isoflurane, decrease alveolar epithelial liquid clearance and thus decrease the threshold for the development of pulmonary edema in rats (Rezaiguia-Delclaux et al., 1998; Laffon et al., 2002). In addition, isoflurane inhibits mitochondrial oxidation, leading to a decrease in the production of ATP in type II alveolar cells and thus stimulates the production of lactate in these cells. It also decreases the synthesis of phosphatidylcholine and induces the apoptosis of type II alveolar cells, so the surfactant is being damaged (Mollieux et al., 1999). Some authors have thus expressed their concern over the usage of halogenated inhalation anesthetics (Wiener-Kronisch and Gropper, 1998). This negative side effect is not observed with the use of intravenously injected anesthetics such as pentobarbital or ketamine-xylazine (Mollieux et al., 1999). The development of neurogenic pulmonary edema in our experiments can thus be caused by a combination of three factors: (i) the negative influence of isoflurane on type II alveolar cells (ii) increased intracranial pressure caused by the rapid inflation of the balloon and additional impairment of nuclei in the medulla oblongata, and (iii) most importantly, a low degree of anesthesia unable to fully suppress the stress of the animal and thus decreasing the threshold for the activation of the sympathetic system and the onset of a catecholamine storm. This latter possibility was supported by our finding that ganglionic blockade prevented the development of neurogenic pulmonary edema.

The worse neurological outcome of animals with neurogenic pulmonary edema (Fig. 4) is probably related to the generally worse health of these animals. It has been previously shown that patients with spinal cord injury who developed neurogenic pulmonary edema had a much worse prognosis that those who did not develop it, although the pulmonary edema is resolved either spontaneously or by using one of several treatment-supportive strategies within days (Fontes et al., 2003; Macleod, 2002). From spinal cord injury studies, it is known that the first hours after the injury are the most critical for the patient (Bracken et al., 1992; Syková et al., 2006b). When the initial stage after the injury is complicated by neurogenic pulmonary edema, it might thus slow down the recovery of the neural pathways, as reflected in our experiment by the BBB and plantar test scores.

We conclude that lower concentrations of isoflurane promote the development of neurogenic pulmonary edema in spinal cord injured rats. For experiments involving balloon-induced spinal cord injury, a concentration of 2.5–3% isoflurane in air (flow 300 mL/min) would be optimal. In addition, it is necessary to keep the concentration of isoflurane constant in all animals of a study.

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A new model of severe neurogenic pulmonary edema in spinal cord injured rat

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Abstract

We describe a new model of neurogenic pulmonary edema in spinal cord injured Wistar male rats. The pulmonary edema was elicited by an epidural thoracic balloon compression spinal cord lesion, performed under a low concentration of isoflurane (1.5 or 2%) in air. Anesthesia with 1.5% isoflurane promoted very severe interstitial and intraalveolar neurogenic pulmonary edema with a significantly increased thickness of the alveolar walls and massive pulmonary hemorrhage. In this group, 33% of animals died. Anesthesia with 2% isoflurane promoted severe interstitial and intraalveolar neurogenic pulmonary edema with less thickening of the alveolar walls and pulmonary hemorrhage. For evoking severe neurogenic pulmonary edema in spinal cord injured rats, 2% isoflurane anesthesia would be more suitable. However, if very severe neurogenic pulmonary edema needs to be evoked, spinal cord injury under 1.5% isoflurane anesthesia could be used, but one-third of the animals will be lost.
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Keywords: Isoflurane; Neurogenic pulmonary edema; Spinal cord injury; Rat; Model

Neurogenic pulmonary edema is an acute, life threatening complication following spinal cord or brain injury. It has been characterized as marked pulmonary vascular congestion, extravasation of protein-rich edema fluid and intraalveolar hemorrhage [12,13,15,16]. Epidemiological data of neurogenic pulmonary edema are scarce; its morbidity in patients with severe central nervous system injury has been reported to be between 40 and 50% and its mortality around 9% [1,6,7].

Several models of neurogenic pulmonary edema have been proposed. In rats, the injection of fibrin (fibrinogen + thrombin) into the cisterna magna has been reported to induce pulmonary edema [11]. In dogs, the injection of veratrine [14,17], and in sheep, the injection of aconitine [19], both into the cisterna magna, are also able to induce neurogenic pulmonary edema. However, the development of neurogenic pulmonary edema in these types of models has been considered to result from a cholinergic mediated increase in vascular permeability [2] rather than severe sympathetic discharge, the most suspected cause of neurogenic pulmonary edema development in human patients [7]. Another model of pulmonary edema in dog uses an intravenous injection of oleic acid [5].

Experimentally, neurogenic pulmonary edema should be induced by spinal cord injury. Leal Filho et al. [16] were the first who developed a model of neurogenic pulmonary edema in which the spinal cord is injured, thus mimicking the clinical situation. However, their model achieved a pulmonary index (the relative pulmonary weight) of only 0.639, presenting as a moderate neurogenic pulmonary edema, which might be insufficient if one wishes to test the treatment modalities of severe neurogenic pulmonary edema.

In preliminary experiments, we observed that lower concentrations of isoflurane anesthesia promote the development of neurogenic pulmonary edema in rats with balloon-induced spinal cord injury. The aim of the current study was to evaluate whether such an experimental design can be used as a model of severe neurogenic pulmonary edema, i.e. whether it is possible to achieve a pulmonary index above 0.7 in rats in which pulmonary edema develops as the result of spinal cord injury.

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We used 51 male Wistar rats (Velaz, Prague, Czech Republic) with body weights between 300 and 330 g. This study was performed in accordance with the European Communities Council Directive of 24th November 1986 (86/609/EEC) regarding the use of animals in research and was approved by the Ethics Committee of the Institute of Experimental Medicine ASCR, Prague, Czech Republic. Animals were anesthetized by 1.5 or 2% isoflurane in room air (flow 300 ml/min), and an epidural spinal cord balloon compression lesion at the Th10 level, using a 2F French Fogarthy catheter filled with 15 μl of distilled water for 5 min [26], was performed. To verify the injury procedure, the balloon was inflated before and immediately after the injury procedure to confirm inflation of the balloon in the spinal canal. To verify whether the pulmonary edema was of neurogenic origin, we performed the same procedure under 1.5 or 2% isoflurane anesthesia without the inflation of the balloon. Controls were healthy animals that did not undergo the injury procedure, sacrificed immediately after the induction of anesthesia.

Animals were sacrificed 10 min after the removal of the catheter. The lungs were immediately removed and weighed. The level of pulmonary subpleural bleeding was evaluated macroscopically as "Absent", "Grade I", "Grade II" or "Grade III", as described previously [20]. Each lung was evaluated separately. To estimate the liquid gain of the lungs, both lungs were weighed and the pulmonary index (lung weight/body weight × 100) calculated. The pulmonary index has been previously considered as a very sensitive indicator of the level of pulmonary edema [15,16,18,19]. The lungs were immediately fixed in 4% paraformaldehyde in phosphate buffer (pH 7.4) for 2 days, embedded in paraffin and stained with hematoxylin and eosin. Sections (5 μm) were cut, and the thickness of the alveolar walls was measured using Neurolucida software (MicroBrightField, Inc., USA), as described previously [20].

To estimate the time of onset of neurogenic pulmonary edema, we performed the whole procedure under conditions enabling visual inspection of the lung surface in three animals from the 1.5% group. After the skin incision, the superficial thoracic muscles were cut in anatomical layers and shifted aside. Then, all intercostal muscles and ribs were removed in segments T7-9 and the translucent pleura thus cleaned. Special care was taken not to cause a pneumothorax by disruption of the pleura. Subsequently, a balloon compression lesion was performed as described above. The time of the beginning of lung darkening and the time when hemorrhages became apparent were noted.

To estimate the level of development of neurogenic pulmonary edema in vivo, we used X-ray imaging with the Image Station In-Vivo FX System (Eastman Kodak Company, USA). Animals were anesthetized with 1.5 or 2% isoflurane and a balloon compression lesion was made. Before arousal, pentobarbital (30 mg/kg) was injected, the animals were put into the Image Station and routine X-ray images were taken.

Values of the group p-index and the diameter of the alveolar wall are reported as mean ± S.E.M. Intergroup differences were analysed using a non-paired Student's t-test. Statistically significant differences are marked by an asterisk ($p < 0.05$).

The spinal cord lesioning procedure was performed in all animals without any unexpected complications. The inflation of the balloon was accompanied by skeletal muscle contractions in all cases, which was considered as a normal reaction to injury and was in accordance with our previous results [22,23,25]. Respiratory arrest, accompanying spinal cord compression, followed the onset of the injury procedure in 30% of cases (9 of 30 operated animals), independently of the concentration of isoflurane anesthesia used. The duration of respiratory arrest ranged from 5 to 40 s, with a mean duration of 21.56 ± 9.11 s. Although all the animals were subjected to an autopsy, no hemorrhage in any other examined organ (brain, heart, thymus, liver, intestine, kidney, spleen and urinary bladder) was found.

All animals anesthetized with 1.5 or 2% isoflurane developed neurogenic pulmonary edema and marked pulmonary subpleural bleeding. In 77% of lungs, Grade III subpleural bleeding was present. In the remaining lungs, either Grade II (17%) or Grade I (6%) subleural bleeding occurred (Table 1, Fig. 1). In these animals, the pulmonary index differed significantly from controls: in the 2% isoflurane group, it was 64% higher in comparison with animals from the control group, while in the 1.5% group it was even more elevated—89% higher.

Six of 18 animals (33%) in the 1.5% group died within 8.45 ± 2.82 min (range from 4 to 12 min) after the beginning of balloon inflation (Table 1). A few minutes before death, their breathing frequency started to increase slowly, and they began to develop a so-called "death rattle". Subsequently, their ventilation stopped and after several seconds, gaseous blood came out of their noses, followed by the cessation of their heart beat. The p-index values of non-surviving animals from the 1.5% group (1.07 ± 0.12) were significantly higher than the p-index values from surviving animals from the 1.5% group (0.77 ± 0.14), both significantly higher than the p-index values of control animals. All lungs of these animals exhibited Grade III subpleural bleeding. In contrast, no animal from the 2% group in which the balloon compression procedure was performed, died (Table 1).

Microscopic examination of the lungs showed that lower concentrations of isoflurane caused edema of the alveolar wall, perforation of thin capillary walls, massive bleeding and the leakage of intravascular fluid into the alveoli. The combination of interstitial and intraalveolar leakage of transsudate with intraparenchymal hemorrhage, consequent to spinal cord injury, was considered as the picture of neurogenic pulmonary edema (Fig. 1). In the 1.5% group, the thickness of the alveolar wall was 264% larger in comparison with controls, in the 2% group, 199% larger than in controls (Table 2). In addition, in vivo X-ray imaging showed diffuse hyperintensive infiltrates in both lungs, mainly around the hilus regions (Fig. 1). Thus, lower concentrations of isoflurane promote the development of massive neurogenic pulmonary edema in spinal cord injured rats.

Neurogenic pulmonary edema developed rapidly in the 1.5% isoflurane group. The first darkening of the lung surface appeared in 6.67 ± 0.47 min, while apparent hemorrghages first appeared in 8.00 ± 0.82 min, both after the inflation of the balloon.

Animals anesthetized with 1.5 or 2% isoflurane but without the inflation of the balloon developed no signs of neurogenic pulmonary edema. Their mean pulmonary index was comparable to controls, and they exhibited no signs of subpleural bleeding.

Table 1
The impairment of lung function after spinal cord injury in animals anesthetized with 1.5 or 2% isoflurane (with/without inflation of the balloon) and control groups

Isoflurane	N	Absent (% of 2N)	Grade I (% of 2N)	Grade II (% of 2N)	Grade III (% of 2N)	p-index	Died (% of N)
1.5%	18	–	–	1 (3%)	35 (97%)	0.85 ± 0.19*	6 (33%)
1.5% no SCI	3	6 (100%)	–	–	–	0.45 ± 0.01	–
2%	12	–	3 (13%)	7 (29%)	14 (58%)	0.74 ± 0.11*	–
2% no SCI	3	6 (100%)	–	–	–	0.44 ± 0.02	–
Control	12	24 (100%)	–	–	–	0.45 ± 0.02	–

The absence or presence of subpleural bleeding (evaluated as Grade I–III) in different groups (the total number of lungs in each group – the right and left lung were considered separately – and the percentage of all lungs in the corresponding group). A significant elevation of the p-index (mean values ± S.E.M.) in comparison with controls is indicated by an asterisk (*$p < 0.05$). The occurrence of death is shown as the total number of deaths in each group and as the percentage of all animals in the group. Control animals are animals without spinal cord injury, sacrificed immediately after the onset of anesthesia. N—number of rats, 2N—number of lungs.
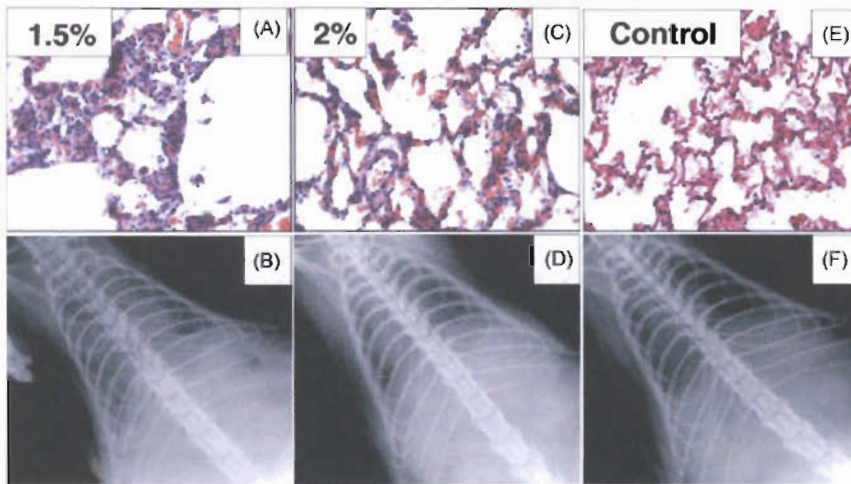

Fig. 1. Neurogenic pulmonary edema in groups anesthetized with 1.5 or 2%, isoflurane. (A) Histology showing a massive neurogenic pulmonary edema with a thickening of the alveolar walls, interstitial edema and massive bleeding in a rat anesthetized with 1.5% isoflurane. (B) X-ray image showing a diffuse shadow in both lungs, mainly around the hilus regions, indicative of neurogenic pulmonary edema in a rat anesthetized with 1.5% isoflurane. (C) Histology showing a developed neurogenic pulmonary edema with a thickening of the alveolar walls and occassional bleeding areas in a rat anesthetized with 2% isoflurane. (D) X-ray image showing a diffuse shadow in both lungs, mainly around the hilus regions, indicative of neurogenic pulmonary edema in a rat anesthetized with 2% isoflurane. (E) Histology of the lungs of a control animal. (F) X-ray image of normal lungs in a control animal.

The thickness of their alveolar walls was comparable with that of controls. No animal from these groups died.

We have developed a new model of neurogenic pulmonary edema in spinal cord injured rats. Although several models of neurogenic pulmonary edema have been proposed [11,14,16,17], a model of severe neurogenic pulmonary edema, in which its development is elicited by spinal cord injury,

Table 2
The thickness of the alveolar wall (μm) in the lungs of animals with SCI anesthetized with 1.5 or 2% isoflurane (with/without inflation of the balloon) and control groups

Group	Diameter of alveolar wall (μm)
1.5%	119.78 ± 32.30*
1.5% no SCI	33.42 ± 11.34
2%	98.30 ± 39.24*
2% no SCI	34.12 ± 13.16
Control	32.89 ± 12.50

Statistical significance in comparison with controls is denoted by an asterisk (*$p < 0.05$). Control animals are animals without spinal cord injury, sacrificed immediately after the onset of anesthesia.

has not yet been published. Our model differs in the development of significantly more severe neurogenic pulmonary edema (pulmonary index above 0.7), which we elicited by balloon compression spinal cord injury under low levels of isoflurane anesthesia. The advantages of this model include the severity of the experimental neurogenic pulmonary edema and the better correlation with the clinical situation than the injection of an exogenic toxic substance next to the bottom of the fourth ventricle, which might also exhibit unexpected side-effects. The "neurogenic" origin of the pulmonary edema was also documented.

In the 1.5% isoflurane anesthesia model, severe edema develops, as the mean p-index of 0.85 documents. The fact that full edema develops in 10 min after experimental spinal cord injury supports the possibility to use this model as a model of severe acute-life threatening edema, often developing in patients with spinal cord injury [7]. However, the disadvantage of this model is the 33% mortality seen in the animals used. On the other hand, to decrease the mortality rate might be one of the experimental treatment goals.

When the 2% isoflurane model of neurogenic pulmonary edema is used, no animal dies and the severity of pulmonary

ma is still high (a mean pulmonary index of 0.74). Thus, this del might be more appropriate for larger studies where the mortality of animals from the 1.5% group would not be imal.

Neurogenic pulmonary edema has been characterized as retitial and intraalveolar edema together with intraalveolar morrhage, developing as a result of severe central nervous em injury [7]. There are several theories about the patho- esis of neurogenic pulmonary edema [6,7,15,16]. The most y explanation is a severe systemic sympathetic discharge, ed the "catecholamine storm" [7,24]. This theory proposes activation of the sympathetic centers in the medulla oblon- , leading to generalized vasoconstriction, an increase in emic pressure and the augmentation of central blood vol- . A rapid increase in blood volume in the pulmonary vascular leads to an increase in pulmonary capillary pressure and an lance in the Starling forces. Finally, the extravasation of ravascular fluid and microruptures of the capillary wall cause monary edema and intraalveolar bleeding [7,15,16,24]. The ogenic role of severe sympathetic discharge in the develop- of neurogenic pulmonary edema in balloon-induced spinal injury has been shown in our previous study, in which pressure and heart rate were monitored. The animals with al cord injury and anesthetized with 1.5% isoflurane exhib- greater reactivity to all parts of the spinal cord intervention; example, the peak mean arterial pressure after the infla- of the balloon was 67% higher than baseline values. In tion, sympathetic blockade with pentolinium prevented the lopment of neurogenic pulmonary edema in such animals. previous experiments thus clearly demonstrated that neu- genic pulmonary edema does not develop in the absence of pathetic vasoconstriction [20].

One of the major characteristics of neurogenic pulmonary ma is the rapidity of its development. Leal Filho et al. [15,16] erved the first microscopic signs of edema within 2 min after initiation of spinal cord compression. In extreme cases, the lopment of neurogenic pulmonary edema has been observed in seconds after a neurological injury in casualties from the nam War [21]. In our study, we observed that these changes me morphologically apparent on the lung surface six min- after lesioning. In addition, lung hemorrhages follow edema lopment in another 2 min. Both results correspond to data ined from clinical studies [7,24] and indicate the potential ignity of neurogenic pulmonary edema—the final edema re can develop within minutes after central nervous sys- injury and thus it can rapidly change the clinical status of a ent with an already worsened general health status.

To date, many models of neurogenic pulmonary edema have used in experimental studies. In these models, edema is ced either by central nervous system injury [15,16] or the inistration of an exogenic substance into the cerebrospinal or directly into the nervous tissue [8,9,27]. As extreme ples, pulmonary edema can be caused by the intravenous inistration of epinephrine [3,4] or bilateral cervical vago- [10]. Although the sympathetic system is almost surely lved in the development of neurogenic pulmonary edema although the administration of an exogenic substance leads

to pulmonary edema, we propose that in future experiments edema should be induced only by central nervous system injury.

The use of 1.5–2% isoflurane anesthesia promotes the development of neurogenic pulmonary edema in rats with a compressed thoracic spinal cord. When 1.5% isoflurane is used, a very severe pulmonary edema develops, and one-third of the animals die. In contrast, no animal dies, and pulmonary edema is still severe, when the rats are anesthetized with 2% isoflurane during balloon compression lesioning.

Acknowledgements

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RAPID BUT NOT SLOW SPINAL CORD COMPRESSION ELICITS NEUROGENIC PULMONARY EDEMA IN THE RAT

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Short title: Spinal compression and neurogenic pulmonary edema

Summary

The development of neurogenic pulmonary edema (NPE) can be elicited by an immediate epidural balloon compression of the thoracic spinal cord. To evaluate whether a slower balloon inflation could prevent NPE development, we examined the extent of NPE in animals lesioned with a rapid ($5\ \mu l$ - $5\ \mu l$ - $5\ \mu l$) or slow rate ($3\ \mu l$ - $2\ \mu l$ - $2\ \mu l$ - $2\ \mu l$ - $2\ \mu l$ - $2\ \mu l$ - $2\ \mu l$) of balloon inflation. These groups were compared with the NPE model (immediate inflation to $15\ \mu l$) and with healthy controls. Slow balloon inflation prevented NPE development, whereas the pulmonary index and histology revealed a massive pulmonary edema in the group with a rapid rate of balloon inflation. Pulmonary edema was preceded by a considerable decrease in heart rate during the inflation procedure. Moreover, rapid inflation of balloon in spinal channel to either $5\ \mu l$ or $10\ \mu l$ did not cause NPE. Thus, a slow rate of balloon inflation in the thoracic epidural space prevents the development of neurogenic pulmonary edema, most likely due to the better adaptation of the organism to the systemic blood pressure elevation during the longer balloon inflation period. It should be noted that spinal cord transection at the same level did not cause neurogenic pulmonary edema.

Keywords: neurogenic pulmonary edema, rat, spinal cord injury, blood pressure, heart rate

Introduction

Neurogenic pulmonary edema is an acute life-threatening complication following spinal cord or brain injury (Fontes *et al.* 2003). It is characterized by marked pulmonary vascular congestion, extravasation of protein-rich edema fluid and intraalveolar hemorrhage (Kandatsu *et al.* 2005, Kondo *et al.* 2004, Leal Filho *et al.* 2005a, b). Many pathophysiological mechanisms have been implicated in the development of neurogenic pulmonary edema, but the exact cascade leading to its development is still unclear (Leah Filho *et al.* 2005a, b). Both the release of vasoactive substances and a severe transient sympathetic discharge are thought to participate in this process (Taoka and Okajima 1998, Urdaneta and Layon 2003). These processes lead to the constriction of the pulmonary veins, an increase in pulmonary capillary hydrostatic pressure, damage to the alveolar wall and the leakage of fluid into the intraalveolar space (Fontes *et al.* 2003).

Our previous experiments showed that severe neurogenic pulmonary edema could be experimentally induced by immediate epidural balloon compression of the thoracic spinal cord in the rat, anesthetized by 1.5% isoflurane in air (Šedý *et al.*, 2007a, b). The aim of this study was to evaluate the extent of neurogenic pulmonary edema development in the case of a gradual inflation of the balloon in the spinal channel. For this purpose, the final balloon inflation volume of 15 μl was reached by several greater or numerous smaller steps. In addition, the occurence of neurogenic pulmonary edema was also examined in animals with lower volumes of balloon inflation as well as with thoracic spinal cord transection.

Materials and methods

Animals

We used 65 male Wistar rats (Velaz, Prague, Czech Republic) with body weight between 300-330g. This study was performed in accordance with the European Communities Council Directive of 24th of November 1986 (86/609/EEC) regarding the use of animals in research and was approved by the Ethics Committee of the Institute of Experimental Medicine ASCR, Prague, Czech Republic.

Design of the study

After the induction of anesthesia with 5% isoflurane in room air (flow 300 ml/min), animals were maintained in 1.5% isoflurane anesthesia (flow 300 ml/min) *via* a face mask throughout the operation. This concentration of isoflurane was previously shown to promote neurogenic pulmonary edema development in rats with immediate balloon compression of the thoracic spinal cord (Šedý *et al.* 2007a). All animals were heated to 37°C, and their body temperature was measured by a rectal thermometer to standardize the procedure and to exclude the influence of hypo- or hyperthermia (Cambria *et al.* 1997; Urdziková and Vanický 2006). A catheter was inserted into the common carotid artery to monitor blood pressure and heart rate changes. After the insertion of the catheter, an epidural balloon spinal cord compression lesion (Vanický *et al.* 2001) or complete Th8 transection was performed. The animal was left to recover for 10 min. and then was sacrificed. The lungs were immediatelly removed and analyzed for the presence of neurogenic pulmonary edema. Controls were animals without spinal cord injury, sacrificed immediately after the onset of anesthesia.

Balloon compression spinal cord lesion

To induce a spinal cord injury we used the model of an epidural balloon compression lesion (Vanický *et al.* 2001), as described previously (Šedý *et al.* 2007a). Briefly, under aseptic conditions, a 2 cm median skin incision at the Th10-L1 level was made. The dorsal muscles were shifted laterally, and the Th10 and Th11 spinous processes were removed. A hole was drilled into the Th10 lamina with a dental drill. Then, a 2F French Fogarthy catheter (Baxter Healthcare Corporation, Irvine, CA, USA), which was filled with distilled water and connected to a 50-μl Hamilton syringe, was inserted into the dorsal epidural space 10 mm rostrally, to reach the Th8-Th9 spinal level. Using a micromanipulator, the balloon was inflated with a rapid (5 μl - 5 μl - 5 μl) or a slow rate of inflation (3 μl - 2 μl - 2 μl - 2 μl - 2 μl - 2 μl) or in the standard NPE model immediatelly to 15 μl. In all cases, the final volume of the balloon was 15 μl. The time window between each step of the inflation was 30 s. Thus, the entire balloon inflation procedure lasted one min. during rapid inflation and 3 min. during slow inflation. As an additional experiment, we also inflated the balloon immediatelly to either 5 μl or 10 μl. After inflation to the final volume, the balloon was left in place for 5 minutes in all cases. Subsequently, the balloon was deflated and removed.

The immediate inflation of the balloon in the epidural space of the thoracic spinal channel with 15 μl of distilled water under 1.5% isoflurane anesthesia reliably and reproducibly produces severe neurogenic pulmonary edema (Šedý *et al.* 2007a, b); we therefore used this model for comparison with the other groups in this experiment. The inflation of a balloon to 15 μl in the spinal channel produces an incomplete lesion, so that after 7 weeks, the hindlimbs of the animals are able to support body weight and occasionally forelimb-hindlimb coordination is observed. This state corresponds to a BBB locomotor score (Basso *et al.* 1995) of 9-11 at 7 weeks post-injury (Vanický *et al.* 2001; Šedý *et al.* 2007a). To verify the injury procedure, the balloon was inflated before and immediately after the injury procedure outside of the animal to confirm the inflation of the balloon in the spinal channel.

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The design of the study did not allow *in vivo* verification of the lesion by imaging or behavioral testing methods.

Spinal cord transection

A complete Th8 spinal cord transection was performed in animals anesthetized with 1.5% isoflurane in order to evaluate the role of a thoracic spinal cord lesion *per se* in neurogenic pulmonary edema development. Briefly, under aseptic conditions, a 2 cm median skin incision at the Th6-8 level was made. The dorsal muscles were cut and shifted laterally, and the Th7 lamina was removed. After that, a rapid spinal transection was performed by a sharp razor blade.

Evaluation of neurogenic pulmonary edema

The lungs were immediately removed from sacrificed animals and weighed. In all cases, a mild hematoma, maximally 1 mm in diameter, was found in the hilus area due to the manipulation of the pulmonary vessels during lung removal (not taken into further account). The level of pulmonary subpleural bleeding was evaluated macroscopically as "Absent" (no bleeding on the lung surface), "Grade I" (small bleeding areas, occupying not more than 10% of the lung surface), "Grade II" (medium-sized bleeding areas, occupying 11-50% of the lung surface) and "Grade III" (massive bleeding areas, occupying more than 50% of the lung surface), as described previously (Šedý *et al.* 2007a, b). Each lung was evaluated separately. To estimate the liquid accumulation in the lungs, both lungs were weighed, and the relative pulmonary weight was calculated as the pulmonary index (lung weight/body weight x 100), which has been previously considered to be very sensitive to the degree of pulmonary edema (Leal Filho *et al.* 2005a, b, Mesquita *et al.* 2002, Minnear and Connel 1982, Šedý *et al.* 2007a). The lungs were immediately fixed in 4% paraformaldehyde in phosphate buffer (pH

7.4) for 2 days, embedded in paraffin, cut in 5 µm sections and stained with hematoxylin and eosin. The thickness of the alveolar walls was measured using Neurolucida software (MicroBrightField, Inc., USA). From each lung, three sections (from the inferior, middle and superior parts of the lung) were taken, and all alveolar wall thicknesses in one representative field from each section were measured. A representative field was defined as a site in the non-subpleural lung parenchyma, without any large vessel or bronchus, outside of the hilus region.

Measurement of blood pressure and heart rate changes

Mean arterial blood pressure and heart rate were monitored in all animals in the study using a PowerLab system (ADInstruments, Colorado Springs, USA). Under isoflurane anesthesia, a catheter was inserted into the left common carotid artery and exteriorized in the interscapular region, the animal was put into a prone position and a balloon compression lesion or transection was performed. Mean arterial pressure (mm Hg) and heart rate (bpm) were monitored for 5 min before the procedure, throughout the entire procedure and for 10 min after the procedure. After the initial rise in blood pressure following the injury procedure, we observed a break point in the descending curve, which was termed the „turning point".

The values obtained were: (1) the baseline value, i.e. the value before the inflation of the balloon, (2) the maximum value after inflation with a particular volume with respect to the experimental group (3, 5, 7, 9, 10, 11, 13, 15 µl), (3) the average value between the peak after the inflation to 15 µl and the turning point, and (4) the average value between the turning point and deflation of the balloon.

Statistical analysis

The pulmonary index values, mean arterial pressure values and heart rate values are reported as mean ± S.E.M. The statistical significance ($p < 0.05$) between groups was

compared using the non-paired Student´s t-test. The differences within the groups were examined using the paired Student´s t-test ($p < 0.05$).

Results

The spinal cord lesioning procedure was performed in all animals without any unexpected complications. The inflation of the balloon to 15 µl was accompanied by skeletal muscle contractions in all cases, which was considered as a normal reaction to injury and was in accordance with our previous observations (Syková *et al.* 2005, Syková and Jendelová 2006, Urdziková *et al.* 2006). In addition, a blood pressure increase acompanied the balloon inflation in all cases (Šedý *et al.* 2007a).

The immediate inflation of the balloon in the spinal channel to 15 µl caused severe neurogenic pulmonary edema in all cases. The extent of subpleural bleeding, the pulmonary index, the thickness of the alveolar walls as well as the blood pressure and heart rate values (Tables 1, 2 and 3; Fig. 2) corresponded to the values observed previously for this model of severe neurogenic pulmonary edema (Šedý *et al.* 2007b).

Slow balloon inflation prevented neurogenic pulmonary edema development

When the balloon was gradually inflated slowly (3 µl - 2 µl - 2 µl - 2 µl - 2 µl - 2 µl - 2 µl), neurogenic pulmonary edema did not develop. In 64% of cases, subpleural bleeding was absent. In the remaining cases, there was Grade I subpleural bleeding (Table 1). In addition, the pulmonary index and the mean thickness of the alveolar walls did not differ significantly from control values (Tables 1 and 2). The histological appearance of the lung tissue was comparable to that of the controls – there were almost no signs of intraalveolar or interstitial edema or hemorrhage (Fig. 1). Moreover, the mean thickness of the alveolar wall was not significantly increased (Table 2).

Neurogenic pulmonary edema after rapid balloon inflation

Although the final volume of the inflated balloon was the same as in the previous cases, the rapid inflation (5 µl - 5 µl - 5 µl) of the balloon caused a severe neurogenic pulmonary edema, comparable to that seen in the standard neurogenic pulmonary edema model. Although the occurence of Grade III subpleural bleeding was not so frequent as in our neurogenic pulmonary edema model, the extent of the bleeding was always at least Grade II (Table 1). Similarly, the pulmonary index values in the rapid inflation group did not differ significantly from the values obtained in our neurogenic pulmonary edema model. On the other hand, the pulmonary index values of rapid inflation group were significantly increased when compared with controls ($p = 0.0001$). Microscopic examination of the lungs showed that the rapid inflation of the balloon caused edema of the alveolar membrane, perforation of thin capillary walls, massive bleeding and the leakage of intravascular fluid into the alveoli – a typical picture of neurogenic pulmonary edema (Fig. 1). Moreover, the mean thickness of the alveolar walls in the rapidly inflated group was significantly higher when compared to controls (Table 2).

Blood pressure and heart rate changes during different rates of balloon inflation

All steps of the graded balloon inflation procedure were accompanied by a significant elevation of the mean arterial pressure (Table 3). The graded lesion was accompanied by a typical ascendently undulating mean arterial pressure curve (Fig. 2). No significant differences in mean arterial pressure values during and after the inflation of the balloon to the final volume to 15 µl were observed among the slowly and rapidly inflated groups (Table 3), although the differences in the degree of neurogenic pulmonary edema were highly significant (Table 1).

The extent of neurogenic pulmonary edema was more reflected by the changes of heart rate. In the rapidly inflated group (with NPE), there was a pronounced decrease in heart rate

during the whole balloon inflation procedure, whereas in the slowly and moderately inflated groups, a mild transient heart rate decrease was observed (Table 3). Moreover, the heart rate changes after the maximum balloon inflation to 15 µl were more prominent in the rapidly than in the slowly inflated groups (Table 3). Taken together, the more rapid the balloon inflation, the more pronounced the decrease in heart rate during and after the inflation.

Incomplete balloon inflation prevented neurogenic pulmonary edema

The inflation of the balloon to 10 µl was accompanied by mild skeletal muscle contractions, the inflation to 5 µl caused almost none reaction. The immediate inflation of the balloon in the spinal channel to either 5 µl or 10 µl did not cause neurogenic pulmonary edema. No animal from 5 µl or 10 µl group presented with subpleural bleeding (Table 1). Moreover, the mean pulmonary index and alveolar wall thicknesses of these animals were comparable to controls (Table 1 and 2).

Spinal transection did not cause neurogenic pulmonary edema

Transection of the spinal cord at the same spinal level as the balloon compression lesion did not cause neurogenic pulmonary edema. Subpleural bleeding was absent in all animals and the pulmonary index was comparable to that of the controls. Histology of the lung tissue did not reveal any sign of neurogenic pulmonary edema (Fig. 1, Table 2). Spinal cord transection did not cause any blood pressure or heart rate disturbances; in particular, there was no sharp increase in blood pressure as typically seen after balloon inflation (Fig. 2).

Discussion

We have shown that a slow rate of balloon inflation in the thoracic epidural space prevented the development of neurogenic pulmonary edema, as well as indicated by the absence of significant subpleural bleeding as well as an increase in the pulmonary index and mean alveolar wall thickness. Similarly, incomplete (5 or 10 μl) balloon inflation or spinal transection at the same level did not cause neurogenic pulmonary edema. The differences in NPE development were accompanied by significant changes in heart rate; rats with NPE had a considerably decreased heart rate after the inflation of the balloon to the final 15 μl volume compared to rats without edema, in which heart rates were comparable to baseline values (Table 3).

The development of neurogenic pulmonary edema seems to be based on the hyperactivity of the sympathetic system, in terms of a severe sympathetic discharge, also called a catecholamine storm (Dragosavac *et al.* 1997, Fontes *et al.* 2003, Leal Filho *et al.* 2005a, b). When a ganglionic blockade is performed by pentolinium, administered before spinal cord lesioning, neurogenic pulmonary edema does not develop (Šedý *et al.* 2007a). Our experiments indicate that slower inflation of the balloon in the epidural space is able to prevent NPE development, probably due to the easier adjustment of the organism to gradual hemodynamic changes over a longer time period. Poulat and Couture (1998) showed that the intrathecal injection of endothelin-1 causes the activation of the endothelin receptor in sympathetic spinal neurons. Their activation subsequently causes a sympathetic discharge, a massive release of catecholamines, intense pulmonary alpha-adrenergic vasoconstriction, an increase in pulmonary vascular permeability and pulmonary edema. One explanation of our results might be that a slowly graded spinal cord lesion leads to the release of small amounts of vasoactive substances to which the systemic and pulmonary circulation are able to adjust

whereas the release of these substances is so rapid in the NPE model that the circulation is unable to adjust.

Another hypothesis suggests that the inflation of the balloon in the epidural space causes a rapid increase in intracranial pressure, which leads to the stimulation of sympathetic „neurogenic pulmonary edema trigger zones" in the bottom of the fourth ventricle (Baumann *et al.* 2007, Leal Filho *et al.* 2005a, b, Šedý *et al.* 2008, Šedý *et al.* in press). During the slow inflation of the balloon in the epidural space, the intracranial space has a longer time to "adapt" to these changes. Moreover, after each of the subsequent balloon inflation steps, the cerebrospinal fluid might „leak" around the incompletely obturated spinal channel. This probably does not happen when the balloon is inflated rapidly – from our previous studies we know that only 5% of the spinal cord tissue is spared at the site of the lesion while the rest of the spinal channel is obturated by the balloon during the inflation, when the balloon is inflated to 15 µl (Urdziková *et al.* 2006, Syková *et al.* 2005, Syková and Jendelová 2006, Šedý *et al.* 2007a). The lack of neurogenic pulmonary edema in animals with a transected thoracic spinal cord indirectly supports such a hypothesis.

Our results indicate that the final volume of the ballon is also very important in the development of neurogenic pulmonary edema. To induce neurogenic pulmonary edema in animals anesthetized with 1.5% isoflurane, the balloon must be inflated to the final 15 µl, because the inflation to 10 µl or less does not cause it. Importantly, this volume-based impairment corresponds to the neurological deficit caused by Th8 epidural ballon inflation. Vanický *et al.* (2001), who developed the epidural balloon spinal cord compression model, reported that inflation of the balloon to 10 µl caused mild neurological deficit, which recovered to normal values of BBB locomotor score in few weeks. We observed that the inflation of the balloon to 5 µl caused almost no neurological deficit next day and it recovered to normal values of BBB next week after the surgery in all cases (Šedý, Jendelová, Syková,

unpublished observation). On the other hand, during the experiments published in our first paper concerning the role of isoflurane anesthesia on NPE development (Šedý *et al.* 2007a), we also inflated the balloon to 20 µl in both groups, anesthetized either with 1.5% isoflurane or 3% isoflurane, and the results were comparable to these obtained in animals in which a final volume of the balloon was set to 15 µl (Šedý, Urdziková, Jendelová, Syková, unpublished observation). This indicates that in 1.5% isoflurane group (with NPE) the neurogenic pulmonary edema can not be further augmented, even when we inflate the balloon to 20 µl.

Rapid balloon inflation is associated with major sympathetic activation leading to blood redistribution from splanchnic vessels to the pulmonary vascular bed and with a pronounced rise of systemic blood pressure, causing a heart rate decrease due to baroreflex activation (Šedý *et al.* 2007a, c). In our experiments, the systemic blood pressure increase in rats with a slowly inflated balloon (without NPE) probably did not cause pronounced baroreflex activation. Conversely, in rats with a rapidly inflated balloon (causing NPE), the threshold was exceeded and the baroreflex turned on, so that the reduction in the heart rate could limit the capacity of the heart to pump blood from the pulmonary to the systemic circulation.

Conlusions

A slow rate of balloon inflation in the thoracic epidural space prevents the development of neurogenic pulmonary edema, most likely due to the adaptation of the organism to enhanced sympathetic tone and consequent cardiovascular reactions (blood volume redistribution, blood pressure increase, baroreflex activation) during the longer balloon inflation period. Spinal cord transection at the same level does not cause neurogenic pulmonary edema.

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Table 1 The impairment of lung function after spinal cord injury in the NPE model as well as in animals with a gradually compressed spinal cord, incompletely compressed spinal cord or a spinal transection.

Group	N	Absent (% of 2N)	Grade I (% of 2N)	Grade II (% of 2N)	Grade III (% of 2N)	Pulmonary index
NPE model	19	-	-	1 (3%)	37 (97%)	0.86 ± 0.09*
5-5-5	7	-	-	6 (43%)	8 (57%)	0.77 ± 0.08*
3-2-2-2-2-2-2	14	18 (64%)	10 (36%)	-	-	0.49 ± 0.03
Immediate 5	3	6 (100%)	-	-	-	0.46 ± 0.04
Immediate 10	3	6 (100%)	-	-	-	0.46 ± 0.03
Transection	5	10 (100%)	-	-	-	0.43 ± 0.06
control	14	28 (100%)	-	-	-	0.45 ± 0.02

The absence or presence of subpleural bleeding (evaluated as Grade I-III) in different groups is given as the total number of lungs in each group (the right and left lung were considered separately) and the percentage of all lungs examined in the respective group. A significant elevation ($p < 0.05$) of the pulmonary index (mean values $\pm$ S.E.M.) in comparison with controls is indicated by an asterisk. Control animals are animals without spinal cord injury,

sacrificed immediately after the onset of anesthesia. N - number of rats, 2N – number of lungs.

Table 2 The thickness of the alveolar wall (μm) in the lungs of animals with either a balloon compression lesion (rapid or graded) or a spinal cord transection and control groups.

Group	Thickness of alveolar wall (μm)
NPE model	94.18 ± 4.37 * (+161%)
5-5-5	62.61 ± 4.17 * (+71%)
3-2-2-2-2-2-2	38.62 ± 2.12 (+5%)
Immediate 5	37.32 ± 2.94 (+ 2%)
Immediate 10	35.99 ± 1.95 (-2%)
Transection	39.63 ± 3.02 (+8%)
Control	36.71 ± 2.57

Statistical significance from control rats is denoted by an asterisk ($p < 0.05$). Control animals are animals without spinal cord injury, sacrificed immediately after the onset of anesthesia. Relative changes from controls are shown in parentheses.

Table 3 Baseline values of mean arterial pressure as well as the values found after a particular degree of balloon inflation in rats with different rate of induction of balloon compression lesion.

Mean arterial pressure (mm Hg)			
	NPE model **15**	**Rapid** **5-5-5**	**Slow** **3-2-2-2-2-2-2**
Baseline values	85 ± 6	97 ± 4	97 ± 2
3 μl	-	-	108 ± 4* (+11%)
5 μl	-	113 ± 6* (+16%)	130 ± 6* (+34%)
7 μl	-	-	141 ± 6* (+45%)
9 μl	-	-	143± 5* (+47%)
10 μl	-	153 ± 6* (+58%)	-
11 μl	-	-	147 ± 5* (+52%)
13 μl	-	-	148 ± 4* (+53%)
15 μl	154 ± 7* (+81%)	155 ± 3* (+60%)	150 ± 4* (+55%)
15 μl – turning point	144 ± 6*	138 ± 3*	133 ± 3*

	(+69%)	(+42%)	(+37%)
Turning point - **deflation**	86 ± 5 (+1%)	90 ± 4 (-7%)	97 ± 3 (±0%)

Statistically significant (paired Student's t-test, $p < 0.05$) differences from baseline values are marked with an asterisk. Relative changes as a percentage of baseline values are shown in parentheses.

Table 4 Baseline values of heart rate as well as the values found after a particular degree of balloon inflation in three different groups with a graded balloon compression lesion during the induction of the lesion.

Heart rate (bpm)			
	NPE model	5-5-5	3-2-2-2-2-2-2
Baseline values	385 ± 9	380 ± 14	410 ± 5
3 μl	-	-	384 ± 11 (-6%)
5 μl	-	364 ± 16* (-4%)	383 ± 7* (-7%)
7 μl	-	-	394 ± 6 (-4%)
9 μl	-	-	394 ± 8 (-4%)
10 μl	-	322 ± 30* (-15%)	-
11 μl	-	-	394 ± 8 (-4%)
13 μl	-	-	391 ± 8 (-5%)
15 μl	253 ± 30* (-34%)	222 ± 23* (-42%)	389 ± 11$ (-5%)
15 μl – turning point	313 ± 21* (-19%)	282 ± 16* (-26%)	371 ± 10*$ (-10%)
Turning point -	361 ± 11	336 ± 15*	387 ± 8

deflation	(-6%)	(-12%)	(-6%)

Statistically significant (paired Student's t-test, $p < 0.05$) differences from baseline values are marked with an asterisk. Significant differences (un-paired Student's t-test, $p < 0.05$) from the NPE model are marked with $. Relative changes as a percentage of baseline values are shown in parentheses.

Figure legends

Fig. 1 Pulmonary histological changes in animals with graded compression lesions or spinal cord transection. **A.** Occasional bleeding areas without evident pulmonary edema in a rat with a slowly graded balloon compression lesion. **B.** Occasional bleeding areas without evident pulmonary edema in a rat with a moderately graded balloon compression lesion. **C.** Histology showing a massive neurogenic pulmonary edema with a thickening of the alveolar walls, interstitial edema and massive bleeding in a rat with a rapidly graded balloon compression lesion. **D.** Histology of lungs in animals with a spinal cord transection. No signs of pulmonary edema are present. **E.** Histology showing a massive neurogenic pulmonary edema with a thickening of the alveolar walls, interstitial edema and massive bleeding in a rat with neurogenic pulmonary edema. **F.** Histology of the lungs of a control animal. Scale bar in F = 200 μm.

Fig. 2 The time course of blood pressure before, during and after the balloon inflation in the spinal channel to a final colume of 15 μl. Each inflation step is indicated by an arrowhead in A,B and D. Arrows show the balloon deflation in A, B and D. **A.** Slowly graded balloon compression lesion. **B.** Rapidly graded balloon compression lesion. **C.** The time course of blood pressure before, during and after spinal cord transection (arrowhead). **D.** The time course of blood pressure in the model of neurogenic pulmonary edema (immediate balloon inflation).

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Low degree of anesthesia increases the risk of neurogenic pulmonary edema development

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Summary Neurogenic pulmonary edema is an acute life-threatening complication following central nervous system injury. The exact pathogenic mechanism leading to its development is still unclear. We introduce a new hypothesis that high levels of anesthesia might protect the organism against the development of neurogenic pulmonary edema due to a more pronounced inhibition of the hypothalamic, brainstem and spinal vasoactive sympathetic centers. On the basis of a more pronounced neuronal inhibition of the vasoactive centers, a severe sympathetic discharge does not occur and neurogenic pulmonary edema does not develop. In contrast, an insufficient anesthesia level is not able to inhibit the sympathetic nervous system during an injury of the central nervous system and thus neurogenic pulmonary edema develops. During experiments with central nervous system injury, low-anesthesia-induced neurogenic pulmonary edema might negatively influence the overall recovery of the animal. More importantly, during a neurosurgical intervention, insufficient anesthesia might similarly lead to neurogenic pulmonary edema development in operated patients. Our hypothesis indicates the necessity of precisely monitoring of the level anesthesia during experimental manipulations of the central nervous system in animals or neurosurgical interventions in humans.
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Introduction

Neurogenic pulmonary edema is an acute life-threatening complication following central nervous system damage, such as spinal cord injury, subarachnoid hemorrhage, primary spinal cord hemorrhage, brain trauma, intracerebral bleeding, severe epileptic grand mal seizure or subdural haematoma [1,2]. The occurrence of neurogenic pulmonary edema in patients with multiple sclerosis with medullary involvement, nonhemorrhagic strokes, bulbar poliomyelitis, cerebral gas embolism, electroconvulsive therapy, intracranial

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tumour, enterovirus encephalitis or bacterial meningitis have also been reported [1,2]. The epidemiological data on neurogenic pulmonary edema are scarce; its morbidity in patients with severe central nervous system injury has been stated between 40% and 50%, its mortality around 9% [1–3].

Neurogenic pulmonary edema usually appears within minutes to hours after a severe central nervous system insult. It is characterized by the rapid onset of dyspnea, chest pain, hemoptysis, tachypnea, tachycardia, bilateral basal pulmonary crackels, respiratory distress or failure, pulmonary edema with normal jugular venous pressure, the absence of cardiac gallop and occasionally a fever [4]. The chest radiograph shows a bilateral alveolar filling process and a normal-sized heart. In lung tissue sections, marked pulmonary vascular congestion with perivascular edema, extravasation and intraalveolar accumulation of protein-rich edema fluid and intraalveolar hemorrhage can be observed [5–9].

Many pathophysiological mechanisms have been implicated in the development of neurogenic pulmonary edema, but the exact cascade leading to its development is still unclear [5,6]. Both the release of vasoactive substances and a rapid, transient, and severe sympathetic discharge are thought to participate in this process [10,11].

Hypothesis

We hypothesize that the degree of anesthesia is inversely proportional to the level of the development of neurogenic pulmonary edema. The deeper the inhibition of the hypothalamic, brainstem and spinal vasoactive sympathetic centers (trigger zones), the smaller the extent of neurogenic pulmonary edema development because the excitation of the sympathetic nervous system is primarily responsible for the initiation and development of neurogenic pulmonary edema.

Evaluation of the hypothesis

Why is the pulmonary edema "neurogenic"

Pulmonary edema has many different causes, mostly belonging to two groups – cardiogenic and non-cardiogenic. The group of non-cardiogenic pulmonary edemas include neurogenic pulmonary edema, where an association with the central nervous system has been empirically proven and other causes, such as left heart failure or the reaction

to an exogenic toxic substance, have been excluded. The direct connection of the edema to CNS injury presumes that the neuronal damage directly or indirectly involves the pulmonary vascular bed. The most probable mechanisms of such influence are temporary neural commotion or the mechanical or electrophysiological disconnection of the central nervous system vasomotor centers, which leads to destabilization of the pulmonary autonomic nervous system. Also, the overstimulation of vasomotor centers might be involved. This statement is supported by the fact that severe central nervous system injury is always associated with significant changes in autonomic nervous system function. On the basis of our [9] and other's previous experiments [5,6], we hypothesize that the function of specific neurons of the central nervous system must be impaired during brain or spinal cord injury to destabilize the autonomic system balance so that an imbalance of Starling forces in the pulmonary vascular bed occurs and neurogenic pulmonary edema may develop.

Why is neuronal damage involved

Numerous experiments showed that some interventions in the brain or spinal cord are able to stimulate the peripheral sympathetic nervous system and to produce changes in blood pressure (for review see [12]). These data indicate that the central nervous system sympathetic centers (including trigger zones for neurogenic pulmonary edema) might be influenced by such manipulations. It has been observed that experimental brainstem distortion or ischemia might cause changes in sympathetic vasomotor tone and an increase in blood pressure (for review see [12]). In addition, the elevation of blood pressure and the subsequent development of neurogenic pulmonary edema are prevented by the intrathecal administration of lidocaine [13].

Why is the origin of pulmonary edema in the central nervous system vasomotor nuclei

Several experimental studies indicated that the neurons responsible for severe sympathetic discharge, the most probable cause of neurogenic pulmonary edema, are located in the hypothalamic, brainstem and cervical spinal cord nuclei. These centers represent so called neurogenic pulmonary edema trigger zones [4] and their arrangement corresponds to the organotopy hypothesis (for review see [12]). The most impor-

tant vasomotor centers for its development are thought to be the A1 and A5 groups of neurons, the nuclei of the solitary tract, area postrema, medial reticulated nucleus and dorsal motor vagus nucleus in the medulla oblongata. The hypothalamic centers, i.e. paraventricular and dorsomedial nuclei, also seem to be of some importance. It should also be noted that C1 adrenaline-synthesizing neurons, definitely identified as a key blood pressure center (for review see [12]), might also be involved. Interestingly, casualties from the Vietnam war who had concomitant brain injury and cervical spinal cord injury did not develop neurogenic edema, whereas the majority of casualties with brain injury developed edema [14]. In the first group, the trigger zones were probably disconnected from the rest of the body by cervical spinal cord transection.

Experimentally, bilateral lesions of the nuclei in the medulla produce profound pulmonary and systemic hypertension and pulmonary edema [4]. The intracranial pressure, as well as toxic or ischemic injury of inhibitory neurons, leads to excessive sympathetic neuronal activity, the release of vasoactive substances such as epinephrine, norepinephrine, endothelins or neuropeptide Y and thus to a severe sympathetic discharge followed by neurogenic pulmonary edema development [4,15,16]. For example, neuropeptide Y has been found in alveolar macrophages and edema fluid in the case of neurogenic pulmonary edema, but not in hydrostatic edema or controls [15]. Alpha-adrenergic blockade (with phentolamine) and spinal cord transection at the C7 level prevent the formation of neurogenic pulmonary edema, suggesting an important role for sympathetic activation [17]. On the other hand, the inhibition of central nervous system nitric oxide has a protective role in the development of neurogenic pulmonary edema [18].

Why might an intracranial pressure increase be involved

After CNS injury, prominent hemorrhage into different compartments that correspond to the epidural, subdural, subarachnoid, and intramedular (intracerebral) spaces and consequent damage of the blood–brain barrier occurs. In clinical situations such as subdural or subarachnoid hemorrhage, the extravasation of blood into the corresponding compartments is even the major mechanism. Walder et al. [19] showed that the amount, but not the type, of fluid injected intrathecally had a significant impact on hemodynamic

and respiratory parameters. The main disadvantage of any intracranial or intraspinal hemorrhage is the very rapid increase of intracranial pressure inside the non-expandable bony space with rather limited mechanisms to decrease it. This pressure increase leads to the compression of central nervous system tissue, resulting in a brainstem distortion or ischemia and later in cerebral herniation. It has been shown in experiments in sheep, that elevation of intracranial pressure increases pulmonary artery pressure, cardiac output, lung lymph flow, permeability-surface area product and extravascular lung water volume [20]. This seems to be due to elevated venous return due to excess sympathetic venoconstriction. In addition, when the intracranial pressure is suddenly elevated by subdural balloon inflation, neurogenic pulmonary edema develops [13].

Why is inflammation not involved

The most frequent form of neurogenic pulmonary edema, the so called "early form", usually develops within minutes or, at most, hours after the injury [4,12,14,21]. In our experiments with spinal cord-injured rats, the full picture of neurogenic pulmonary edema developed within 8 min from the onset of our intervention on the spinal cord [9]. In contrast, the cascade of the inflammatory response, which would be able to cause such extravasation of intravascular fluid together with the damage of blood vessel walls leading to intraalveolar hemorrhage, would most likely take a longer time, such as in acute respiratory distress syndrome [22]. It is true that some exogenic substances causing the initiation of an allergic reaction cascade might induce a rapid inflammatory reaction leading to the extravasation of fluid and the development of edema, but neurogenic pulmonary edema is not such a case. In addition, spinal cord injury does not initiate any systemic inflammatory response, as demonstrated by the lack of any damage to other organs except the lungs.

Central nervous system injury is associated with the enhanced production of free oxygen radicals, mainly originating from extravasated blood [23]. If the free oxygen radicals originating from CNS injury would be responsible for the development of neurogenic pulmonary edema, the picture of neurogenic stunned myocardium, known to be caused by free oxygen radicals [24], would probably appear concomitantly, at least in some patients. This is, however, not the case in neurogenic pulmonary edema [1].

Why the sympathetic nervous system transducts the signal

Many direct or indirect experiments have shown that the sympathetic nervous system has a major responsibility for neurogenic pulmonary edema development. It has been shown in animal models of neurogenic pulmonary edema that changes of systolic and diastolic pressure together with heart rate alterations indicate rapid systemic activation of the sympathetic nervous system, which was termed "severe sympathetic discharge" or "catecholamine storm" [1,10,11]. The most likely mechanism of the overactivation of the sympathetic nervous system (sympathoexcitatory reflexes) is the secretion of vasoactive substances from peripheral sympathetic endings, which leads to the sudden increase of systemic blood pressure, generalized peripheral vasoconstriction, a decrease in systemic vascular resistance, augmentation of central blood volume and a reduction of the compliance of the left ventricle. These changes further lead to the constriction of the pulmonary veins, an increase in pulmonary capillary hydrostatic pressure, damage to the alveolar wall and the leakage of fluid into the interstitium and intraalveolar space and hemorrhage — taken together — the typical picture of neurogenic pulmonary edema.

Why is the dose of anesthesia important

Systemic anesthesia is accompanied by the inhibition of the spontaneous and evoked activity of neurons. The first phase of systemic anesthesia is characterized by the inhibition of the activity of cortical neurons, whereas deep anesthesia leads to partial inhibition of subcortical neurons, probably including the sympathetic ones. For example, when we used 1.5% isoflurane (where severe neurogenic edema develops) instead of 3% isoflurane (where no edema develops) for performing a spinal cord lesion in rats, the baseline mean arterial pressure and heart rate values were significantly increased. In addition, animals operated under lower isoflurane anesthesia doses exhibited higher "reactivity", in terms of blood pressure and heart rate changes, to all parts of the surgical intervention [9]. Leal Filho et al. [5,6] performed a similar experiment, but they used pentobarbital or a ketamine–xylazine mixture for the anesthesia of rats, in which a balloon compression lesion was made. They observed severe neurogenic pulmonary edema in pentobabital-anesthetized rats and borderline neurogenic edema in rats anesthetized with ketamine–xylazine. The systolic blood pressure in pentobarbital-anesthetized rats rose to twice the baseline values, whereas it was only 13% higher in ketamine–xylazine-anesthetized rats [5,6], indicating that the pentobarbital-anesthetized rats responded more reactively to spinal compression. However, these authors did not perform dose–response experiments and used only one concentration of each type of anesthesia (pentobarbital 60 mg/kg; ketamine–xylazine 75 and 10 mg/kg, respectively). We can therefore hypothesize that lower doses of ketamine–xylazine would also be able to promote severe neurogenic pulmonary edema and vice versa with pentobarbital. Extreme differences in different doses of isoflurane (3% — no edema vs. 1.5% — massive edema) in our experiments strongly support such a hypothesis [9].

Consequences of the hypothesis and discussion

Our hypothesis might help to understand the pathophysiology of neurogenic pulmonary edema. First, it highlights the crucial role of the connection between the dose of anesthesia and sympathetic excitation in the pathogenesis of neurogenic pulmonary edema. Second, it focuses on the mechanism by which the anesthesia level might influence the neurogenic pulmonary edema trigger zones in the central nervous system.

Our hypothesis indicates the necessity of maintaining precisely the same level of anesthesia during experimental central nervous system manipulations, such as the preparation of brain or spinal cord injury models [25,26], the injection of particular substances into the central nervous system parenchyma [27,28], neurosurgical manipulations such as implantation of hydrogel scaffolds [29], the preparation of animal models of epilepsia [30] and many others. For these purposes, the anesthesia should be precisely and reproducibly dosed. Although this is quite simple with volatile anesthetics such as isoflurane or sevoflurane [7,9], it might be more difficult when intravenous anesthetics such as pentobarbital or ketamine–xylazine are used [5,6]. If possible, intravenous anesthetics should be avoided in experiments where central nervous system tissue is surgically manipulated or the intracranial pressure is changed. If this is not possible, the infusion rate of the anesthetic solution should be carefully controlled.

Today, many models of neurogenic pulmonary edema have been used in experimental studies. In

these models, pulmonary edema is induced either by central nervous system injury [5,6] or the administration of an exogenic substance into the cerebrospinal fluid or directly into the nervous tissue [15,18,19]. Pulmonary edema can also be caused by the intravenous administration of epinephrine, which stimulates vasoconstriction [31,32], or bilateral cervical vagotomy, which inhibits vasodilatation [33]. Although the sympathetic nervous system is almost surely involved in the development of pulmonary edema after the administration of an exogenic substance, we propose that neurogenic pulmonary edema should always be induced by central nervous system injury in future experiments, to be sure of its "neurogenic" origin.

In clinical practice, our hypothesis might also be important. On the basis of our hypothesis and previous experimental data we propose that if, accidentally, the level of anesthesia decreases during a neurosurgical operation, neurogenic pulmonary edema might develop and this might negatively influence the course and the result of the operation. Our previous experiments [9] indicate that the reduction of the anesthesia level to where neurogenic pulmonary edema develops need not be to the arousal stage — in our experiment, all rats were anesthetized so deeply that no corneal, tail pinch or interdigital toe reflexes occurred.

In the future, clinical studies might show that during the acute phase (minutes to hours) after central nervous system injury, the maintenance of the patient under anesthesia might be helpful in preventing the development of neurogenic pulmonary edema. Today, no intervention to prevent the development of neurogenic pulmonary edema is known. For example, the blockade of the sympathetic nervous system [9] or an intrathecal injection of lidocaine [13], which are used in experiments to prevent the development of neurogenic pulmonary edema, would probably not be advisable in human medicine. In addition, any other total or partial modulation of the sympathetic nervous system which would positively influence neurogenic pulmonary edema development, might also significantly worsen the general health status of the patient.

Thus, the major message from our hypothesis to all scientists and medical doctors dealing with the neurogenic pulmonary edema is to be aware of the usage of anesthesia in their animals or patients. This hypothesis might also help us to explain the role of anesthesia in the development of neurogenic pulmonary edema in the future.

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MECHANISMS OF NEUROGENIC PULMONARY EDEMA DEVELOPMENT

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Short title: Neurogenic pulmonary edema development

Summary

Neurogenic pulmonary edema is a life-threatening complication, known for almost 100 years, but its etiopathogenesis is still not completely understood. This review summarizes current knowledge about the etiology and pathophysiology of neurogenic pulmonary edema. The roles of systemic sympathetic discharge, central nervous system trigger zones, intracranial pressure, inflammation and anesthesia in the etiopathogenesis of neurogenic pulmonary edema are considered in detail. The management of the patient and experimental models of neurogenic pulmonary edema are also discussed.

1

Introduction

Neurogenic pulmonary edema is an acute life-threatening complication of severe central nervous system injury. Although long neglected in clinical practice, it has been recognized scientifically for many years; the first report comes from Shanahan (1908). Neurogenic pulmonary edema develops rapidly following the injury and significantly complicates the overall clinical status of the patient. It is characterized by marked pulmonary vascular congestion with perivascular edema, extravasation and intra-alveolar accumulation of protein-rich edema fluid and intraalveolar hemorrhage (Kandatsu et al., 2005; Leal Filho et al., 2005a, 2005b). Although several pathophysiological mechanisms have been proposed, the exact cascade leading to the development of neurogenic pulmonary edema remains unclear (Leal Filho et al., 2005a, 2005b). Both the release of vasoactive substances and a rapid, transient, and severe sympathetic discharge are thought to participate in this process (Urdaneta and Layon, 2003). The aim of this review is to summarize the known data about the pathophysiology of neurogenic pulmonary edema and also highlight the importance of some new data, recently obtained from experimental research of neurogenic pulmonary edema.

Epidemiology of neurogenic pulmonary edema

Although epidemiological data of neurogenic pulmonary edema are scarce and often based on case reports or epidemiological studies with low numbers of enrolled patients or different diagnostic criteria, we can assume its morbidity in patients with severe central nervous system injury to be 40-50% and its mortality around 7% (Fontes et al., 2003; Dragosavac et al., 1997; Antoniuk et al., 2001). The main reason for the low number of published epidemiological

studies is probably the generally poor clinical status of the patient, caused by the primary central nervous system injury, and the necessity of rapid and often complex treatment when neurogenic pulmonary edema develops.

Management of patients with neurogenic pulmonary edema

The signs of neurogenic pulmonary edema are quite non-specific. It presents subjectively with a sudden onset of dyspnea, chest pain, worsening of expectoration, nausea, vomitting, weakness and an awareness of the patient´s own life. During the clinical examination, one finds tachypnoe, tachycardia, basal bilateral pulmonary crackles, respiratory distress or failure, expectoration of sanguinolent sputum or even hemoptysis, hypoxemia, increased systemic blood pressure and decreased heart rate, consciousness disturbances, and pulmonary edema with normal jugular venous pressure (Baumann et al., 2007). Some authors describe a so-called "death rattle" (Fontes et al., 2003). Importantly, no or very few signs of inflammation are present. The most relevant imaging method is the chest X-ray examination, where diffuse hyperintensive infiltrates in both lungs are apparent. The transient increase in pulmonary artery occlusion pressure is usually not found due to the very short duration of its increase and the delay in measurement (Ganter et al., 2006). Although the levels of some substances such as brain natriuretic peptide, blood C-reactive protein and IL-6 are increased, unfortunately none of these can be used as a marker specific for neurogenic pulmonary edema (Baumann et al., 2007). Treatment is based primarily on ventilation with positive end-expiratory pressure and support of the patient´s general health status. Other treatment modalities are still under debate among clinicians and scientists (Baumann et al., 2007).

Role of central nervous system damage or injury in the development of neurogenic pulmonary edema

Neurogenic pulmonary edema has been described following several central nervous system injuries, including spinal cord injury, subarachnoid hemorrhage, primary spinal cord hemorrhage, brain trauma, intracerebral bleeding, severe epileptic grand mal seizure or subdural hematoma (Fontes et al., 2003; Dragosavac et al., 1997; Ochiai et al., 2001; Seric et al., 2004; Baumann et al., 2007; Simmons et al., 1969; Macleod et al., 2002). There are two possible explanations for the association of the edema with central nervous system injury. One presumes that the neuronal damage directly or indirectly involves the pulmonary vascular bed. The most probable mechanisms of such involvement are a temporary neural commotion or either a mechanical or electrophysiological disconnection of the central nervous system vasomotor centres, which leads to the destabilization of the pulmonary autonomic nervous system (Fontes et al., 2003; Baumann et al., 2007). The overstimulation of the vasomotor centres might be an alternative explanation. This is supported by the fact that severe central nervous system injury is always associated with significant changes in autonomic nervous system function (Leal Filho et al., 2005a, 2005b, Šedý et al., 2007a, 2007d). The function of specific neurons of the central nervous system must be impaired during brain or spinal cord injury to destabilize the autonomic system balance so that an imbalance of Starling forces in the pulmonary vascular bed occurs and neurogenic pulmonary edema may develop (Šedý et al., 2007c).

Numerous experiments have shown that some interventions in the brain or spinal cord are able to stimulate the peripheral sympathetic nervous system and to produce changes in blood pressure (Guyenet, 2006). These data indicate that the central nervous system sympathetic centers (including trigger zones for neurogenic pulmonary edema) might be influenced by such manipulations. It has been observed that experimental brainstem distortion or ischemia can cause changes of sympathetic vasomotor tone and an increase of blood pressure.

Moreover, the elevation of blood pressure and the subsequent development of neurogenic pulmonary edema can be prevented by the intrathecal administration of lidocaine (Guyenet, 2006; Hall et al., 2002).

Trigger zones of neurogenic pulmonary edema

Several experimental studies have indicated that the neurons responsible for the severe sympathetic discharge, the most probable cause of neurogenic pulmonary edema, are located in the hypothalamic, brainstem and cervical spinal cord nuclei. These centers represent so-called neurogenic pulmonary edema trigger zones (Baumann et al., 2007), and their arrangement corresponds to the organotopy hypothesis (for review see Guyenet, 2006). The most important vasomotor centres for neurogenic pulmonary edema development are thought to be the A1 and A5 groups of neurons, nuclei of the solitary tract, the area postrema, the medial reticulated nucleus and the dorsal motor vagus nucleus in the medulla oblongata. Some hypothalamic centres (paraventricular and dorsomedial nuclei) also seem to be of some importance. It can be assumed that C1 adrenaline-synthesizing neurons, definitely identified as a key blood pressure centre (for review see Guyenet, 2006), are responsible for the observed sympathetic activation. Interestingly, casualties from the Vietnam war who had concomitant brain injury and cervical spinal cord injury did not develop neurogenic edema, whereas the majority of casualties with brain injury alone developed edema (Simmons et al., 1969). In the first group, the trigger zones were probably disconnected from the rest of the body by cervical spinal cord transection.

In experiments on rabbits, bilateral lesions of the nuclei in the medulla oblongata produced profound pulmonary and systemic hypertension and pulmonary edema (Blessing et al., 1981). The increased intracranial pressure as well as toxic or ischemic injury of inhibitory neurons

cause excessive sympathetic neuronal activity (severe sympathetic discharge) and the release of vasoactive substances such as epinephrine, norepinephrine, endothelins or neuropeptide Y into the circulation, leading to the development of neurogenic pulmonary edema (Baumann et al., 2007; Hamdy et al., 2000; Poulat et al., 1998). For example, neuropeptide Y has been found in alveolar macrophages and edema fluid in the case of neurogenic pulmonary edema, but not in rats with hydrostatic edema (Hamdy et al., 2000). Alpha-adrenergic blockade (with phentolamine) or spinal cord transection at the C7 level prevents the formation of neurogenic pulmonary edema, suggesting an important role for sympathetic activation (Nathan et al., 1975). In addition, the inhibition of central nervous system nitric oxide by the injection of a competitive inhibitor of NO syntase, NG-nitro-L-arginine methyl ester (L-NAME), into the cisterna magna worsened the extent of development of neurogenic pulmonary edema (Hamdy et al., 2001).

Neurogenic pulmonary edema develops on the basis of intracranial pressure increase
After central nervous system injury, a prominent hemorrhage into different compartments that correspond to the epidural, subdural, subarachnoid and intramedular (intracerebral) spaces and consequent damage to the blood-brain barrier occur. In clinical situations such as subdural or subarachnoid hemorrhage, the extravasation of blood into the corresponding compartments is the major mechanism. Walder et al. (2002) showed that the amount but not the type of fluid injected intrathecally had a significant impact on hemodynamic and respiratory parameters. The main disadvantage of any intracranial or intraspinal hemorrhage is the very rapid increase of intracranial pressure inside a non-expandable bony space with rather limited mechanisms to decrease it. This pressure increase leads to the compression of nervous tissue, resulting in a brainstem distortion or ischemia and later even in cerebral herniation. It has been shown in experiments on sheep that the elevation of intracranial

pressure increases pulmonary artery pressure, cardiac output, lung lymph flow, permeability-surface area product and extravascular lung water volume (Peterson et al., 1983). This seems to be due to elevated venous return due to excess sympathetic venoconstriction. In addition, when the intracranial pressure is suddenly elevated by subdural balloon inflation, neurogenic pulmonary edema develops (Hall et al., 2002).

Our experiments with a model of severe neurogenic pulmonary edema, elicited by an epidural balloon compression spinal cord lesion under lower concentrations of isoflurane anesthesia, strongly support the crucial role of intracranial hypertension in the development of neurogenic pulmonary edema (Šedý et al., 2007b). When the balloon is rapidly inflated in the enclosed space of the thoracic part of the spinal channel, the rapid increase in intracranial pressure apparently has an etiopathogenic role in the development of neurogenic pulmonary edema (Šedý et al., 2007a, 2007d). This suggestion is supported by our recent findings that other types of thoracic spinal cord lesions such as transection, hemisection or contusion do not lead to the development of neurogenic pulmonary edema (Šedý et al., unpublished data).

Inflammation is not involved in the development of neurogenic pulmonary edema
The most frequent form of neurogenic pulmonary edema, the so-called "early form", usually develops within minutes or, at the most, hours after the injury (Baumann et al., 2007; Guyenet, 2006; Simmons et al., 1969; Seric et al., 2004). In our experiments with spinal cord-injured rats, the full picture of neurogenic pulmonary edema developed within 5-12 minutes from the onset of our intervention on the spinal cord (Šedý et al., 2007a,b,d). It should be pointed out that the cascade of the inflammatory response, which would be able to cause such extravasation of intravascular fluid together with the damage of blood vessel walls leading to intraalveolar hemorrhage, will take a longer time, as in acute respiratory distress syndrome

(Gattinoni et al., 1993). It is true that some exogenous substances causing the initiation of an allergic reaction cascade might induce a rapid inflammatory reaction leading to the extravasation of fluid and the development of edema, but neurogenic pulmonary edema is not such a case. In addition, spinal cord injury does not initiate any systemic inflammatory response, as demonstrated by the lack of any damage to other organs except the lungs (Šedý et al., 2007a).

Role of the sympathetic system in the development of neurogenic pulmonary edema

Many experiments have shown, directly or indirectly, that the sympathetic nervous system has a major responsibility for neurogenic pulmonary edema development. It has been shown using animal models of neurogenic pulmonary edema that changes in systolic and diastolic pressure together with heart rate alterations indicate a rapid systemic activation of the sympathetic nervous system, which has been termed "severe sympathetic discharge" or "catecholamine storm" (Fontes et al., 2003; Taoka and Okajima, 1998; Urdaneta and Layon, 2003). The catecholamine storm has its parallel in the older "blast theory" of Theodore and Robin (1976), which proposes that a neurally induced transient rise in intravascular pressure may damage the endothelium, causing protein-rich plasma to escape into the interstitial and alveolar spaces. The overactivation of the sympathetic nervous system is associated with the enhanced secretion of catecholamines from peripheral sympathetic nerve endings, which leads to peripheral vasoconstriction, an increase in systemic vascular resistance and subsequently to an increase in systemic blood pressure together with the augmentation of central blood volume and a reduction in the compliance of the left ventricle. These changes are followed by the constriction of the pulmonary veins, an increase in pulmonary capillary hydrostatic pressure, damage to the alveolar wall and the leakage of fluid into the interstitium and intraalveolar space and hemorrhage resulting in the typical picture of neurogenic pulmonary edema.

Dose-dependent influence of anesthesia on the development of neurogenic pulmonary edema

Systemic anesthesia is accompanied by the inhibition of both spontaneous and evoked activity of neurons. The first phase of systemic anesthesia is characterized by the inhibition of the activity of cortical neurons, whereas deep anesthesia leads to the partial inhibition of subcortical neurons, probably also including the sympathetic ones. When we used 1.5% isoflurane (with which severe neurogenic edema develops) instead of 3% isoflurane (with which no edema develops) for performing a spinal cord lesion in rats, the baseline mean arterial pressure and heart rate values were significantly increased. In addition, animals anesthetized with lower concentration of isoflurane anesthesia exhibited higher "cardiovascular reactivity", in terms of blood pressure and heart rate changes, to any of the particular procedures during the whole surgery (Šedý et al., 2007a, 2007d). Leal Filho et al. (2005a, 2005b) performed similar experiments, but they used pentobarbital or a ketamine-xylazine mixture for anesthetising rats in which a balloon compression lesion was made. They observed severe neurogenic pulmonary edema in pentobabital-anesthetized rats and borderline neurogenic edema in rats anesthetized with ketamine-xylazine. The systolic blood pressure in pentobarbital-anesthetized rats rose to twice the baseline values, whereas it was only 13% higher in ketamine-xylazine rats (Leal Filho et al., 2005a, 2005b), indicating that the pentobarbital-anesthetized rats responded more strongly to spinal compression. However, these authors did not perform dose-response experiments and used only one concentration of each type of anesthetic (pentobarbital 60 mg/kg; ketamine-xylazine 75 mg/kg and 10 mg/kg, respectively). Perhaps lower doses of ketamine-xylazine would be able to promote severe neurogenic pulmonary edema also. Similarly, higher doses of pentobarbital would be able to prevent its development. The extreme differences in response to different concentrations of

isoflurane (3% - no edema vs. 1.5% - massive edema) seen in our experiments strongly support such hypothesis (Šedý et al., 2007a, 2007d).

The above data indicate the necessity to maintain precisely the same level of anesthesia during experimental central nervous system manipulations, such as brain or spinal cord injury models (Vanický et al., 2001; Syková et al., 2005a), the injection of particular substances into the central nervous system parenchyma (Syková et al., 2005b; Syková et al., 2006), neurosurgical manipulations such as the implantation of hydrogel scaffolds (Lesný et al., 2002), the preparation of animal models of epilepsia (Bender et al., 2004) and many others. For these purposes, the anesthesia should be precisely and reproducibly dosed. Although this is quite simple with volatile anesthetics such as isoflurane or sevoflurane (Kandatsu et al., 2005; Šedý et al., 2007a), it might be more difficult when intravenous anesthetics such as pentobarbital or ketamine-xylazine are used (Leal Filho et al., 2005a, 2005b). If possible, intravenous anesthetics should be avoided in experiments in which central nervous system tissue is surgically manipulated or the intracranial pressure is changed. If this is not possible, the infusion rate of the anesthetic solution should be carefully controlled.

In the future, clinical studies might show that during the acute phase (minutes to hours) after central nervous system injury, the maintenance of patients under anesthesia might be helpful in preventing the development of neurogenic pulmonary edema. Presently, no intervention to prevent the development of neurogenic pulmonary edema is known. For example, the blockade of the sympathetic nervous system (Šedý et al., 2007a) or the intrathecal injection of lidocaine (Hall et al., 2002), which are used in experiments to prevent the development of neurogenic pulmonary edema, would probably not be advisable in human medicine. In addition, any other total or partial modulation of the sympathetic nervous system that would

positively influence neurogenic pulmonary edema development might also significantly worsen the clinical status of the patient.

Experimental models of neurogenic pulmonary edema

Today, many models of neurogenic pulmonary edema are used in experimental studies. In these models, the pulmonary edema is induced either by central nervous system injury (Leal Filho et al., 2005a, 2005b; Šedý et al., 2007b) or by the administration of exogenous substances into the cerebrospinal fluid or directly into the nervous tissue (Hamdy et al., 2000, 2001; Walder et al., 2002). In rats, the injection of fibrin (fibrinogen + thrombin) into the cisterna magna has been reported to induce pulmonary edema (Ishikawa et al., 1988). In dogs, the injection of verathrin (Lane et al., 1998; Maron, 1985), and in sheep, the injection of aconitine (Minnear and Connell, 1981), both into the cisterna magna, are also able to induce neurogenic pulmonary edema. However, the development of neurogenic pulmonary edema in these types of models has been considered to result from a cholinergic-mediated increase in vascular permeability (Bosso et al., 1990) rather than from severe sympathetic discharge, the most suspected cause of neurogenic pulmonary edema development in human patients (Fontes et al., 2003). Another model of pulmonary edema in dogs uses an intravenous injection of oleic acid (Dauber and Weil, 1983). Pulmonary edema can also be caused by the intravenous administration of epinephrine, which stimulates vasoconstriction (Dai et al., 1993a, 1993b), or bilateral cervical vagotomy, which inhibits vasodilatation (Iazzetti et al., 1988). Although the sympathetic nervous system is almost surely involved in the development of pulmonary edema after the administration of an exogenous substance, we propose that neurogenic pulmonary edema should always be induced by central nervous system injury in future experiments in order to ensure that the edema is, in fact, "neurogenic" in origin.

Conclusions

Neurogenic pulmonary edema is a rapidly developing, life-threatening complication of central nervous system injuries. It significantly worsens the general health status of the patient. For neurogenic pulmonary edema, the rapid onset of dyspnea and several other non-specific signs are typical. Most valuable for diagnosis is a chest X-ray, while the most valuable modality in treatment is ventilation with positive-end expiratory pressure. A specific marker for neurogenic pulmonary edema has not yet been found, and a specific treatment protocol has not yet been developed. Most probably, such edema develops on the basis of a rapid systemic sympathetic discharge, leading to pulmonary vascular congestion with perivascular edema, extravasation and the intra-alveolar accumulation of protein-rich edema fluid and intraalveolar hemorrhage. There exists evidence that intracranial pressure is also of some importance. The level of anesthesia might be crucial for the extent of neurogenic pulmonary edema development. There are several models of neurogenic pulmonary edema; however, those in which neurogenic pulmonary edema is induced by central nervous system injury should be preferred.

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5.6. Přehled dosažených výsledků

1. Balónková kompresní léze je vhodný model traumatického míšního poranění u laboratorního potkana, použitelný i pro studium vlivu anestezie na rozvoj neurogenního plicního edému.

2. Nízké koncentrace isofluranu (1,5 % a 2 %) mají zásadní význam pro rozvoj neurogenního plicního edému u zvířat s poškozením míchy. Neurogenní plicní edém se rozvíjí u 100% těchto zvířat.

3. Zvířata narkotizovaná 1,5% isofluranem mají při vytváření míšního poranění více než 33% pravděpodobnost úmrtí na následky neurogenního plicního edému.

4. Bezpečná koncentrace isofluranu pro provádění traumatického poškození míchy je 2,5 – 3 % isofluranu ve vzduchu při proudění 300 ml směsi/min. Koncentrace isofluranu 4% a více způsobí předávkování pokusného zvířete a není proto vhodná k pokusu.

5. Zvířata narkotizovaná nižšími koncentracemi isofluranu vykazují nižší práh reaktivity na všechny části chirurgického zákroku ve smyslu změn krevního tlaku a tepové frekvence. Neurogenní plicní edém u zvířat narkotizovaných nižšími koncentracemi isofluranu se rozvíjí díky nedostatečné inhibici sympatiku.

6. Neurogenní plicní edém snižuje stupeň návratu motorických i sensitivních neurologických funkcí. Zatímco se tato skutečnost dobře odráží v behaviorálních testech, není pozorovatelná morfologicky.

7. Získaná pokusná data je možné využít pro přípravu modelu těžkého neurogenního plicního edému u potkanů s poškozením míchy. Z tohoto hlediska se nejlépe jeví využití anestezie 2% isofluranem.

8. Postupné a rovněž neúplné vytváření balónkové léze dokáže zabránit rozvoji neurogenního plicního edému.

9. Neurogenní plicní edém nevzniká na podkladě míšní transekce nebo balónkové kompresní léze s menším objemem balónku.

10. Nízký stupeň inhibice sympatiku v důsledku nízké anestezie pravděpodobně potencuje rozvoj neurogenního plicního edému.

6. Diskuse

V této práci jsme prokázali, že nízká koncentrací isofluranu potencuje rozvoj neurogenního plicního edému u potkanů s poškozenou míchou. Rovněž jsme nalezli bezpečnou koncentraci isofluranu, při které k rozvoji neurogenního plicního edému nedochází. Neurogenní plicní edém vzniká na základě excesivní reakce sympatiku. Rozvinutý neurogenní plicní edém ovlivňuje návrat neurologických funkcí po poškození míchy, proto je nutné se jej při experimentálních studiích na potkanech vyvarovat, neboť může zkreslovat výsledky tím, že může nepředvídatelně ovlivňovat regeneraci poškozených oblastí. Získaných poznatků lze také využít při přípravě modelu těžkého neurogenního plicního edému.

6.1. Centrum vzniku neurogenního plicního edému

Jak vyplývá z definice i názvu, vzniká neurogenní plicní edém v návaznosti na poškození tkáně centrálního nervového systému (Leal Filho et al., 2005a, 2005b; Kandatsu et al., 2005). Patofyziologický mechanismus vzniku neurogenního plicního edému však stále není uspokojivě analyzován. Bylo prokázáno, že neurogenní plicní edém vzniká při těžkých poškozeních nejrůznějších částí mozku i míchy (Kandatsu et al., 2005; Leal Filho et al., 2005a, 2005b; Macmillan et al., 2002; Ochiai et al., 2001). Není tedy a priori splněna podmínka, že poškození určitého centra způsobí rozvoj neurogenního plicního edému. To však neznamená, že vznik neurogenního plicního edému není vázán na změny vyplývající z poškození určitého centra, jednak díky působení zvýšeného intrakraniálního tlaku, jednak díky vyplavení neurohumorálních působků z poškozené tkáně centrálního nervstva. Experimentální i klinické práce ukazují, že vznik neurogenního plicního edému vychází z vasomotorických center ventrolaterální části prodloužené míchy – adrenergní area A1 a A5, *nucleus tractus solitarii, nucleus dorsalis nervi vagi* a *area postrema* a ventrální medulární raphe (Walder et al., 2002; Hirabayashi et al., 1996; Macmillan et al., 2002; Ochiai et al., 2001; Carruth et al., 1992; Keegan a Lanier, 1999; Geiger et al., 1998; Brown et al., 1986). Bilaterální léze těchto jader způsobuje extensivní sympatickou reakci, aktivaci plicních C vláken, systémovou hypertenzi a vznik plicního edému (Carruth et al., 1992; Walder et al., 2002). Experimentální model plicního edému u potkanů je indukován vpravením fibrinu

(fibrinogen+trombin) do *cisterna magna* (Ishikawa et al., 1988), model plicního edému u psů je indukován vpravením veratrinu (směs alkaloidů z rostlin čeledi lilkovitých; název získal podle kýchavice bílé – *Veratrum album*) opět do cisterna magna (Lane et al., 1998; Maron, 1985), tedy v obou případech do bezprostřední blízkosti spodiny čtvrté mozkové komory a výše uvedených jader.

Pokud jsou tato jádra skutečně významná pro vznik neurogenního plicního edému, podporuje to teorii o nadměrné aktivaci sympatiku jako příčině neurogenního plicního edému, popsané v úvodu (kap. 1.7.). Preventivní působení alfa-adrenergních blokátorů (fentolamin) proti vzniku neurogenního plicního edému tuto teorii jen potvrzuje (Nathan and Reis, 1975). Tato teorie by pak vysvětlovala i rozvoj neurogenního plicního edému u zvířat s nízkou koncentrací anestetika, u kterých, jak se domníváme, působí operační zákrok mnohem větší stres, než u zvířat v hluboké anestezii.

6.2. Role intrakraniálního a systémového tlaku

Epidemiologická data ukazují, že neurogenní plicní edém vzniká zejména při rozsáhlejších poškozeních centrálního nervového systému, s výrazným podílem případů se zvýšeným intrakraniálním tlakem (Weir, 1978; Graf a Rossi, 1975; Fontes et al., 2003; Vinš, 2003; Stocker a Burgi, 1998; Brito et al., 1995; Dragosavac et al., 1997; Antoniuk et al., 2001; Urban et al., 2001). V experimentu bylo prokázáno, že při náhlém zvýšení intrakraniálního tlaku dojde ke zvýšení systémového tlaku a mírnému zpomalení srdeční frekvence během 1 minuty (Leal Filho et al., 2005a). Při injekci plné krve nebo roztoku albuminu do cisterna magna umírá 50 % pokusných zvířat na komplikace spojené se vznikem neurogenního plicního edému, které jsou obdobné úmrtím 33% zvířat v naší skupině narkotizované 1,5% isofluranem. Přitom množství vpravené tekutiny je mnohem důležitější než její složení (Walder et al., 2002). Toto zjištění může mít paralelu s našimi výsledky u potkanů, kde jsme balónek v míšním kanále nafukovali postupně a kde neurogenní plicní edém nevzniká. Jiní autoři prokázali, že při provedení bilaterální adrenalektomie před zákrokem na míše ke zvýšení systémového tlaku ani změnám srdeční frekvence nedojde a plicní edém nevzniká (Nathan a Reis, 1975). Naopak, oboustranná vagotomie nemá žádný protektivní efekt při vzniku neurogenního plicního edému, rozsah edému navíc ještě zhoršuje (Bosso et al., 1990). Zvýšený intrakraniální tlak

prokazatelně poškozuje centra v prodloužené míše, která jsou podezřívána ze spuštění sympatické bouře (Walder et al., 2002). Klinické studie ukazují, že subarachnoideální krvácení z ruptury aneurysmatu v povodí arteria vertebralis indukuje vznik neurogenního plicního edému častěji, než ruptura aneurysmatu v jiné lokalizaci (Ochiai et al., 2001). Námi použitý model balónkové kompresní léze zapadá do tohoto konceptu, traumatické poškození míchy je způsobeno náhlým nafouknutím balónku v těsném prostoru míšního kanálu a jeho udržení po dobu 5 minut. Po celou tuto dobu je nepochybně intrakraniální tlak nad místem uložení balónku významně zvýšen, neboť v příslušném místě nebyla provedena laminektomie a páteř zde proto není destabilizována (Vanický et al., 2001; Urdziková, 2006). Naopak postupné nafukování balónku umožňuje adaptaci systému na zvyšování intrakraniálního tlaku. Etiopatogenetickou roli intrakraniálního tlaku rovněž podporuje naše zjištění, že transekce hrudní míchy k rozvoji neurogenního edému nevede. Tyto pokusy mohou vysvětlit, proč se v klinické praxi, během neurochirurgického výkonu, neurogenní plicní edém prakticky nevyskytuje, i když se zde používá jako anestetikum isofluran. Během operace totiž nikdy není tak výrazné zvýšení intrakraniálního tlaku jako při úrazu nebo v experimentu při použití balónkové kompresní léze. Předkládané úvahy dále podporuje zjištění, že vzniku neurogenního plicního edému je možné zabránit hypovolémií (Minnear a Connel, 1982).

6.3. Role pulmonálního kapilárního hydrostatického tlaku

Je nepochybné, že změny kapilárního hydrostatického tlaku v plicních kapilárách se zásadním způsobem uplatňují při vzniku neurogenního plicního edému. V důsledku zvýšené sympatické aktivity se zvyšuje systémový tlak, coz způsobí centralizaci oběhu a tím i k zvýšené množství krve v plicním řečišti. Na tak náhle zvýšené nároky zřejmě nemůže levá srdeční komora dostatečně rychle zareagovat tak, aby přečerpala potřebné množství krve do velkého oběhu . Tak nastává městnání krve v plicích. V našich pokusech (publikace 3) dochází ke vzniku NPE zejména tam, kde po nafouknutí balónku v páteřním kanále nastává výrazný pokles srdeční frekvence. Tato situace nastává zejména v povrchové anestezii, kdy je zachován baroreflex, snižující srdeční frekvenci při náhlém vzestupu krevního tlaku. Toto tvrzení podporuje i paralelní zjištění nižšího krevního průtoku v aortě u experimentálních zvířat s NPE (Theodore a Robin, 1976; Wasowska-Krolikowska et al., 2000). Náhle enormně narůstá

kapilární hydrostatický tlak. Vzniklá nerovnováha Starlingových sil, působících na stěnu kapilár, vede k exsudaci tekutiny a při překročení určitých hodnot i k poškození až popraskání stěn kapilár a vzniku krvácení, které je častým doprovodným znakem neurogenního plicního edému a může být příčinou úmrtí (Chang et al., 2005; Leal Filho et al., 2005a, 2005b). V našich experimentech způsobilo toto krvácení úmrtí více než 33% zvířat s nízkou (1,5 %) anestezií isofluranem ve velmi krátké době. Hlavním nebezpečím neurogenního plicního edému je tedy zejména jeho náhlý a velmi razantní rozvoj, který může během minut ukončit život pacienta. Vznik neurogenního plicního edému může být navíc potencován plicní kapilární vasokonstrikcí (Fontes et al., 2003) nebo změnami ve stěnách plicních cév v důsledku systémové choroby, jak bylo popsáno v případě systémového lupus erythematodes (Chang et al., 2005; Liu et al., 1998; Schwab et al., 1993). Neurogenní plicní edém bývá u této systémové choroby navíc doprovázen akutním masivním plicním krvácením, které může být příčinou úmrtí pacienta (Chang et al., 2005; Liu et al., 1998; Schwab et al., 1993).

6.4. Role permeability kapilár a anestezie

Jak bylo řečeno v úvodu, isofluran indukuje reversibilní snížení clearance alveolární epiteliální tekutiny a snižuje tak práh pro vznik plicního edému (Rezaiguia-Delclaux et al., 1998; Laffon et al., 2002; Wiener-Kronisch a Gropper, 1998). Bylo prokázáno, že isofluran inhibuje mitochondriální oxidaci, což snižuje produkci ATP v pneumocytech II. typu a stimuluje tak produkci laktátu v těchto buňkách. Navíc snižuje syntézu fosfatidylcholinu a indukuje apoptózu pneumocytů II. typu, což zásadním způsobem poškozuje tvorbu surfaktantu (Mollieux et al., 1999). U intravenózních anestetik typu pentobarbitalu nebo kombinace ketaminu-xylazinu tento nežádoucí efekt zjištěn nebyl (Mollieux et al., 1999). Použití isofluranu tedy primárně vytváří podmínky pro vznik neurogenního plicního edému. Navíc, nízké koncentrace isofluranu zřejmě zvyšují stres pokusného zvířete a vznik neurogenního plicního edému je dále potencován. V našich měřeních reagovala zvířata narkotizovaná 1,5% isofluranem mnohem výrazněji nejen na poranění míchy, ale prakticky ve všech časových úsecích předcházejících vytvoření míšní léze.
Při použití řady jiných anestetik, jako je např. lidokain, ketamin, xylazin, pentobarbital, halothan a sevofluran byl během vytváření míšní léze

v experimentu rovněž pozorován rozvoj neurogenního plicního edému, doprovázeného krvácením (Laffon et al., 2002; Leal Filho et al., 2005a, 2005b; Pandey et al., 2000; Kandatsu et al., 2005; Molliex et al., 1998). Zvýšení tlaku v nízkotlakém plicním řečišti navíc působí barotrauma endotelu plicních kapilár a poruchy kapilární permeability jsou tak dále stupňovány. Výsledkem je exsudace tekutiny až protržení kapilární stěny a krvácení, které jsme pozorovali i my. Podle nás je vznik neurogenního plicního edému v našich experimentech způsoben primárním lehkým poškozením plic vlivem isofluranu, v kombinaci se sníženou anestezií u 1,5% a 2% skupin, která dovolí rozvoj sympatické bouře a vznik edému. Pokud je experimentálně do plic vpraven neuropeptid Y, tedy látka známá tím, že je společně s noradrenalinem vylučována sympatickými nervy, která zvyšuje stupeň plicní vasodilatace a plicní vaskulární permeabilitu (Widdicombe, 1991), reaguje organismus pokusného zvířete zvýšením plicní vaskulární permeability a vznikem neurogenního plicního edému. Neuropeptid Y přitom působí přímo na endotelové buňky (Hirabayashi et al., 1996).

6.5. Morfologické posouzení plicního edému

Rutinní hematoxylin-eosinové vyšetření je považováno za zlatý standard a velmi levný způsob detekce plicního edému. Klasická histologická technika velmi dobře odráží morfologii alveolární stěny, tvaru a velikosti pneumocytů a krevních elementů a morfologie cévních stěn. Rutinní histologické zpracování v naší studii skutečně velmi dobře odráželo těžké morfologické poškození u zvířat narkotizovaných 1,5% a 2% isofluranem, včetně významného zesílení alveolárních stěn. Zůstává však pravdou, že proces dehydratace a rehydratace v průběhu zpracování může zkreslit výsledné množství tekutiny v plicních alveolech (Leal Filho et al., 2005a, 2005b). Pro stanovení množství tekutiny jsme proto zvolili techniku stanovení plicního indexu, neboli relativní váhy plic, který je přes svou jednoduchost mimořádně citlivým ukazatelem (Leal Filho et al., 2005a, 2005b. Pro naše experimenty bylo proto stanovení plicního indexu více než výhodné, neboť jsme neobdrželi žádné hraniční výsledky. Technika stanovení plicního indexu navíc nijak nepoškozuje plicní tkáň a umožňuje následné histologické zpracování tkáně. Jednou z dalších možností by bylo například srovnání mokré a suché váhy plic.

6.6. Rychlost vzniku neurogenního plicního edému

Jednou z hlavních charakteristik neurogenního plicního edému je vysoká rychlost, s jakou se vyvine. Leal Filho et al. (2005a, 2005b) pozorovali první mikroskopické známky neurogenního plicního edému již 2 minuty po provedení kompresní míšní léze. Z doby Vietnamské války pochází případ, kdy neurogenní plicní edém vznikl krátce po poranění (Simmons et al., 1969). V naší studii jsme pozorovali, že tyto změny začínají být na povrchu plic patrné již 6 minut po lézi. Navíc, plicní hemorrhagie, projevující se jako subpleurální sufuze, začnou být morfologicky patrné za další 2 minuty. Oba výsledky odpovídají klinickým studiím (Fontes et al., 2003; Urdaneta and Layon, 2003) a ukazují na jednu z hlavních hrozeb neurogenního plicního edému – může se vytvořit krátce po poranění centrálního nervového systému, konečný obraz edému plic se rozvine během minut a může takto rychle zhoršit již tak nedobré postavení pacienta v těžkém zdravotním stavu.

6.7. Neurogenní plicní edém jako příčina smrti

V našich experimentech zemřela více než třetina zvířat z 1,5% isofluranové skupiny na komplikace neurogenního plicního edému. V podstatě se jednalo o náhlé udušení v důsledku masivního plicního edému, spojeného s intraalveolárními hemorrhagiemi. Klinické práce popisují podobný stav, které označují termínem náhle vzniklý „smrtelný chropot", provázený dušností, hemoptýzou a pacientovým strachem o vlastní život (Macleod, 2002; Keegan a Lanier, 1999). Hlavním nebezpečím neurogenního plicního edému je tedy zejména náhlý vznik a rychlá progrese (Macleod, 2002; Keegan a Lanier, 1999), což dokumentoval i náš experiment, kdy jsme pozorovali plicní tkáň přes translucentní pleura parietalis během vytváření míšní léze, kdy se krvácení objevilo velmi záhy po poranění míchy a navíc rychle progredovalo.

6.8. Návrat neurologických funkcí u zvířat s neurogenním plicním edémem

Prokázali jsme, že při rozvinutém neurogenním plicním edému dochází ke zpomalení návratu neurologických funkcí ve druhém a třetím týdnu po poranění Toto poškození může souviset s rolí glutamátu při vzniku neurogenního plicního edému. Kondo et al. (2004) prokázali, že při lokální injekci excitotoxického glutamátu do čtvrté mozkové komory se rozvíjí neurogenní plicní edém. Naopak, při inhibici NMDA a AMPA glutamátových receptorů je vznik neurogenního plicního edému potlačen (Kondo et al., 2004). Glutamát, uvolněný z prodloužené míchy při vzniku neurogenního plicního edému může tedy působit na zvýšení rozsahu sekudárního míšního poškození. Horší návrat neurologických funkcí může rovněž souviset s horším celkovým stavem zvířat s neurogenním plicním edémem. V dřívějších pracech bylo ukázáno, že pacienti s neurogenním plicním edémem měli mnohem horší prognózu ve srovnání s pacienty, kteří edém neměli, ačkoli edém odezněl během několika dní, a to buď spontánně nebo s použitím různých terapeutických strategií (Fontes et al., 2003; Macleod, 2002). Z klinických studií míšního poranění je zřejmé, že nejkritičtější je právě několik prvních hodin po úrazu (Bracken et al., 1992; Syková et al., 2006b). Pokud je iniciální stav komplikován neurogenním plicním edémem, může se zpomalit regenerace nervových drah, což se v našem experimentu projevilo signifikatním zhoršením BBB a plantar testu právě v druhém a třetím týdnu po poranění.

6.9. Model neurogenního plicního edému

V naší studii jsme vytvořili nový model těžkého neurogenního plicního edému, který vzniká na základě poranění míchy. Ačkoli již bylo několik modelů popsáno (Ishikawa et al., 1988; Lane et al., 1998; Maron, 1985; Leal Filho et al., 2005a), model těžkého plicního edému, kde hodnota plicního indexu přesahuje 0,7 a kde zároveň edém vzniká na základě míšního poranění, nebyl dosud publikován. Hlavní výhodou tohoto modelu je fakt, že plicní edém vzniká přímo na základě poranění tkáně centrálního nervstva, nikoli na základě vpravení cizorodé toxické substance do organismu, což samo o sobě může mít vedlejší účinky.

Při použití 1,5% isofluranu je vytvořen model velmi těžkého neurogenního plicního edému, jak dokumentuje průměrný plicní index 0,85. Skutečnost, že úplný obraz plicního edému se vyvine do 8 minut po míšním poranění ukazuje

na jeho podobnost s klinickou situací, kde se jedná rovněž o rychle se rozvíjející a život ohrožující záležitost (Fontes et al., 2003). Nevýhodou tohoto modelu je však více než třetinová úmrtnost pokusných zvířat. Na druhé straně, jedním z cílů studie může být terapeutické ovlivnění zdravotního stavu zvířat ve smyslu snížení mortality.

Při přípravě modelu neurogenního plicního edému použitím 2% isofluranu ve vzduchu neumírá žádné zvíře a neurogenní plicní edém je stále těžkého stupně, jak dokládá průměrný plicní index 0,74. Tento model bude zřejmě mnohem lepší pro rozsáhlejší studie, kde by vysoká mortalita pokusných zvířat při použití 1,5% isofluranu nebyla optimální.

Do dnešního dne byla v experimentu použita řada modelů neurogenního plicního edému. Plicní edém zde vzniká buď na základě poranění tkáně centrálního nervstva (Leal Filho et al., 2005a, 2005b) nebo vpravením exogenní substance do mozkomíšního moku nebo přímo do nervové tkáně (Hamdy et al., 2000, 2001; Walder et al., 2002). V extrémním případě může být edém indukován intravenózní aplikací adrenalinu (Dai et al., 1993a, 1993b) nebo bilaterální cervikální vagotomií (Iazetti and Maciel, 1988). Ačkoli je sympatikus zodpovědný za rozvoj neurogenního plicního edému, jak mimo jiné ukazují i naše výsledky, preferujeme použití modelů, u kterých je neurogenní plicní edém vyvoláván poškozením tkáně centrálního nervstva bez jakéhokoli vpravení exogenních substancí. Naše dva modely takové uspořádání nabízejí.

6.10. Hypotéza vzniku neurogenního plicního edému

Naše hypotéza protektivního vlivu anestezie na rozvoj neurogenního plicního edému může pomoci porozumět patofyziologii neurogenního plicního edému. Její význam může být v tom, že u experimentů, zabývajících se neurogenním plicním edémem, zaměří úhel pohledu směrem k anestezii. Navíc se zabývá sympatickou hyperaktivitou, tj. mechanismem, kterým může stupeň anestezie ovlivnit rozvoj neurogenního plicního edému.
Naše hypotéza naznačuje nutnost reprodukovatelnosti a zachování adekvátní koncentrace anestezie při experimentálních manipulacích v oblasti centrálního nervstva, jako je příprava modelů mozkové a míšní léze (Vanický et al., 2001; Syková et al., 2005), injektáž exogenních látek nebo buněk do parenchymu

centrálního nervstva (Syková and Jendelová, 2005, 2006), neurochirurgické manipulace, např. ve smyslu implantace hydrogelů (Lesný et al., 2002), příprava a manipulace s experimentálními modely epilepsie (Bender et al., 2004) a mnohé další. Pro tyto účely by bylo vhodné, aby byla anestezie přesně a reprodukovatelně dávkována. Ačkoli je tento cíl poměrně snadno dosažitelný u inhalačních anestetik jako je isofluran nebo sevofluran (Kandatsu et al., 2005), u injekčních anestetik typu pentobarbitalu nebo směsi ketamin-xylazin (Leal Filho et al., 2005a, 2005b) může být tento úkol nesnadný. Pokud je to možné, je z našeho hlediska lépe se u podobných manipulací intravenózním anestetikům buď vyhnout, nebo použít například infuzi anestetika, kde je možné jeho množství přesně dávkovat (injektomat).

V klinické praxi může být naše hypotéza rovněž přínosem. Na základě ní je možné předpovědět situaci, kdy se v průběhu neurochirurgické operace změní hloubka anestezie a rozvine se tak neurogenní plicní edém. Naše výsledky ukazují, že snížení hloubky anestezie nemusí nutně znamenat probuzení pacienta – zvířata s plicním edémem, narkotizovaná 1,5% isofluranem rovněž nebyla při vědomí ani nereagovala reflexními podněty.

6.11. Využití poznatků v klinické praxi

Klinicky zaměřené práce ukázaly, že neurogenní plicní edém vzniká na základě subarachnoideálního krvácení v důsledku míšního poranění (Stocker a Burgi, 1998), primárního míšního krvácení (Inobe et al., 2000), ruptury aneurysmatu (Brito et al., 1995; Weir, 1978), mozkového traumatu (Dragosavac et al., 1997), intracerebrálnímu krvácení (Dragosavac et al., 1997), těžkého epileptického záchvatu typu grand mal (Antoniuk et al., 2001; Wasowska-Krolikowska et al., 2000), meningitidy nebo tumoru, spojených s rozvojem hydrocefalu (Wagle et al., 1990), intrakraniálního tumoru (Keegan a Lanier, 1999) a subdurálního hematomu (Rubin et al., 2001). Kromě toho však může neurogenní plicní edém vzniknout velmi vzácně i iatrogenně při neurochirurgických zákrocích (Urban et al., 2001; Keegan a Lanier, 1999; Simon, 1993). Z velmi malého počtu klinických prací, z nichž navíc většinu tvoří kasuistiky, je zřejmé, jak je tato klinická jednotka podceňována. V extrémním případě ji někteří dokonce vyhrazují pouze jako diagnózu post-mortem, což bylo opakovaně kritizováno jako nepřípustné (Fontes et al., 2003). Epidemiologická data, byť je jich stále

nedostatek, naznačují přesný opak, totiž že neurogenní plicní edém se vyskytuje u pacientů s poškozením centrálního nervového systému relativně často (Leal Filho et al., 2005a, 2005b; Fontes et al., 2003; Macleod, 2002). Jeho diagnóza není snadná a obvykle probíhá per exclusionem (Macleod, 2002; Keegan a Lanier, 1999). Nejvíce imituje aspirační pneumonii (Macleod, 2002; Keegan a Lanier, 1999). Naše výsledky naznačují závažnost nedostatečné anestezie při vzniku neurogenního plicního edému a ukazují, že isofluran je jedním z anestetik, které mohou jeho vznik potencovat. Rovněž je ukázáno, že při rozvinutém neurogenním plicním edému dochází k horšímu návratu neurologických funkcí u zvířat. Mělo by tedy být snahou kliniků neurogennímu plicnímu edému maximálně přecházet. Je však pravdou, že včasné odhalení neurogenního plicního edému může být v podmínkách již takto kritického celkového klinického stavu pacienta velmi obtížné. Z jakékoli náhle vzniklé dušnosti a zhoršení ventilačních parametrů u pacienta s poškozením CNS má proto být na prvním místě podezřírán neurogenní plicní edém.

7. Závěry

1. Epidurální balónková kompresní míšní léze v anestezii nízkou koncentrací isofluranu (1,5%-2%) vyvolává rozvoj neurogenního plicního edému, nebo dokonce úmrtí na následky neurogenního plicního edému u laboratorního potkana (publikace 1,2).

2. Při koncentracích isofluranu vyšších než 4% včetně, dojde k předávkování anestetikem a úmrtí potkanů, které však není v žádném vztahu k neurogennímu plicnímu edému (publikace 1).

3. Nejbezpečnější koncentrace isofluranu pro provádění zákroků na míše u potkana je koncentrace isofluranu 2,5 – 3% ve vzduchu, při proudu 300 ml inhalační směsi za minutu (publikace 1).

4. Neurogenní plicní edém oslabuje návrat motorických i sensitivních neurologických funkcí. Zatímco se tato skutečnost dobře odráží v behaviorálních testech - BBB testu a plantárním testu, morfometricky prokazatelná není (publikace 1).

5. Získaná experimentální data je možné využít pro přípravu modelu těžkého neurogenního plicního edému u potkanů s poškozením míchy. Z tohoto hlediska se nejlépe jeví využití anestezie 2% isofluranem (publikace 2).

6. Postupné vytváření balónkové kompresní míšní léze zabrání rozvoji neurogenního plicního edému. Transekce míchy nezpůsobuje rozvoj neurogenního plicního edému (publikace 3).

7. Na základě dosažených výsledků a podrobného studia literárních pramenů byla formulována hypotéza o patofyziologickém mechanismu možného vlivu nízkých koncentrací anestetika na rozvoj neurogenního plicního edému (publikace 4).

8. Dosažené výsledky byly shrnuty v přehledovém článku (publikace 5).

8. Souhrn

Neurogenní plicní edém (NPE) je akutní, život ohrožující komplikace poranění centrálního nervového systému (CNS). Anestetika mohou rozvoj NPE stimulovat nebo inhibovat. Zkoumali jsme vliv isofluranu u potkanů samců kmene Wistar, narkotizovaných 1,5 - 3% isofluranem ve vzduchu, na rozvoj NPE po balónkové kompresní míšní lézi. Rozvoj neurogenního plicního edému byl posuzován *in vivo* a na histologických řezech plicní tkáně. Stupeň návratu neurologických funkcí u zvířat narkotizovaných 1,5% a 3% isofluranem byl sledován s použitím BBB a plantar testu po dobu 7 týdnů po míšním poranění. Stupeň zachování šedé a bílé hmoty míšní byl zkoumán pomocí morfometrie. Kromě toho byla hodnocena úloha postupně vytvořené míšní léze a úloha transekce míchy na rozvoj NPE. U všech zvířat narkotizovaných 1,5 - 2% isofluranem se rozvinul NPE. Téměř 42% zvířat zemřelo v důsledku těžkého plicního krvácení a udušení; RTG vyšetření, plicní index a histologické řezy ukázaly masivní NPE. Více než 71% zvířat narkotizovaných 2,5 - 3% isofluranem nemělo žádné známky NPE. Krevní tlak stoupal po kompresi míchy více u 1,5% skupiny než u 3%; tato hypertenzní reakce byla způsobena hyperaktivitou sympatiku. Zvířatům ze 3% skupiny se motorické a sensitivní funkce navrátily mnohem rychleji než u zvířat z 1,5% skupiny; morfometrie a magnetická rezonance míšních lézí neprokázaly žádný rozdíl. Postupné nebo neúplné vytvoření balónkové léze zabránilo rozvoji NPE. Na modelu transekce míchy NPE nevznikal. Nízké koncentrace isofluranu umožňují rozvoj NPE u potkanů s poraněnou míchou a signifikantně komplikují návrat jejich neurologických funkcí. Klíčovým mechanismem rozvoje NPE je pravděpodobně nadměrná aktivace sympatiku na podkladě náhlého zvýšení intrakraniálního tlaku v uzavřeném prostoru nad místem léze. Aktivaci sympatiku může být zabráněno hlubokou anestezií. Optimální koncentrace isofluranu pro provádění balónkové kompresní míšní léze je mezi 2,5 – 3% isofluranu ve vzduchu (proud směsi 300 ml/min). Skutečnosti, že se NPE rozvíjí na podkladě balónkové léze v povrchové isofluranové anestezii, může být využito pro přípravu modelu těžkého NPE, který vzniká na podkladě poranění CNS. Práce přispívá k objasnění etiopatogeneze neurogenního plicního edému a popisuje význam anestezie na rozvoj NPE, což může v budoucnu vylepšit terapii tohoto onemocnění.

9. Summary

Neurogenic pulmonary edema (NPE) is an acute life-threatening complication of the central nervous system (CNS) injury. Anesthetics can either promote or inhibit the NPE development. We examined the role of different concentrations of isoflurane anesthesia (1.5 - 3%) on the development of NPE in rats with balloon compressed spinal cord. The development of NPE was examined *in vivo* and on histological sections of lung tissue. Neurological recovery in animals anesthetized with 1.5% or 3% isoflurane was monitored using BBB and plantar tests for 7 weeks post-injury. The grade of the spinal gray and white matter sparing was evaluated using morphometry. The role of gradually developed spinal cord lesion and spinal cord transection in the development of NPE were evaluated also. NPE developed in all animals anesthetized with 1.5-2% isoflurane. Almost 42% of animals died due to massive pulmonary bleeding and suffocation; X-ray imaging, pulmonary index and histological sections showed massive NPE. More than 71% of animals anesthetized with 2.5-3% isoflurane had no signs of NPE. Blood pressure rose more rapidly in animals from 1.5% group than in 3% group; this hypertensive reaction was caused by the sympathetic hyperactivity. Animals from 3% group recovered their motor and sensory functions more rapidly than animals from 1.5% group; morphometry and MRI did not showed significant difference. Gradual or incomplete spinal cord compression prevented the NPE development. NPE did not develop in the model of spinal cord transection. Low concentrations of isoflurane promote the NPE in rats with spinal cord injury and significantly complicate the recovery of neurological functions. The most likely mechanism of NPE development is the severe sympathetic discharge on the basis of increased intracranial pressure in an enclosed intracranial space above the spinal cord lesion site. This sympathetic hyperactivity might be prevented by a deeper anesthesia level. Optimal concentration of isoflurane anesthesia for the performance of balloon compression spinal cord lesion is between 2.5-3% of isoflurane in air (flow of the anesthetic mixture 300 ml/min). The fact that the NPE development develops on the basis of balloon compression lesion under low degree of anesthesia might be employed for the development of severe NPE model, based on the CNS injury. Our study helps to understand etiopathogenesis of NPE and describes the role of anesthesia in the NPE development, which might improve the therapy of NPE.

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Acute and delayed implantation of positively charged 2-hydroxyethyl methacrylate scaffolds in spinal cord injury in the rat

Laboratory investigation

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Object. Hydrogels are nontoxic, chemically inert synthetic polymers with a high water content and large surface area that provide mechanical support for cells and axons when implanted into spinal cord tissue.

Methods. Macroporous hydrogels based on 2-hydroxyethyl methacrylate (HEMA) were prepared by radical copolymerization of monomers in the presence of fractionated NaCl particles. Male Wistar rats underwent complete spinal cord transection at the T-9 level. To bridge the lesion, positively charged HEMA hydrogels were implanted either immediately or 1 week after spinal cord transection; control animals were left untreated. Histological evaluation was performed 3 months after spinal cord transection to measure the volume of the pseudocyst cavities and the ingrowth of tissue elements into the hydrogels.

Results. The hydrogel implants adhered well to the spinal cord tissue. Histological evaluation showed ingrowth of connective tissue elements, blood vessels, neurofilaments, and Schwann cells into the hydrogels. Morphometric analysis of lesions showed a statistically significant reduction in pseudocyst volume in the treated animals compared with controls and in the delayed treatment group compared with the immediate treatment group ($p < 0.001$ and $p < 0.05$, respectively).

Conclusions. Positively charged HEMA hydrogels can bridge a posttraumatic spinal cord cavity and provide a scaffold for the ingrowth of regenerating axons. The results indicate that delayed implantation can be more effective than immediate reconstructive surgery. (DOI: 10.3171/SPI-08/01/067)

KEY WORDS • hydrogel • nerve tissue engineering • scaffold • spinal cord injury • spinal surgery • 2-hydroxyethyl methacrylate

THE treatment of SCIs requires a complex strategy to restore the function of the damaged tissue. Currently, treatment of SCIs includes early methylprednisolone administration according to the protocol of NASCIS

III.[4] stabilization surgery, and early and intensive rehabilitation. A primary spinal cord insult causes axonal degeneration followed by secondary tissue damage accompanied by glial scar formation, mesenchymal scarring (mostly represented by deposition of chondroitin sulfate proteoglycans), and posttraumatic pseudocyst cavities. The astroglial scar consists of a loose network formed by astrocytic processes attached by tight junctions.[3] Mesenchymal scarring is made up of fibrous connective tissue and collagen, forming a tight barrier. In addition to creating a mechanical barrier, both types of scars may adversely affect neuronal regeneration by producing neuroinhibitory molecules. Pseudocystic cav-

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ities, which develop in necrotic regions, are formed by a thin astrogliotic lining filled with extracellular fluid and macrophages. Such an environment is hostile for tissue regeneration and the restoration of function. Tissue engineering is focused on constructing a permissive environment at the injury site and thus supporting axonal regeneration.

Hydrogels are biomaterials characterized by a porous 3D structure with physical and chemical parameters that may be tailored. The use of various biomaterials has been proven to be effective in providing a cellular framework for regenerating tissue.[25,29,32,33] In contrast to uncharged hydrogels, those with a positive or negative charge provide a wide range of possibilities for modification of their chemical and physical properties.[19,20] Our previous results show that positively charged HEMA-based hydrogels are characterized by intensive ingrowth of connective tissue elements compared with negatively charged HEMA hydrogels.[15]

Most experiments using various biomaterials to treat SCI are designed in such a way that the implant is applied immediately after experimental spinal cord injury. In clinical settings, however, this approach is not applicable. Any therapeutic approach to treating a damaged spinal cord will rather be possible only after a certain delay following the primary insult. Several reports have shown that therapy delayed by weeks to months may still stimulate regeneration of the nervous tissue, and in some cases the results seem to be superior to those obtained using an acute experimental approach.[8,28]

In the present study, we studied the effectiveness of positively charged HEMA hydrogels to bridge a spinal cord lesion in rats. We compared the integration of hydrogel scaffolds implanted into the spinal cords of animals either immediately or 1 week after injury.

Materials and Methods

Hydrogel Synthesis

Macroporous hydrogels based on HEMA with MOETACl were prepared by radical copolymerization of monomers (HEMA 0.67 g, MOETACl 0.12 g, and ethylene dimethacrylate 0.019 g as crosslinker) in the presence of fractionated particles of NaCl with a diameter of 50–90 μm (10.02 g) and the solvent polyethylene glycol (MW 400, 3.79 g) using an initiator 2,2'-azo-bis-isobutyronitrile (0.0067 g) for 8 hours at 80°C. Polymerization was performed in a pelleting apparatus as described previously.[13] After polymerization the hydrogels were washed with water and physiological saline solution once a day for 5 days. The hydrogels thus obtained had communicating pores (2×10^6 pores per cm^3) with an average size of 80 μm, specific pore volume of 0.45 (combined volume of all pores in 1 cm^3 of hydrogel).[20]

Hydrogel Implantation and Animal Care

A complete spinal cord transection was selected as a model for evaluating in vivo the effect of hydrogel implantation on SCI immediately after lesion creation and after a 1-week delay. Twenty-three 8-week-old Wistar rats (Velaz, Ltd.) each weighing 300–350 g were used. Anesthesia was induced by means of an intraperitoneal injection of pentobarbital (30 mg/kg). Atropine (0.2 ml administered subcutaneously) and gentamicin (0.05 ml administered intramuscularly) were used preoperatively. A T-9 laminectomy was performed under a surgical microscope using aseptic surgical technique. The dura mater was opened and a 2-mm thick segment of spinal cord was dissected out to produce the SCI. Using a surgical microscope, we ensured that no remaining tissue was left in this segment.

In 10 animals the dura was sutured using 10-0 Ethilon (Ethicon, Johnson & Johnson), followed by muscle and skin closure. These animals served as a control group. In 7 animals, we inserted a 2 × 2 × 2–mm block of HEMA-MOETACl hydrogel immediately after the transection, followed by suturing of the dura, muscles, and skin. These animals served as the acute treatment group (referred to in this paper as the acute group). In the other 6 animals, we reopened the surgical site 1 week later, removed the debris from the lesion, and implanted a block of hydrogel inside the lesion. The dura was closed again, followed by suturing of the muscle and skin. These animals served as the delayed treatment group (delayed group). In all 3 groups, bladder expression was performed until the recovery of sphincter control, and gentamicin was administered intramuscularly for 5 days to prevent urinary infection. All animals were kept in cages with food and water ad libitum. Behavioral evaluation was performed once a week for 3 months in both groups of hydrogel-treated animals (acute and delayed) using the Basso-Beattie-Bresnahan rating scale in order to evaluate the functional effect of hydrogel implantation. This study was performed in accordance with the European Communities Council Directive of November 24, 1986 (86/609/EEC) regarding the use of animals in research and was approved by the Ethics Committee of the Institute of Experimental Medicine, Academy of Sciences of the Czech Republic in Prague.

Tissue Processing and Histology

Animals were killed on postoperative Day 14 (2 control animals), Day 28 (2 control animals), and Day 90 (6 control animals and 13 animals treated with hydrogel) by means of an intraperitoneal injection of pentobarbital (which initially induced a deep state of anesthesia). They were then perfused with physiological saline followed by 4% paraformaldehyde in 0.1 M phosphate buffer. The spinal cords were left in bone overnight, then removed and postfixed in the same fixative for at least 24 hours. Next, 3-cm long segments of the thoracic region of the spinal cords including injury epicenters with implanted hydrogels were carefully dissected out, frozen, and cut in 40-μm sections on a cryostat. The sections were stained with H & E, Luxol fast blue, and cresyl violet using standard protocols. For immunohistochemical studies, the following primary antibodies and dilutions were used: GFAP-Cy3 (1:200, Sigma-Aldrich) to identify astrocytes, NF 160 (1:200, Sigma-Aldrich) to identify neurofilaments, p75 (1:100, Chemicon International) to identify Schwann cells, RECA-1 (1:50, Abcam.) to identify endothelial cells of blood vessels, ED-1 (1:100, Invitrogen) for macrophages, CS-56 (1:50, Sigma-Aldrich) to identify chondroitin sulfate, and CD4 (1:800, Abcam). Alexa Fluor 488 goat anti–rabbit IgG (1:200, Invitrogen), IgM Cy3 (1:100, Chemicon International), and Alexa Fluor 594 goat anti-rabbit IgG (1:500, Invitrogen) were used as secondary antibodies.

Cavity Measurement

For measurements of the cavity volume, the Luxol fast blue and cresyl violet sections were examined. For morphometry, every fifth section was selected and its image was captured by means of a digital camera; high-resolution images were used to trace the areas of the cavities. The identified areas in individual sections were measured using image analysis software (Neurolucida, version 5.05.4). The volume of the spared tissue was assessed as the sum of cross-section areas multiplied by the distance between them. The sizes of the cavities in different groups were compared using the Student unpaired t-test (probability values ≤ 0.05 were considered statistically significant).

Magnetic Resonance Imaging

Spinal cords inside a vertebral column were scanned ex vivo on an MR spectrometer (Bruker Biospec 47/20, 4.7 tesla, 20 cm horizontal bore) equipped with a 200-mT/m gradient system and the homemade quasi-TEM (transverse electromagnetic) mode operating microstrip surface coil for spinal cord imaging.[7] Sagittal images (matrix 512 × 160, field of view 10 × 3 cm, slice thickness 0.5 mm, contiguous slices, TE/TR 70/2500 msec) and axial images (matrix 256 × 128, field of view 4 × 2 cm, slice thickness 0.5 mm, slice

0.5 mm, TE/TR 70/2800 msec) were acquired using an ordinary RE sequence with a RARE factor of 8.

Results

ion Development in Untreated Animals

Observation of the lesion site over time revealed the gradual development of pseudocysts during the first 3 months stinjury. At 2 weeks postinjury, the lesion site was dominated by a glial scar forming a barrier between the 2 segments of the spinal cord. Minor pseudocysts were found at lesion site (Fig. 1A). One month after SCI, however, the eudocysts already dominated the injured area (Fig. 1B). ree months after SCI, the lesion site consisted primarily a major pseudocyst, which had developed by a gradual cess of the individual cavities merging together (Fig.).

mparison of Acute and Delayed Implantation of drogel

We compared the results of hydrogel implantation in the groups of hydrogel-treated animals, acute (implanted immediately after cord transection) and delayed (implanted 1 ek postinjury). Both groups showed good hydrogel integration inside the lesion site although pseudocyst cavities re found at the implant–tissue borders. Care was taken to sure that the hydrogel fitted well inside the transection vity during implantation, in both the acute and the deed conditions. The mechanical properties of the scaffold

allowed it to adhere to the spinal cord stumps without the need for excessive pressure on the nervous tissue. Even when the hydrogel firmly adhered to the spinal cord tissue, in some cases pseudocysts were found at the border site; rats treated by implantation of hydrogel immediately after injury (acute group) developed large pseudocysts (Fig. 1D), whereas the hydrogel in the rats treated by delayed implantation (delayed group) firmly adhered to the tissue and the volume of pseudocyst cavities in these animals was significantly smaller (Fig. 1E).

Magnetic Resonance Imaging

We obtained T2-weighted MR images of 3 spinal cords treated with hydrogel (1 acute and 2 delayed). The positively charged HEMA hydrogels showed a hypointense signal on T2-weighted images, while the pseudocyst cavities were hyperintense. The spinal cord from the animal in the acute group showed a hypointense signal indicating hydrogel and a large residual area of a hyperintense signal around the hydrogel (pseudocyst cavity; Fig. 1F). In contrast, the spinal cord from the animal in the delayed group showed only a weak signal or no hyperintense signal around the hydrogel (Fig. 1G). The MR imaging findings were confirmed by our histomorphometric findings, which excluded the possibility that the cavities could represent histological artifacts.

Cavity Measurement

At 3 months after implantation we found significant between-group differences in the volume of the pseudocyst
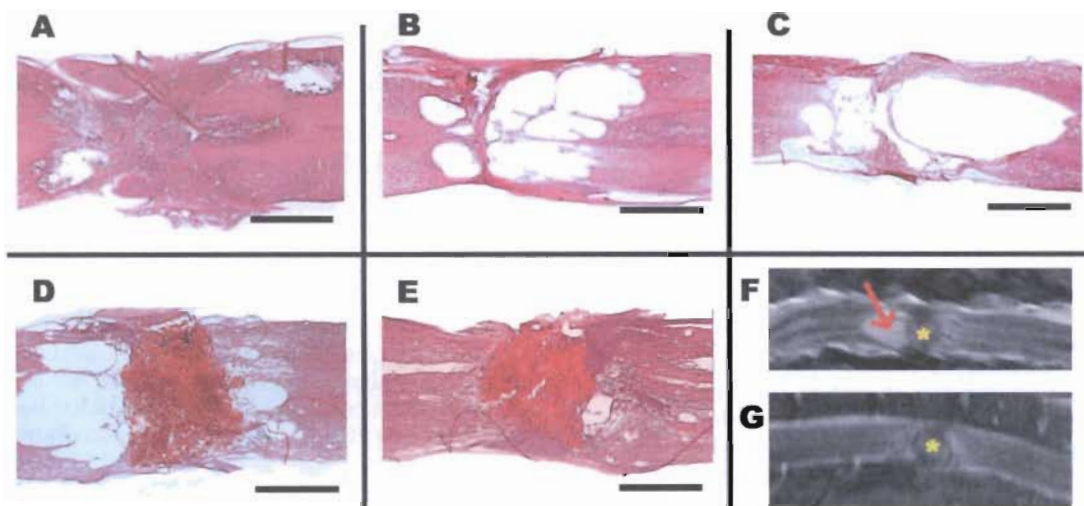

FIG. 1. Representative photomicrographs (A–E) and MR images (F and G) illustrating SCI development after complete transection at the T-9 level without treatment (control group, A–C) and with HEMA-MOETACl hydrogel implantation performed immediately (acute group, D and F) or 1 week (delayed group, E and G) after transection. A: Spinal cord lesion 2 weeks after transection. The lesion is dominated by mesenchymal and glial scarring. B: Lesion site 4 weeks after transection is composed of several posttraumatic pseudocysts. C: Lesion site 12 weeks after transection is dominated by a major posttraumatic pseudocyst, and the integrity of the spinal cord is severely damaged. D: Spinal cord from an animal in the acute group, 2 weeks after injury. Even though the lesion site is partially restored, several pseudocyst cavities are still present on the implant–spinal cord border. E: Spinal cord from an animal in the delayed group, 12 weeks after injury. Spinal cord integrity is restored, and the hydrogel forms a bridge between both stumps of the spinal cord. F and G: Spinal cord lesions treated with immediate (F) and delayed (G) implantation of HEMA-MOETACl hydrogel (asterisk). Notice the pseudocyst cavity (arrow) formed at the hydrogel–tissue border in the acutely treated animal. In contrast, no cavity is evident and the hydrogel adheres well to the spinal cord tissue after delayed hydrogel implantation. H & E, bar = 200 μm.

cavities. The mean volume of the pseudocyst cavities was largest in the control group and significantly smaller in the implanted animals. Comparison of the 2 groups of rats with implanted scaffolds showed that the mean volume of the pseudocyst cavities was larger in the acute group than in the delayed group (Fig. 2).

Histological Evaluation of Hydrogel Integration

Three months after SCI, some pseudocysts were found at the implant–tissue border; the hydrogel pores were mostly filled with connective tissue elements, such as fibroblasts, collagen, blood vessels, and chondroitin sulfate (Fig. 3A–D). Cellular infiltration in the hydrogel pores consisted mostly of macrophages, as seen in H & E–stained sections and confirmed by means of ED-1 immunohistochemical studies, together with a few CD4-positive lymphocytes. Histologically, we specifically searched for any inflammatory response or adverse reaction of the tissue to the implanted material. Except for occasional foreign body granulomas seen at the implant–tissue borders, no purulent inflammatory reaction was observed.

Neurofilaments grew from both the proximal and distal ends of the lesion toward the implant, some crossing the border and entering the porous structure of the hydrogel scaffold (Fig. 3E and F). Neurofilaments were found not only at the tissue–implant border but also in the central part of the implant, and in most cases they infiltrated the whole volume of the hydrogel (Fig. 3G). Analysis of GAP-43 immunostaining showed that the regenerating neurofilaments grew in parallel with the long ascending and descending tracts. We assume that these neurofilaments represent regenerating long axonal tracts and local neuronal sprouting. Schwann cells (p75-positive cells) were also found at the lesion site, growing from spinal cord roots towards the hydrogel. They readily crossed the nervous tissue–implant border and infiltrated the hydrogel scaffold. When double staining (NF 160 and p75) was used, we found Schwann cells following neurofilaments; processes of both cells ran parallel to each other (Fig. 3H). Outside the hydrogel, most p75-positive cells were found at the spinal root zone, the most probable source of these Schwann cells. Astrocytes, on the other hand, rarely crossed the nervous tissue–implant border (Fig. 3I). Only rarely were astrocytic processes found on the edge of the hydrogel implant. On the tissue–implant border, the astrocytes formed a thin layer of a dense network, constituting a glial scar. It was not significantly different from glial scarring in the control group.

We found no between-group differences in the number of neurofilaments or Schwann cell projections inside the hydrogels. Both groups showed extensive ingrowth of neurofilaments inside the hydrogel pores and Schwann cells penetrating the implants from the spinal cord–spinal nerve junction zone and accompanying the neurofilaments.

Behavioral Testing

We tested both groups of animals with implanted hydrogels (acute and delayed). Immediately after SCI, the mean Basso-Beattie-Bresnahan score in both groups dropped to 0. We did not find any significant difference between the 2 groups of hydrogel-treated animals, as most animals in both groups remained paraplegic during the 3-month observation period.
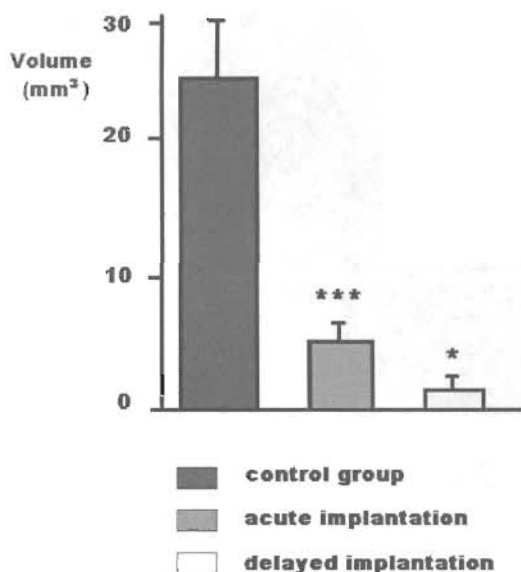

Fig. 2. Bar graph illustrating mean pseudocyst cavity volumes (with standard deviations) for the control animals and the acute and delayed treatment groups. *p < 0.05, ***p < 0.001, Student unpaired test.

Discussion

In anamniotic vertebrates, such as the eel, the axons readily cross a transection lesion,[11] but in higher vertebrates the site is marked by glial scarring and pseudocyst cavities, forming an almost impenetrable barrier between the cranial and the caudal segments of the spinal cord. While stem cell therapies lead to some functional improvement in animals with SCI, they do not lead to reconstitution of functional tissue in large lesions due to cavity formation.[1,24,27] Thus "bridging the gap" is one of the most crucial steps in spinal cord lesion repair. Various biomaterials have been used to date in order to bridge the tissue defect after SCI.[13,14,17]

Advances in the chemistry of biomaterials allow us to use hydrogels in experimental approaches to SCI. Some of the properties of hydrogels make hydrogel scaffolds the most promising materials for neural tissue engineering: 1) They can be synthesized and produced in large quantities. 2) Their chemical and physical properties can be easily modified, allowing them to be prepared for immediate use in the operating room. 3) The diffusion parameters within implanted hydrogels attain values similar to those of developing neural tissue.[5] 4) Their tissue reconstruction properties may be improved using stem cells, neurotrophins, or signaling sequences.[17,24,25,34] 5) They do not raise controversy such as is seen in association with stem cells, particularly embryonic tissue implants. With their 3D porous structure, hydrogels provide an acellular mechanical framework for the ingrowth of supportive tissue, and together with other strategies supporting regeneration (for example, the use of growth factors or stem cells) they may contribute to regeneration after SCI.

The advantage of using artificial implants is that we can modify their properties in a controlled fashion. The hydro-
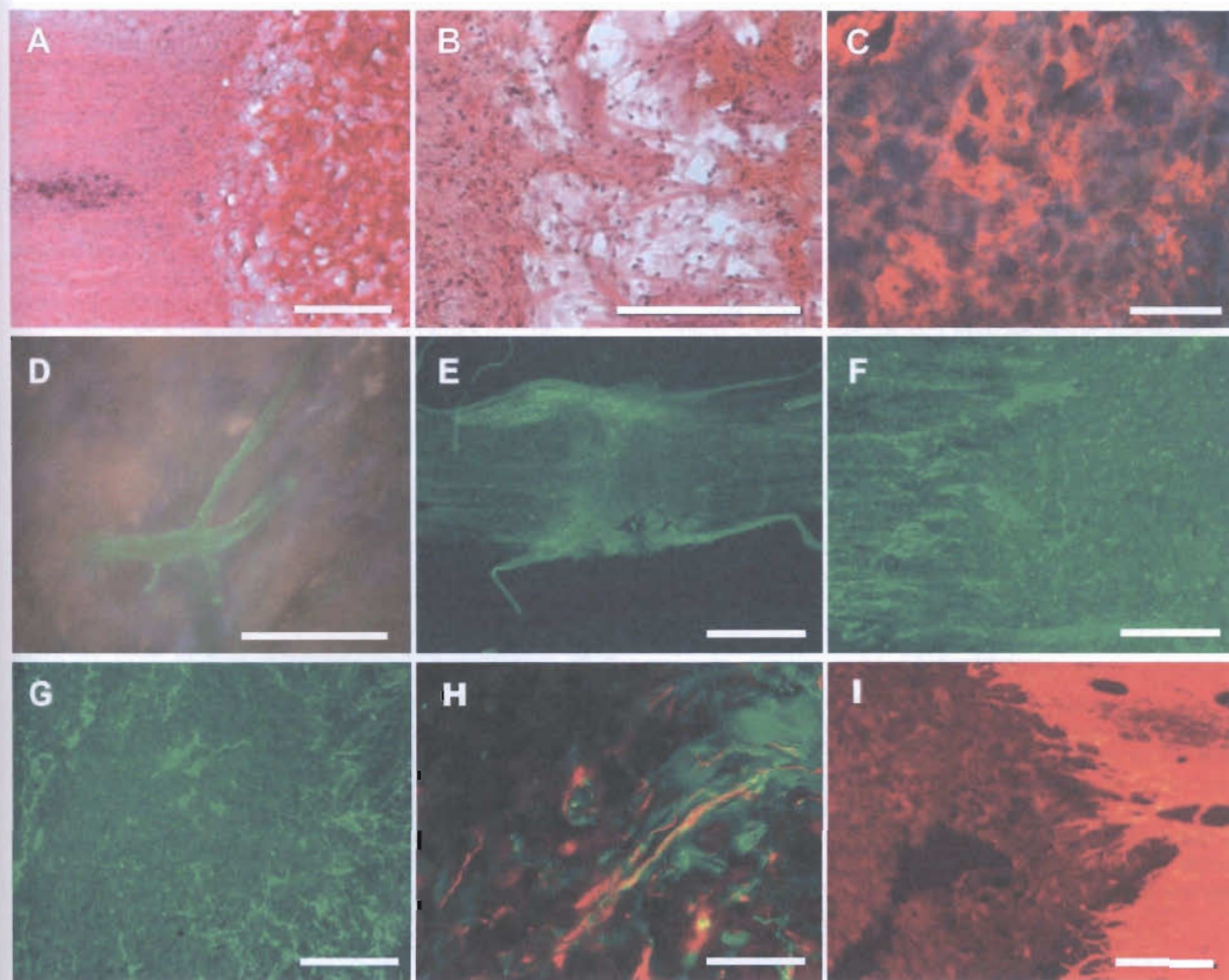
FIG. 3. Photomicrographs illustrating spinal cord tissue restoration using a positively charged HEMA scaffold. The transected spinal cord is bridged using a HEMA-MOETACl hydrogel which serves as a scaffold for tissue ingrowth and cell infiltration. A: The cranial spinal cord adheres well to the hydrogel. H & E, bar = 100 μm. B: Loose connective tissue forms the bridges between spinal cord tissue and porous hydrogel. H & E, bar = 100 μm. C: Chondroitin sulfate infiltrates the porous structure of the hydrogel in both acute and delayed hydrogel treatment groups. CS56 immunostaining, bar = 50 μm. D: Blood vessels grow and branch inside the hydrogel. RECA-1 immunostaining, bar 50 = μm. E: Neurofilaments grow from the spinal cord, cross the hydrogel–spinal cord border, and infiltrate the hydrogel pores. NF 160–g488 immunostaining, bar = 500 μm. F and G: Neurofilaments readily cross the lesion–scaffold border and grow inside the pores, also infiltrating the central parts of the scaffold. NF 160–g488 immunostaining, bar = 100 μm. H: Schwann cells from the dorsal root area grow inside the hydrogel following the growth of the neurofilaments inside the hydrogel pores. Double staining with p75 and NF 160–g488, bar = 100 μm. I: In contrast, astrocytes only barely cross the spinal cord–implant border. Immunostaining with GFAP-Cy3, bar = 100 μm.

gels have a highly porous structure, with a pore size mostly between 10 and 100 μm.[2,16,21,31,32] Several groups have attempted to create oriented pores in order to promote directed axonal regeneration.[9,21] Prang and colleagues[21] showed that after implanting a scaffold with oriented pores in an acute SCI, these alginate-based hydrogels induced directed axon regeneration. Moreover, adding adult neural progenitor cells inside the pores promoted cell contact–mediated axon regeneration in vitro. Another approach to improving axonal regeneration involves adjusting the adhesion properties. Woerly et al.[30] studied an HPMA hydrogel modified with a peptide cell–binding peptide sequence (RGD [ar-

ginine-glycine-aspartic acid]). The HPMA-RGD implant showed stronger adhesion to the host tissue and promoted ingrowth and spread of astrocytes and neurofilaments inside the hydrogel. The adhesion properties of hydrogels may also be modified using various functional groups with a positive or a negative charge. The use of DRG cultured on an agarose hydrogel with a covalently bound chitosan (polycationic polysaccharide) supported a significant increase in the length of regenerating neurites.[10] Another important factor is the mechanical stability of the hydrogel. In the early 1990s, Marchand et al.[18] showed that a collagen scaffold stable for only 2–3 months fails to provide long-

term support for axonal regeneration. Extending the stability of the collagen gel by cross-linking improved the mechanical properties of the matrix and ensured axonal regeneration over a 6-month period. This finding is especially important in light of the efforts to fabricate biodegradable implants.

Combining scaffold implantation with the use of neurotrophic factors or stem cell treatment may lead to improved results. Loh et al.[17] found that modifying an HPMA-RGD hydrogel with either brain-derived neurotrophic factor or ciliary neurotrophic factor significantly increases the ingrowth of axons into the implant compared with the results achieved with unmodified hydrogels. In another study,[35] modifying an agarose gel with the extracellular protein laminin or nerve growth factor-releasing microcylinders significantly enhanced neurite extension from dorsal root ganglia. Tsai et al.[26] used a pHEMA-methylmethacrylate hydrogel with a combination of various matrices and growth factors in a spinal cord transection. The results of the study showed that specific combinations may lead to selective improvement in the regeneration of selected brainstem tracts.

The HEMA-based hydrogels represent another group of polymers used in SCI research.[2,9] These hydrogels have proved to be biocompatible. Modifying the surface charge of the hydrogel using copolymers and polyelectrolyte complexes further modifies its mechanical properties or the water content in an equilibrium-swollen state.[20] The pronounced ingrowth of connective tissue elements was found in positively charged HEMA copolymers associated with nervous tissue elements, such as NF 160–positive neurofilaments and Schwann cell projections. In addition, in experiments evaluating the growth of cells in 3D hydrogel structures, bone marrow stromal cells were found to grow more abundantly in positively charged hydrogels than in negatively charged ones.[16]

Transplantation of Schwann cells has been studied as a therapeutic tool in animal models of SCI.[5,6] Woerly et al.[32] reported that Schwann cells infiltrated HPMA hydrogel myelinated regenerating neurofilaments. Bunge[6] found regenerating neurons growing inside a Schwann cell bridge after spinal cord transection in rats. In our study, we found Schwann cells growing from the spinal root area, invading the lesion area, and infiltrating the implanted hydrogel together with nerve cell processes.

Various hypotheses concerning the development of posttraumatic cavities have been suggested.[12] Our results show that when an HEMA hydrogel is implanted after a 1 week delay, the stumps of the spinal cord are bridged together leaving significantly smaller pseudocyst cavities compared with the results of immediate treatment. Delayed treatment for SCI has already proven effective in some cases.[8,28] Coumans et al.[8] found that using embryonic spinal cord tissue may improve histological and functional outcome even 2 or 4 weeks after spinal cord transection. Woerly et al.[33] showed an improvement in behavioral and histological outcomes when NeuroGel (Organogel Canada Ltée) was implanted 3 months after a contusion lesion. The situation with respect to the effect of delayed therapy in SCI is still obscure, but some elucidation may be provided. The first days following central nervous system injury are characterized by an influx of inflammatory cells, which is especially pronounced in the spinal cord.[22] This influx of inflammatory cells results in the formation of a deleterious environment. After the end of the first week postinjury, however, the concentrations of most of the inflammatory cells are already on the decline, and the spinal cord tissue is therefore more suitable for treatment by transplantation. Moreover, excessive manipulation of edematous tissue may result in damage to the areas adjacent to the lesion. With delayed transplantation there may be less damage of this kind and, as a result, improved adherence to the spinal cord stumps.

Our results show that delaying hydrogel implantation until 1 week after an SCI may be preferable to implanting hydrogel immediately after injury. In clinical practice, the implantation of a bridging scaffold during acute posttraumatic surgical stabilization may not be beneficial.

Because we did not find a behavioral effect in association with scaffold implantation such as has been found in association with stem cell implantation,[1,23,24,27] it might be useful to combine the 2 strategies. Further study is required to assess the combination of scaffold implantation and stem cell implantation in SCI treatment.

Conclusions

Our results show that HEMA-based positively charged hydrogel is suitable for spinal cord repair. The hydrogel forms a bridge between both stumps of the spinal cord following a complete transection. The porous structure is infiltrated with blood vessels, neurofilaments, and Schwann cells. Delayed implantation of such a scaffold inside a transection lesion is associated with a statistically significant decrease of posttraumatic cavity volume in comparison with the results of acute implantation.

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Review

Methods for behavioral testing of spinal cord injured rats

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Abstract

Behavioral outcome in rats with spinal cord injury (SCI) is the most important factor for evaluating the extent of injury and treatment efficacy. For this purpose, a number of behavioral testing methods can be used. In this review, 35 individual locomotor, motor, sensory, sensory–motor, autonomic or electrophysiological behavioral tests, their weaknesses and strengths, testing conditions, the need for habituation, pre-training and/or food deprivation, methods for increasing the animals' skills, systematic testing protocols and methods for selecting the proper behavioral tests for particular injury models are discussed on the basis of a retrospective analysis of scientific studies published from 1995 to 2007. This review is primarily targeted towards researchers outside the field or to researchers new to the field of SCI.
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Contents

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1. Introduction

Spinal cord injury (SCI) is a severe, often life-threatening and debilitating clinical condition, with an incidence of 40 new cases per million people throughout the world each year, affecting mainly young people with a mean age of 28.6 years (Basso. 2004; Grill. 2005; Kwon et al., 2002). It is characterized by a complex of motor, sensory and autonomic dysfunctions, the degree of which is characteristic for the severity of the SCI. In experiments, the most often used experimental animal is the rat (*Rattus norvegicus*), in which several models of complete or incomplete

SCI can be performed, such as transection, hemisection, contusion, compression, ischemia, excitotoxic lesions or crush injuries of the spinal cord (for review, see Grill, 2005; Kwon et al., 2002). Ideally, the behavioral responses in animal models should be relevant to the clinical signs of SCI in human patients (Muir and Webb, 2000; Syková et al., 2006b).

The final functional status of the animal depends upon the extent of neuronal damage in the gray matter at the injury site, the loss of ascending and descending axons in the white matter, and the reorganization of the remaining nervous system. A proper evaluation of the regenerating nervous tissue at the site of injury can be done using several morphological methods, including immunohistochemical staining of regenerating neurons and axons or retrograde and anterograde staining. The most important factor for future clinical studies is, however, the functional outcome, evaluated experimentally by a number of behavioral tests (Basso, 2004) (Table 1). They are used to determine the lesion severity and location, to document the extent of recovery following SCI and to identify the integrity of specific motor and sensory pathways that may be the substrate of recovery after SCI. Some tests are simple, not requiring any special training or equipment, while others are more sophisticated and require specialized and/or expensive equipment (Table 2). According to the type of data collected, these tests can be categorized as: (i) endpoint measures, in which behavior is scored according to some goal to be reached, e.g. the time to cross a beam or the number of pellets eaten; (ii) kinematic measurements, which can range from a qualitative description of movement, such as weight support or toe clearance in the BBB test, to continuous kinematic measurements, such as distances, angles or velocities of body parts during movement; (iii) kinetic measurements, which quantify or describe the force produced by a limb or limbs, for example, during weight support; and (iv) electrophysiological measurements, where muscle or sensory system activity is detected and measured (Muir and Webb, 2000). It should be noted that one test could fall into more than one category. For example, in the limb hanging test, the grasping itself can be evaluated as an endpoint measure, while hanging can be considered as a kinetic measurement (Table 1).

In this review, we have categorized the behavioral tests as: (i) locomotor tests, testing the locomotor apparatus of the animal; (ii) motor tests, analyzing the strength, coordination and other abilities of the skeletal muscles; (iii) sensory tests, evaluating proprioception, touch, pain or temperature sensing; (iv) sensory–motor tests, testing the proper connection between the sensory and motor systems; and (v) autonomic tests, evaluating the function of the sympathetic and parasympathetic systems. In addition, (vi) reflex-response based tests are considered separately (Tables 1 and 3). It should be noted that the categorization into particular groups is not absolute; for example, the limb hanging test mainly analyzes motor function, but proper grasping is not possible without the sensory signals from the skin of the paws.

This review was designed to discuss a range of testing methods suitable for the behavioral testing of rats with SCI. Testing conditions, the strengths and limitations of each test and methods for preparing an appropriate study, based on the authors' own experiences, are discussed. Moreover, the review contains recommendations for the use of different behavioral testing methods in different injury models and describes the particular advantages and disadvantages of each testing method.

To date, only a few papers have focused on these questions, even though the call for a comprehensive overview to help in the appropriate selection of tests for individual studies, together with the standardization of behavioral testing methods across labs, remains strong (Basso, 2004; Muir and Webb, 2000; Wahlsten, 2001). Although this review is primarily designed to provide researchers outside the field of SCI or researchers new to the field with a quick orientation to this broad and often controversial topic, it might also help specialists with a heretofore somewhat narrow focus to expand the horizon of their knowledge (Tables 1, 3, 4 and 5).

2. Data analysis

All recommendations are based on an analysis of 553 original papers or short communications, published in the English language from January 1995 to July 2007 in journals with a defined impact factor. A particular publication was included in the analysis when the experiment was performed on rats with any type of SCI, which were subsequently tested behaviorally. The search for such papers was performed using Medline, Web of Science, OVID and Google Scholar. The parameters studied were the strain of the rats, their gender, the type of injury and the behavioral testing methods used. Data were analyzed using MS Excel. Any discussion related to the behavioral testing method was taken into account, if appropriate for this review.

3. Testing conditions

To obtain reliable, reproducible and worthwhile data, the testing procedure must follow several rules that are established before beginning the study or in preliminary experiments. These rules are often described as testing conditions. Once established, they must not be changed throughout the study.

3.1. Environment

Behavioral testing should be done in a quiet, warm and ventilated room with minimal distractions, where both sufficient light and the possibility of darkening are available. The temperature of the room should be the same as in the animal care facility. The relative humidity

Table 1

List of the main behavioral methods for testing spinal cord injured rats

Test	Type	Reflects	S/O	Range of scale	Scale	Reference
Locomotor tests						
Primary open-field	Kinematic	Locomotion	S	Different	Ordinal	Tarlov (1954) Bohlman et al. (1981)
BBB	Kinematic	Locomotion	S	0–21	Ordinal	Basso et al. (1995)
Open field activity	Endpoint Kinematic	Locomotion Exploring activity	O	1–5	Ordinal	Bignami (1996)
Automated walkway	Kinematic	Locomotion	O	Complex	Ordinal	Hamers et al. (2001)
Footprint analysis	Kinematic	Motor coordination	O	%	Ordinal	Chan et al. (2005)
Kinematic analysis	Kinematic	Locomotion	O	%	Ordinal	Metz et al. (1998)
Thoracolumbal height	Kinematic	Weight support	O	%	Ordinal	van de Meent et al. (1996)
Swim test	Kinematic	Swimming ability	S	0–17	Ordinal	Smith et al. (2006a)
EW notation	Kinematic	Locomotion	O	Complex	Ordinal	Eshkol and Wachmann (1958)
Motor tests						
Inclined plane	Kinetic	Muscle strength	O	Angle	Ordinal	Gale et al. (1985)
Limb hanging	Endpoint	Grasping	O	Yes/no	Ordinal	Diener and Bregman (1998)
	Kinetic	Muscle strength		Time (s)	Continuous	
Limb grip strength	Kinetic	Muscle strength	O	Time (s)	Continuous	Pearse et al. (2005)
Forelimb asymmetry	Kinematic	Paw preference	O	%	Ordinal	Schallert et al. (1986)
Rearing	Kinematic	Paw preference	S	No. of rears	Ordinal	Arvanian et al. (2006)
Food pellet reaching	Kinematic Endpoint	Motor coordination	O	0–9 Time (s)	Ordinal Continuous	Whishaw (2000)
Sensory tests						
Hot plate	Endpoint	Temperature	O	Up to 60 s	Continuous	Gale et al. (1985)
Cold spray	Kinematic	Temperature	S	0–3	Ordinal	Yu et al. (1998)
von Frey	Kinematic	Mechanical allodynia	O	0.008–300 N or 2.5–125 g	Ordinal	Liebscher et al. (2005)
Paw compression	Endpoint	Pain	O	Force (N)	Continuous	Randall and Selitto (1957)
Withdrawal reflexes	Endpoint	Reflex	O	Time	Continuous	Gale et al. (1985)
Sensory–motor tests						
Rope walk	Endpoint	Balance	O	% or 0–4	Ordinal	Kim et al. (2001)
Narrow beam	Endpoint	Balance	O	0–6	Ordinal	Hicks and D'Amato (1975)
Grooming	Kinematic	S–M connections	S	0–5	Ordinal	Bertelli and Mira (1993)
Foot slip	Endpoint	S–M coordination	O	%	Ordinal	Metz and Whishaw (2002)
	Kinematic	Motor function	S	0–6		
Grid walking	Kinematic	Sensory–motor coordination	O	0–20	Ordinal	Behrmann et al. (1992)
	Endpoint					
Reflex–response based tests						
Toe spread reflex	Endpoint	Reflex	S	Absent–normal–abnormal	Ordinal	von Euler et al. (1997)
Contact placing response	Endpoint	Reflex	S	%	Ordinal	Kunkel et al. (1993)
Righting reflex	Kinematic Endpoint	Reflex	S	0–3	Ordinal	Gale et al. (1985)
Autonomic tests						
Ex copula erection	Kinematic	Erection	S	1–5	Ordinal	Holmes et al. (1988)
Non-contact erection	Kinematic	Erection	S	1–3	Ordinal	Sachs et al. (1994)
Mating	Kinematic	Erection	S	1–6	Ordinal	Nout et al. (2007)
Cystometric monitoring	Electro-physiology	Micturition erection	O	–	–	Nout et al. (2005)
Autonomic dysreflexia	Endpoint	Autonomic dysreflexia	O	BPM and mmHg	Continuous (yes/no)	Cameron et al. (2006)
Special tests						
Evoked potentials	Electro-physiology	Sensory and motor pathways	O	–	–	Fehlings et al. (1987)
MRI	Imaging	Neural pathway function	O	–	–	Hofstetter et al. (2003)

The table shows the method name, the type of the test, the neurological function it reflects, whether it is subjective (S) or objective (O), the range of the scale, the type of the scale, when the test was developed and the primary reference(s). BPM—beats per minute.

Table 2
Categorization of behavioral testing methods according to complexity

Simple	Moderate	Complex
Primary open field	BBB	E–W notation
Rope walk	Open field activity	Automated walkway test
Beam walk	Thoracolumbal height	Footprint analysis
Inclined plane	Swim test	Kinematic analysis
Limb hanging	Limb grip strength	Foot pellet reaching
Rearing	Forelimb asymmetry	Ex copula erection
Cold spray	Hot plate	Non-contact erection
Paw compression	Von Frey	Telemetric monitoring
Grooming	Foot slip	Autonomic dysreflexia
Toe spread reflex	Grid walk	Electrophysiology
Contact placing response	Mating	fMRI
Withdrawal reflexes		
Righting reflex		

should be maintained in a range between 45% and 65%; values outside this range can favor the proliferation of certain airborne pathogens resulting in health problems. An air change rate of at least 15 times per hour is important for the animal, but also for the scientist to help reduce staff exposure to harmful allergens present in rat fur. Generally, noise in the testing room should be minimized, including high-frequency noises undetectable by the human ear; noise levels should never exceed 50 dB. In several laboratory animals, the use of a constant noise, such as background music, whenever the animals are in the testing environment, has been described to assist in masking other loud and/or unexpected sounds. In contrast, there is no scientific evidence for the need to use such constant noise with rats (Tatlisumak and Fisher, 2006). It is very important to avoid excessive light, which might cause retinal damage to the animals and thus impair their health, abilities and behavioral performance. Albino rats are more sensitive to light than other strains of rats (Tatlisumak and Fisher, 2006). Appropriate behavioral testing apparatuses should be installed and their reliability checked before the experiment starts. All parts of the equipment should be frequently cleaned, but without the use of any cleaning materials that could impair the behavior of the animal. If a startled animal emits a fear scent onto the testing apparatus, other animals can easily detect it, and a marked decline in the behavioral performance of subsequent animals might result (Basso, 2004; Wahlsten, 2001).

3.2. Animals

Several kinds of rat inbred strains, such as Wistar, Louis, Long-Evans, Fisher or Sprague-Dawley, are suitable for SCI studies. In the past 12 years, the most favored rat strain in SCI studies has been Sprague-Dawley, followed by Wistar (Table 4). In any given study, only one strain should be used if differences between the different strains are not the subject of the study, because there exists

considerable variability in locomotor and sensory recovery rates between strains. For example, the locomotor recovery of Sprague-Dawley rats is quicker than that of Wistar rats, while the recovery of Wistar rats is quicker than that of Long-Evans rats (Mills et al., 2001). The animals should be of the same age or, even better for SCI studies, of the same weight. Differences in the capacity for behavioral recovery between young and aged animals are well known (Brailowsky and Knight, 1987). For example, in motor function differences in recovery may be due in part to an increase in age-related impairments in balance and coordination (Wallace et al., 1980) that correlate with the loss of cerebellar noradrenergic function (Bickford, 1993). The weight of the animal is more important for the type of injury than for the behavioral testing procedure. For SCI studies, on adult animals the weight is usually set between 300 and 350 g. In addition, the temperature of the animal during the injury procedure should be maintained at 37 °C, to prevent hypo- or hyperthermia (Urdzíková and Vanický, 2006; Vanický et al., 2001).

Both male and female rats are used in SCI studies worldwide; both have advantages and disadvantages. Our analysis showed that females were used in 39% of SCI studies, males in 33% and animals of any gender in the remaining 28%. The main reasons for the more frequent use of females are the easier urinating procedure, the more rapid onset of an automatic urinary bladder and the lower occurrence of lower urinary tract infections. All these advantages are based on the presence of a significantly shorter and straighter urethra in female rats in comparison with males. On the other hand, female rats exhibit hormonal instability, thus for SCI studies, the performance of the lesioning procedure during the same part of the estrous cycle should be required (Chaovipoch et al., 2006; Tatlisumak and Fisher, 2006). This requirement stems from reports of the neuroprotective effects of estrogen and progesterone (Chaovipoch et al., 2006; Roof and Hall, 2000). However, new reports have indicated that estrogen (Hall et al., 2005; Swartz et al., 2007) and progesterone (Fee et al., 2007) do not, in fact, have any neuroprotective effects.

3.3. Housing of the animals

We prefer to house the animals in cages of two, because separation might increase the stress of the rat. For some behavioral tests, a known room-mate in the home cage might be used as an attractant. On the other hand, housing three or more spinal cord injured rats in one standard-sized cage would not be reasonable because of the increased risk of transferring infections, a shortage of space for movement and an increased need for cleaning and maintenance. Nevertheless, increasing the number of animals in one cage is the easiest way of enriching the environment (Tatlisumak and Fisher, 2006). The substrate in the cages of the animals should be soft, smooth, and absorbent to avoid complications

Table 3

A list of the main behavioral tests showing the necessity of previous habituation and/or pre-training, the most popular type of spinal cord injury for which the behavioral method was used in the previous 12 years, spinal cord injury severity for which the test is applicable (L—low, M—moderate, S—severe) and the main advantages and disadvantages of each method

Test	Habituation/ training	Most popular injury type	Lesion severity	Advantages	Disadvantages
Locomotor tests					
Primary open-field	+/−	Contusion	M, S	Simple, cheap	Low sensitivity
BBB	+/−	Contusion	L, M, S	Simple, cheap	Subjective
Open field activity	+/−	Contusion	L, M	Unique data are obtained	Depends on the rats' motivation
Automated walkway	+/+	Hemisection	L, M	Precise	Equipment
Footprint analysis	+/+	Cervical	L, M	Precise	Environment-dependent
Kinematic analysis	+/+	Contusion	L, M	Detailed	Equipment
Thoracolumbal height	+/−	Contusion	M	Examines only one characteristic	Equipment
Swim test	+/+	Contusion	L, M	Spontaneous locomotion	Subjective
E-W notation	−/−	Cervical	L, M	Detailed	Requires training of the scientist
Motor tests					
Inclined plane	−/−	Compression	L, M, S	Simple, cheap	Not standard among laboratories
Limb hanging	+/−	Contusion	L, M	Unique data	Not for severely injured animals
Limb grip strength	+/−	Contusion	L, M	Precise	Requires equipment
Forelimb asymmetry	+/+	Cervical	L, M	Sensitive to chronic deficits	Not for severely injured animals
Rearing	+/+	Contusion	L, M	Sensitive to selective limb use	Not for severely injured animals
Foot pellet reaching	+/+	Cervical	L	Testing of fine motor function	Food deprivation
Sensory tests					
Hot plate	−/−	Contusion	L, M, S	Simple	Risk of injury False positivity
Cold spray	−/−	Excitotoxic	L, M, S	Simple	Low sensitivity
Von Frey	−/−	Contusion	L, M, S	Simple	High chance of mistakes
Paw compression	−/−	Transection	L, M, S	Simple, cheap	Low sensitivity
Withdrawal reflexes	−/−	Contusion	L, M, S	Simple	Low sensitivity
Sensory–motor tests					
Rope walk	+/+	Cervical	L	Simple, cheap	Requires training
Narrow beam	+/+	Contusion	L	Uncovers discrete changes	Requires training
Grooming	+/−	Cervical	L, M	Simple, cheap	Subjectivity
Foot slip	+/+	Contusion	L, M	Uncovers discrete changes	Requires training
Grid walking	+/+	Contusion	L, M	Uncovers discrete changes	False-positives or negatives
Reflex-response based tests					
Toe spread reflex	−/−	Contusion	L, M, S	Simple, cheap	Low sensitivity
Contact placing response	−/−	Hemisection	L, M, S	Simple, cheap	False positivity
Righting reflex	−/−	Contusion	L, M	Simple, cheap	Low sensitivity
Autonomic tests					
Ex copula erection	+/−	Contusion	L, M, S	Unique data	Subjectivity
Non-contact erection	−/−	Contusion	L, M, S	Unique data	Low sensitivity
Mating	−/−	Contusion	L, M, S	Unique data	Subjectivity
Telemetric monitoring	−/−	Contusion	M, S	Precise	Equipment
Autonomic dysreflexia	−/−	Compression	M, S	Unique data	Equipment
Special tests					
Evoked potentials	−/−	Contusion	L, M, S	Precise	Equipment
MRI	−/−	Hemisection	L, M, S	Precise	Expensive
CBS	Different	Contusion	L, M, S	Complex	The same score might reflect different recoveries

The lesion severity corresponds to the BBB scale (severe—BBB 0-7; moderate—BBB 8-14; low—BBB 15-21). If not specified as "Cervical", the type of injury is thoracic.

of the skin, joints and bones. Washing the animals might also be helpful to prevent infections (Santos-Benito et al., 2006). The bedding should be changed periodically. Too frequent changes of bedding might result in the long-term removal of scent markers, which might impair the psychological and social comfort of the animal. Cages should have sufficient bedding to prevent decubiti or pressure sores (Kim et al., 2002).

Table 4
The five most commonly used behavioral testing methods, strains and lesion types in the past 12 years

No.	Test	Strain	Lesion type
1.	BBB	Sprague-Dawley	Contusion
2.	Electrophysiology	Wistar	Transection
3.	Hot plate	Lewis	Hemisection
4.	Von Frey	Long-Evans	Compression
5.	Grid walking	Fisher	Cervical injury

Table 5
Recommendations concerning the use of behavioral testing methods for different types of injury based on an analysis of studies published during the past 12 years

	First choice	Second choice	Third choice
Cervical	Forelimb assymetry	Footprint analysis	BBB
Th compression	BBB	Hot plate	Inclined plane
Th contusion	BBB	Electrophysiology	Von Frey Hot plate
Th hemisection	BBB	Electrophysiology	Hot plate Von Frey
Th transection	BBB	Electrophysiology	Kinematic analysis
Th excitotoxic	Hot plate	Cold testing	Von Frey
Th ischemic	BBB	Electrophysiology	Inclined plane Hot plate
Other injury	BBB	Electrophysiology	Hot plate Grid walk

All housed animals should be provided with five freedoms (Tatlisumak and Fisher, 2006). Freedom from malnutrition becomes important during the restriction of the diet required by some behavioral tests. Freedom from injury and disease indicates the necessity of keeping the environment clean and safe in terms of sharp cage edges, free wires, etc. This kind of freedom includes providing the animals with preventive antibiotics, if preliminary or similar studies indicate their need. Freedom from thermal and physical discomfort becomes important mainly during the first phase after the SCI procedure. Animals shortly after an injury procedure demand a higher temperature to prevent cold. The simplest way to achieve this is by the addition of extra bedding material to the animal's cage. Freedom from fear and stress is absolutely crucial for the proper reflection of neurological functions during the subsequent testing sessions; a stressed, startled or depressed animal will fight for its life and try to escape instead of performing the required task. The freedom to express most normal patterns of behavior is mainly dependent on the companionship of conspecifics and, if possible, housing in an enriched environment. Cage-mates provide opportunities for play, grooming and other social activities, which might be important when the mate is subsequently used as an attractant. It should be noted that rats in particular are extremely gregarious animals and will exhibit marked changes in behavior, such as aggression during handling or destructive behavior, if deprived of companionship. When individual housing is unavoidable, which is fortunately not very often in SCI studies, at least some form of shelter should be introduced into the animal's environment (Tatlisumak and Fisher, 2006).

3.4. Handling animals

Most often, a rat is held by its tail or beneath its body. In rats with SCI, the latter method is usually preferred, to avoid damage to the spinal cord. Also, the provocation of pathologic reflexes and the irritation of the perineum are thus avoided, so the result of behavioral testing is not distorted. In addition, rats are much more "friendly" animals than mice, so holding them by their body is not as dangerous as with mice. Rats should feel comfortable during the testing; if the animals are stressed, their reactions are adversely affected. To eliminate any possible effect of a full urinary bladder on behavior, the bladder should be expressed before testing. Rats enrolled in an SCI study should be repeatedly cleaned to prevent urinary and breathing infections caused by microorganisms and ammonia, which rest in the bedding of the cage. The severely injured animal has decreased weight support and locomotion, which reduces the extent of the space in which the animal moves and also decreases the mean distance between the bottom of the animal's trunk and the ground. The need for frequent cleaning increases with the severity of the injury and the length of the study (Santos-Benito et al., 2006).

3.5. Animal's behavior during testing

During the testing session, it is important to take into account the type of behavior shown by the animal, developed on the basis of the testing device. Murray (2004) categorized the animal's behavior during the testing session into (i) spontaneous behaviors, (ii) triggered behaviors, and (iii) trained behaviors. In tests based on the spontaneous activity of the animal, such as exploration or locomotion, any triggering to enhance the movement of the animal should be avoided. A typical example of such action is irritating or pushing the animal's back or tail by the scientist to enhance the movement of the rat in an open field. In tests based on triggered behaviors, the response is elicited by a standard stimulus. Here, the experimenter should make sure that the stimulus is always the same, applied to the same part of the animal's body with the same intensity and that the animal has the "physical and psychological space" to respond properly. For example, the animal will probably not be interested in food pellets if the pellets are standard rat chow, the animal is not food-deprived or the animal is stressed due to unexpected noise produced by the scientist. When trained behavior is tested, the animal should be able to accomplish the task without any problems before the injury. For example, when beam

walking has not been trained previously, the post-injury behavioral results might be more dependent on the ability of the animal to learn than on its sensory–motor coordination.

6. Video-monitoring

Several tests require video-monitoring for training the testers, monitoring the scoring sessions and/or proper evaluation of the testing sessions. In addition, video-monitoring allows the investigator to store each session's data, to evaluate the data afterwards, to evaluate the data repeatedly and to compare and send the data between investigators or laboratories. Basso (2004) describes in her review a situation in which a running wheel was placed into a rat's home cage overnight and its revolutions measured. The number of rotations was exceptionally high, so the question arose as to whether the rat could have been running so far. A video camera resolved this problem—the rats were lying beside the running wheel, spinning it with their forelimbs. This example nicely illustrates the necessity of video-monitoring during the testing or training in situations where the examiner is not able to see the animal or its particular outcomes.

7. Pre-training

In the majority of behavioral tests, the pre-training and/or acclimatization of animals are necessary (Table 3). The main reason for this is to familiarize the animal with the testing procedure, to avoid stressing the animal during the subsequent sessions and to verify the normal health of the animal. Pre-training might also be required to make sure the animal understands the required task. To minimize the inter-individual variability in some tests, the pre-training values are considered to be the normal values and the post-injury values are calculated as deviations (in percentages). In addition, assessing the performance of trained behaviors will eliminate differences in motivation between animals and will provide more detailed and specific measures of functional abilities (Muir and Webb, 2000).

8. Lab diet, motivators and attractants

Most tests do not require any food restriction or addition, so animals have access to rat chow and water ad libitum. It has been observed that rats need 20–26 g of chow per day, depending on the weight (age) of the animal. In contrast to guinea pigs or primates, rats do not require a daily dietary source of vitamin C. Interestingly, rats obtain an additional source of vitamin B by eating fecal pellets— they are coprophagic. Although rats are able to cope with reduced water intake and conserve fluids by producing very concentrated urine, the amount of clear water intake is not restricted throughout an SCI study (Tatlisumak and Fisher, 2006).

Some tests require the restriction of food in order to increase the motivation of the rats to look for and reach food. In these tests, the restricted diet is usually around 13 g per animal per day (Chan et al., 2005). On the other hand, some scientists feel that food deprivation or restriction represents an unreasonable health risk to severely impaired animals with SCI (Basso, 2004). However, cyclic food restriction with no weight loss is suggested as a method of choice. Instead of deprivation, animals can be pre-trained to eat attractive motivators, such as raspberry syrup, cereal such as fruit loops or apple jacks, or other substances. Chocolate, alcohol and caffeine should be avoided in the rat diet, because they can cause serious medical problems. Chocolate contains theobromine, which in large amounts has diuretic effects, relaxes smooth muscles, and stimulates the heart and central nervous system (Aboel-Zahab et al., 1997). Caffeine has hypercholesterolemic effects and negatively influences the development of the nervous system (Fears, 1978; Ohta et al., 2002). The negative effects of ethanol are multiple, similar to those observed in humans (Barr et al., 2005; Rivier, 1995). Conversely, some substances such as creatine or cyclosporine A might have neuroprotective effects (Rabchevsky et al., 2001, 2003).

During a long-term study, the lab diet dose should be adjusted individually to maintain the body weights of all animals in a similar range, usually 270–330 g (Santos-Benito et al., 2006). It is important to know that animal body weight decreases by about 10% during the first week after SCI and starts to raise thereafter (Van Meeteren et al., 2003). Immediately after the injury procedure, the scientist should make sure the animals can reach the food and water (Tatlisumak and Fisher, 2006). With gnawing animals, food and water are usually offered in an elevated wire-mesh hopper. In our experiments, we place food pellets and an additional water flask on the bottom of the cages of all animals until they are able to reach the hopper (Šedý et al., 2007a; Syková et al., 2006a).

In several tests, such as open-field based tests, where food motivators are not optimal, other attractants, such as the presence of a familiar housing mate or a close relation or the smell of the home cage, can be used. When using immunosupressive drugs, the quality of the lab diet might be enriched, using a high-energy diet such as Ensure (Archer et al., 2005). In the majority of tests, the use of attractants is not necessary, because rats are tireless explorers and are interested in all new stimuli.

3.9. Pharmacological treatment

For several testing methods, some pharmacological treatment is necessary to avoid stress or injury of the animal or to prevent unwanted reactions or inflammation. To limit post-operative pain, analgesics such as metacam (5 mg/kg/day), or buprenorphine hydrochloride (0.1 mg/kg/day, s.c.) should be administered for the first 24–48 h (Baldridge et al., 2002; Kim et al., 2002; Roussos et al., 2005; Xu et al., 1999). Antibiotics, including gentamycin sulfate (1–12 mg/kg/

day, i.m., i.p. or s.c.), ampicilin (100–150 mg/kg/day, i.m.), enrofloxacin (2.5 mg/kg/day, s.c.), cefazolin sodium (50–100 mg/kg/day, s.c.) or approximately 15 ml of an oral suspension of sulfamethoxazole (40 mg/ml)–trimethoprim (8 mg/ml) are used for the prevention of urinary infections (Baldridge et al., 2002; Roussos et al., 2005; Xu et al., 1995, 1999). Immunosuppressive drugs, such as cyclosporine A (5–10 mg/kg/day, i.p.) or FK506 (initial bolus 2 mg/kg, followed by 0.2 or 0.5 mg/kg/day), can be used to prevent the rejection of implanted cells or biomaterial (Akgun et al., 2004; Diaz-Ruiz et al., 2004; Lopez-Vales et al., 2005). In addition, ascorbic acid (approx. 10 mg/rat/day) can be administered to prevent bacterial growth and to support the general health of the animal (Roussos et al., 2005; Xu et al., 1995). To encourage rats to drink more, sucrose can be added to the drinking water for the first few days (Kim et al., 2002). If an animal's general health is not very good, small amounts of Ringer-Lactate, saline or 5% dextrose might be injected; such an intervention should be written into the experimental protocol. Some experimenters give 10 ml of saline s.c. immediately after the lesioning procedure to compensate for the poor oral water intake in the perioperative period (van de Meent et al., 1997). Other laboratories prefer an injection of 5% saline–glucose (Baldridge et al., 2002; Roussos et al., 2005). When minor autophagia of an impaired hindlimb occurs, some scientists dip the limb into 1% picric acid to give the skin a bitter taste (Kim et al., 2002).

For more sophisticated testing methods, such as evoked potential monitoring, some other drugs might be necessary. These include volatile (e.g. isoflurane) or intravenous anesthetics (e.g. pentobarbital or ketamine–xylazine), myorelaxation drugs (e.g. pancuronium bromide), atropine (to diminish secretions in the respiratory tract) or ophthalmic ointment. The intramuscular injection of drugs into a hindlimb should be avoided due to muscular atrophy, thus the forelimb musculature should be used instead (Santos-Benito et al., 2006). Generally, the less medication that is used in an experimental study, the fewer cross-reactions and false results will occur. On the other hand, some drugs are necessary to minimize the stress and pain of the animals or are even required by ethical committees or grant agency policies, thus their use cannot be avoided. Importantly, the pharmacokinetics of several drugs, such as gentamycin (Segal et al., 1988) or cyclosporine A (Ibarra et al., 1996), have been shown to be altered in spinal cord injured mammals. Thus, when new drugs are introduced, it should be taken into consideration that the dose–response curve might be different from other types of injury.

3.10. Time factors

There are several time factors that must be decided upon before the study starts, including the time of testing, the frequency of testing, the length of testing, the number of repetitions of individual testing and the periods between them. A preliminary or so-called pilot study utilizing a few animals is a great tool for establishing the time factors without losing significant numbers of animals, time or money. At the beginning of an SCI study, less might be more. In order to minimalize the role of circadian rhythms, the testing time should be consistent and should not be changed during the study (Kriegsfeld et al., 1999). It should also be taken into account that rats are naturally crepuscular or nocturnal animals, so they are more active during the night. Testing is usually performed in the morning or in the evening, depending on the schedule of the scientist, in order to ensure sufficient time in which to perform the testing. Also, the day-night cycle can be reversed for the purpose of the experiment, if needed.

The frequency of testing depends on the required sensitivity of the data to be obtained. If no gross abnormalities are expected, the interval between testing periods can be longer than in the case where the clinical state of the animal is expected to change rapidly. For example, in the first 5–6 weeks after SCI or the start of treatment, testing of the animals is usually performed 1–2 times per week, while after this period, testing 1–2 times per month might be sufficient. Generally, the animals would be tested before SCI (treatment), several hours to one day after SCI (treatment), once per week for the first six weeks and then once per month until the end of the study. Testing the animals for more than six months is not typical in many studies; however, Ramon-Cueto et al. (2000) observed locomotor improvement even seven months after injury. Long-term experiments with chronically injured animals need additional special care, including special feeding, housing, cleaning, control of health, etc. (for review, see Santos-Benito et al., 2006).

In animals with central nervous system injury, the question of when the testing, and more importantly, the training sessions should begin, remains crucial. It has been shown that the pattern of gene expression is influenced by both the severity of injury and the time after injury (Li et al., 2004). Genes encoding molecules for cellular signaling, synaptic plasticity, metabolism, ion channels and transporters are up-regulated following severe injury, but down-regulated following moderate injury. Furthermore, moderate injury is associated with an increase in the number of responsive genes, whereas a severe injury is associated with a decrease during the same post-injury period (Li et al., 2004). Similarly, when voluntary exercise is delayed by two weeks, an increase in BDNF expression and an improvement in behavioral outcome can be observed (Griesbach et al., 2007). Generally, early time point testing (less than two weeks post-injury) is unnecessary unless the hypothesis posits changes during this early period; testing is stressful and may negatively influence the recovery of the animals. These papers indicate the necessity of properly timing the "behavioral testing window" and more importantly the "training window". However, as not much is known about the timing of these windows and as

the timing might be different in particular SCI models, a quite large field remains open to investigation.

How long the animals should be tested depends primarily on the type of test. In some tests, a minimal testing period is required to see the full picture of the testing modality, while in others the maximal testing period serves to prevent injury to the animals. The question of how many times an individual test should be repeated also depends mainly on the type of test. Usually, a test is done once or three times by each examiner, but can range between 1 and 20 times. The period between individual testings depends mainly on the time the animal requires to reach a level of physical and psychological comfort similar to that before the start of testing.

3.11. Role of the scientist

In the interest of objectivity, the testing should be performed by two to three independent scientists who are familiar with the testing procedure. When two or more testers are included into a study, the difference between the values obtained by each examiner should vary by no more than 5% (Basso, 2004). To blind the study, the tested animals should be marked by code, so that the scientists are not able to recognize to which group the animals belong, even after repetitive testing. The recording sheets should also be free of group identification marks. As it is important to use the same tester(s) throughout the experiment, vacations, conferences, etc. should be planned in advance. Also, if the scheduled tester is sick, the testing day might be postponed by 1–2 days rather than being performed by another individual. The frequent changing of testers throughout a study might completely ruin the study. All scientists have a small level of individual bias. However, the existence of the same small relative error throughout an entire study will not affect the absolute error, while the existence of several different relative errors of several different scientists will certainly create an absolute error, without any chance to correct it after the end of the study. Finally, feelings of stress, frustration or anxiety on the part of the examiner can negatively impact the behavioral performance of the animals, which become very apprehensive. Also, a shortage of time and hurrying might influence the animals' behavior. Therefore, it is important for the tester to leave all external issues outside the door of the testing room.

3.12. Overall recovery of the animal

The overall recovery of the animal should be included in the testing process, because it provides the investigator with additional significant data. Overall recovery can be evaluated by weekly weighing of the animal (Urdzíková and Vanický, 2006) and measurements of the thigh circumference, reflecting the leg muscle mass, usually before the beginning of a testing session (Vaquero et al., 2006). In addition, the general locomotor activity of the

animal could also be graded as normal, reduced or minimal (Farooque, 2000).

3.13. Experimental design of the study

During the preparation of the study, several aspects such as animal weight and gender, injury procedure, post-injury care and the proper selection of testing methods should be carefully taken into account. The testing strategy, once started, should not be changed during the experiment (Metz et al., 2000). If some other factor, such as treadmill training or an enriched environment, is included in the study, it should be applied to all animals in the study (Fouad et al., 2000). One of the most important factors is the selection of proper, sensitive, standardized and generalizable behavioral tests to produce reliable, reproducible and worthwhile data. Most importantly, the behavioral test must match the hypothesis and it must have proven performance standards—it must be shown that the test produces reliable results across sessions, examiners and labs, as was done for the BBB test (Basso et al., 1996). Without evidence of reliability, it is impossible to attribute behavioral changes to biological events or interventions. In addition, the standardization of behavioral equipment and testing procedures is necessary. Although no ideal behavioral test has yet been developed, the weaknesses of one test can be compensated by the strengths of another test. For example, the BBB test reflects open-field locomotion in detail, but fails to analyze body balance, which can be easily monitored during beam walking. Conversely, beam walking in isolation will not be able to distinguish, for example, between an animal's status corresponding to BBB 1 and 8—in both cases, the animal falls off the beam.

One of the most important questions during the selection of the proper testing method is whether the investigator wants to know "whether" or "why" behavior changed. This problem was previously discussed in an excellent review by Basso (2004). She concludes that experiments concerned with "why" behavioral changes occur should rely on precise, quantitative behavioral tests, although studies focused on "whether" a change occurred can use more general measures of behavior without invalidating the results. Another question is the required sensitivity, i.e. the ability of a test to detect changes in behavior that actually exist. Rats are well known for compensating to overcome lesion-induced deficits, thus it is important to choose tests that will target the deficit directly and be minimally affected by compensatory behaviors. If the test is influenced by compensatory mechanisms, it may not be clear if the effect of the therapy is ameliorating the deficit itself or enhancing motor learning mechanisms that allow the development of compensatory behavior (Schallert and Woodlee, 2003). Dichotomous or trichotomous tests, with broad categories such as yes/no or absent/normal/abnormal, have a lower level of sensitivity because they are unlikely to detect small changes. On the other hand, they

are usually less time and money consuming. In contrast, the most sensitive tests, such as kinematic analysis, usually require special equipment, educated personnel and much time. Fundamentally, (i) the more general the test, the broader its scope and the lower its precision and (ii) the greater the test's applicability, the less sensitive it tends to be. If using more than one or a battery of tests, the order and timing of the individual tests should be preserved throughout the study (Basso, 2004).

4. Locomotor tests

Locomotor tests are usually open-field based tests in which the locomotor apparatus, especially the proximal forelimb, hindlimb and tail striated muscles, are tested. Besides limb muscle function, locomotion also requires the proper coordination and strength of the involved muscle groups. Locomotor recovery is recognized as the most important modality in patients suffering from SCI (Syková et al., 2006b). For the purpose of behavioral testing of spinal cord injured animals, locomotion is usually divided into overground locomotion, horizontal or inclined treadmill locomotion and locomotion during swimming. The crucial break point for locomotion is the onset of weight support, on which depends proper hindlimb stepping. In addition, the spatiotemporal details of the locomotor pattern, including step size and swing duration, vary as a function of the weight support provided (Timoszyk et al., 2005). Experimental lesions are usually performed at the mid-thoracic level, so only the hindlimb and tail muscles are affected. The lesion is usually not made above the C5 level, so the phrenic motor pool, responsible for respiration, is spared (McKenna et al., 2000).

4.1. Primary open-field tests

Open-field behavioral testing of the locomotor performance of rats with an SCI is probably the oldest such testing. It is simple, reproducible and does not need any special devices. The developed rating scales are based on the observation of defined leg movements, reflecting the activation of spinal networks that are able to produce a coordinated stepping pattern (Rossignol and Dubuc, 1994). In the past, the most commonly used test of this kind was Tarlov's open-field test, which ranks hindlimb movements and weight support in five categories (Tarlov, 1954). However, this method has been found to be more sensitive when the animal is able to hindlimb weight support and is less reliable when used to score hindlimb movements without weight support (Broton et al., 1996; Metz et al., 2000). Subsequently, the test was modified, resulting in the so-called Tarlov's modified open-field test, which has a scale from 0 (complete paralysis) to 6 (normal locomotion) (Guizar-Sahagun et al., 2004). Additional modifications of Tarlov's scale are, for example, the motor performance score (MPS), developed by von Euler et al. (1996, 1997), the motor deficits score (MDS), used mainly in

an ischemic model of SCI developed by Maršala and Yaksh (1994), the open field motor test (OFT), developed by Behrmann et al. (1992) and Bohlman's motor evaluation score (Bohlman et al., 1981). None of these scales is, unfortunately, very sensitive; so they have been improved by increasing the number of categories for all hindlimb motor features in the BBB score (Basso et al., 1995). In contrast to these testing methods, the evaluation of one isolated function modality during open-field testing has been proposed several times; for example, Houle et al. (2006) examined the angle of forelimb swing movement and classified it using their own scale, ranging from 1 (angle more than 90°) to 5 (grooming motion above the level of the eyes).

4.2. BBB test

The BBB test, named after the first letters of its developers, Basso, Beatie and Bresnahan, is probably the most commonly used test of locomotor function in spinal cord injured rats worldwide (Basso et al., 1995). It is a modified open-field test, based on grading hindlimb locomotion from 0 (no spontaneous locomotor activity) to 21 (normal movement—coordinated gait with parallel paw placement) (Table 6). Scores from 0 to 7 indicate the return of isolated movements in the hip, knee and ankle joints. Scores from 8 to 13 indicate the return of paw placement and coordinated movements with the forelimbs. Scores from 14 to 21 show the return of toe clearance during stepping, predominant paw position, trunk stability and tail position (Table 6). Rats are usually tested on a non-slippery surface, able to reveal toe clearance disturbances, in a circular arena, about 90 mm in diameter and 30 cm high. The use of a video camera is recommended especially for training the testers and comparison between testers from different experimental groups. However, the BBB was primarily designed to be based on live, subjective observation. The video-camera is thus not recommended for testing, mainly due to limited parts of the animal body seen and the inability to evaluate the toe clearance (D. Michelle Basso, personal communication). The testing is based on an analysis of the movements of individual hindlimb joints, sweeping without weight support, weight-supported dorsal stepping, plantar placement of the paw with or without weight support, forelimb–hindlimb coordination, internal or external rotation and parallelness of the paws in the predominant paw position at initial contact and when the paw is lifted off, toe clearance (occurrence of pathologic acoustic phenomena during hindlimb locomotion), elevation of the tail during locomotion and trunk stability (Basso et al., 1995). One advantage of this scale is that pre-operative training of the animals is not necessary; however, pre-operative gentling and exposure to the testing field are highly recommended. The BBB score was originally designed for contusion injuries, but it also works well for other types of injuries such as a balloon compression lesion or hemisection (Metz et al., 2000; Syková and Jendelová, 2005; Syková et al., 2006a; Šedý

Table 6

Basso, Beatie and Bresnahan locomotor rating scale, reprinted from the Journal of Neurotrauma (Basso et al., 1995) with permission from Mary Ann Liebert, Inc

	No observable hindlimb movement
	Slight movement of one or two joints, usually the hip and/or knee
	Extensive movement of one joint or extensive movement of one joint and slight movement of one other joint
	Extensive movement of two joints
	Slight movement of all three joints of the HL
	Slight movement of two joints and extensive movement of the third
	Extensive movement of two joints and slight movement of the third
	Extensive movement of all three joints of the HL
	Sweeping with no weight support or plantar placement of the paw with no weight support
	Plantar placement of the paw with weight support in stance only (i.e. when stationary) or occasional, frequent or consistent weight-supported dorsal stepping and no plantar stepping
	Occasional weight-supported plantar steps; no FL–HL coordination
	Frequent to consistent weight-supported plantar steps and no FL HL coordination
	Frequent to consistent weight-supported plantar steps and occasional FL–HL coordination
	Frequent to consistent weight-supported plantar steps and frequent FL HL coordination
	Consistent weight-supported plantar steps; consistent FL–HL coordination, and predominant paw position during locomotion is rotated (internally or externally) when it makes initial contact with the surface as well as just before it is lifted off at the end of stance; or frequent plantar stepping, consistent FL–HL coordination, and occasional dorsal stepping
	Consistent plantar stepping and consistent FL–HL coordination and no toe clearance or occasional toe clearance during forward limb advancement; predominant paw position is parallel to the body at initial contact
	Consistent plantar stepping and consistent FL–HL coordination during gait and toe clearance occurs frequently during forward limb advancement; predominant paw position is parallel at initial contact and rotated at lift-off
	Consistent plantar stepping and consistent FL–HL coordination during gait and toe clearance occurs frequently during forward limb advancement; predominant paw position is parallel at initial contact and lift-off
	Consistent plantar stepping and consistent FL–HL coordination during gait and toe clearance occurs consistently during forward limb advancement; predominant paw position is parallel at initial contact and rotated at lift-off
	Consistent plantar stepping and consistent FL HL coordination during gait, toe clearance occurs consistently during forward limb advancement, predominant paw position is parallel at initial contact and lift-off, and tail is down part or all of the time
	Consistent plantar stepping and consistent coordinated gait, consistent toe clearance, predominant paw position is parallel at initial contact and lift-off, and trunk instability; tail consistently up
	Consistent plantar stepping and consistent gait, consistent toe clearance, predominant paw position is parallel throughout stance, and consistent trunk stability; tail consistently up

al., 2007a, b; Urdzíková et al., 2006). It is thought to be less sensitive to clip compression injuries (von Euler et al., 1997). Some authors propose that there are potential limitations to the BBB score due to the subjectivity of the test (Broton et al., 1996; Metz et al., 2000), but the experiences of our and other groups show that rigorous

training of the testers minimizes subjectivity, especially when the evaluation is made by two independent scientists (Basso et al., 1996; Syková and Jendelová, 2005; Urdzíková et al., 2006). Metz et al. (2000) concluded that an important drawback of the test lies in the fact that the ordinal BBB rating system is not linear; the lower part of the scale concerns gross aspects of locomotion, while the upper part of the scale includes rather discrete movement aspects that do not represent major improvements in the animal's motor ability. However, this problem occurs in the majority of tests and probably cannot be easily solved. The BBB score is now widely used and has been shown to provide reproducible results, so it remains the gold standard (Basso et al., 1996).

4.3. BBB sub-scoring scale

After the publication of the BBB scale (Basso et al., 1995), several modifications and improvements have been proposed (Lankhorst et al., 1999, 2001; Metz et al., 2000; Popovich et al., 1999; von Euler et al., 1997). The BBB sub-scoring scale improves the sensitivity of the BBB scale by scoring each of the behavioral attributes independently and then adding them together to yield a single score, using a process we call sub-scoring (Basso, 2004; Lankhorst et al., 1999, 2001; Popovich et al., 1999; Van Meeteren et al., 2003). The justification for sub-scoring is the assumption that when a treatment is applied, it will affect some but perhaps not all aspects of locomotion. For instance, a treatment may improve toe clearance or trunk stability without having any impact on forelimb–hindlimb coordination (for details, see Basso, 2004).

4.4. Open-field activity test

This simple test, originally described by Bignami (1996), is sensitive to a wide range of injuries, including SCI. To assess exploratory behavior, rats are tested in an open field (100×80 cm) subdivided into nine fields. Animals are observed individually for 5 min. The number of fields crossed during this observation interval is noted, and a ratio of the animals' performance in comparison to baseline data (taken as 100%) is modified to a 5-point score. A value of 0–50% is rated as 1 point, 51–90% as 2 points, 91–110% as 3 points, 111–200% as 4 points and more than 200% of baseline values is rated as 5 points (Bignami, 1996; Metz et al., 2000).

Generally, the open field activity test is a good measure of gross motor behavior and general health in spinal cord injured rats. Exploratory activity is especially sensitive to individual differences among animals with a low locomotor capacity, and even severely damaged rats can show significant locomotor activity. However, spontaneous exploratory activity is influenced by motivational factors such as anxiety, which can cause freezing behavior in rodents, thus reducing the rate of exploration (Gerlai and Clayton, 1999). It is also important to leave appropriate

intervals between individual test sessions to avoid habituation to the testing environment. Furthermore, locomotor training after SCI can enhance exploratory activity (Fouad et al., 2000; Metz et al., 2000).

4.5. Automated walkway test

In the automated walkway test (CatWalk® test, CatWalk®-assisted gait analysis), first described by Hamers et al. (2001, 2006), the animal is first trained to cross a 100–120 cm long glass walkway, at least 6 mm thick, with black Plexiglas walls spaced 8 cm apart and a ceiling above. In a dark room, the light from an encased fluorescent bulb is transmitted through the glass surface of the walkway. Paw contact causes light to exit the floor and illuminate the paw print, which is monitored by a video camera connected to a computer with the CatWalk® program, which acquires, compresses and stores the data for further analysis. The analysis is based on step sequence distributions, inter-limb coordination (regularity index), the total floor area contacted with the paw (print area), the distance between two hind paws (base of support), the duration of the swing and stance phases and hind paw pressure (Gensel et al., 2006; Hamers et al., 2001). This test overcomes the difficulty of analyzing all phases of the movements of rapidly locomoting animals in an open field, especially in the evaluation of forelimb–hindlimb coordination; it can thus remove the subjectivity encountered with other tests, such as the BBB, from the evaluation of forelimb–hindlimb coordination. In addition, the animals are not forced to walk, thus eliminating undesired influences on the results.

In contrast to other locomotor tests, Catwalk analysis provides the experimenter with a very large amount of data concerning different locomotor patterns. One interesting aspect of this test is the fact that different quantitative or qualitative outcome measures are differently sensitive to different SCI models. For example, the regularity index nicely reflects the neurological impairment resulting from complete transection or contusion injury, but it fails with dorsal column transection. This problem was discussed in detail by Hamers et al. (2006). The animals need to be pre-trained to cross the Catwalk runway without any hesitation or stress reactions. Importantly, the gait velocity should be controlled by the fact that training of the animals results in runs with a stable crossing time of the runway between 1 and 2 s, as described by Deumens et al. (2007). This requirement is based on the fact that different gait velocities—for example, trot and pace, have a different order of paw placings, i.e. the regular step patterns. Thus, the regularity index might be different in an animal when it runs at a different speed or, more importantly, changes its speed throughout the runway. Other pitfalls of the CatWalk® analysis were recently reviewed by Hamers et al. (2006). It should be noted, that the CatWalk system is still under development, and new methods of obtaining additional data are expected to be developed in the near future (Hamers et al., 2006).

4.6. Footprint analysis test

One of the earliest descriptions of walking tract footprint analysis was published by de Medinaceli et al. (1982). Later, several modifications were developed (Chan et al., 2005; Metz et al., 2000). This test is based on a walking analysis of paw-colored animals across a narrow, paper-covered wooden beam, 1 m in length and 7 cm wide. Pre-training of the animals is required. A different color dye is used for forelimbs and hindlimbs to distinguish between them. The distance between corresponding steps, the angle of the paws (an indicator of walking stability and body balance) and the ability of the paw to support the animal's body are evaluated (Chan et al., 2005; de Medinaceli et al., 1982; Metz et al., 2000). A series of at least eight sequential steps is used to determine the mean values for each measurement of limb rotation, stride length and base of support. The base of support is determined by measuring the core-to-core (midpoint) distance of the central pads of the hind paws. The limb rotation is defined by the angle formed by the intersection of a line through the print of the third digit and a line through the central pad parallel to the walking direction. Stride length is measured between the central pads of two consecutive prints on each side.

It is unfortunately true that individual and motivational factors can greatly interfere with the quantification. In addition, walking velocity influences the length of single steps (Metz et al., 2000). In animals that have only partial weight support and little plantar placement, measurements of angle and stride length are almost impossible. It has been shown that footprint analysis can serve to refine observations made using the BBB or other motor tests for weight support trunk stability and foot placement (Metz et al., 2000). The use of only footprint analysis for evaluating recovery in SCI rats would probably not be optimal.

A recently developed system for gait analysis—the DiGiGait™® system (Mouse Specifics, Inc., Boston, USA)—overcomes the imperfections of footprint analysis. No dyes are used, and the data are obtained and subsequently analyzed by a computer. The device includes a transparent treadmill belt, which allows a camera and subsequently image processing software to identify and analyze the paw contact with the treadmill surface. The system is able to analyze horizontal or inclined gait dynamics, treadmill and overground locomotion and coordination. The analysis of treadmill locomotion is possible at different speeds (range 0–100 cm/s). Although it was also developed for behavioral analysis of rats or guinea pigs, up to now it has been used mainly in gait analysis of spinal cord injured mice (Li et al., 2005).

4.7. Kinematic analysis

The kinematic measurement of step cycles provides an assessment of the individual components of limb movement in two or three dimensions (Metz et al., 1998;

Vesterga and Gramsbergen, 1990). Two-dimensional kinematic analysis is usually performed because the predominant motion of the hindlimbs can be assumed to be planar; therefore, movements of the rat hindlimb joints distal to the hip are primarily those of flexion and extension and movements in the coronal and transverse planes are often neglected (Gasc, 2001; Gillis and Biewener, 2001). For kinematic analysis, the tested animal's hindlimb is shaved and the iliac crest, greater trochanter, lateral malleolus and the fifth metatarsophalangeal joint are marked with ink visible in normal or infra-red light or with glued-on circular light-reflecting discs (Filipe et al., 2006). While the position of the knee joint is obscured by loose skin coverage, it is calculated by using hip and ankle joint positions and external individual measurements of femur and tibia length. The proper position of the markers is crucial–if the marker deviates by as little as 2 mm in placement, the result can be as much as a 10° change in angular joint measures (Basso, 2004). Moreover, soft tissue movement over the limb joints might significantly alter the position of the marker, which will subsequently lead to misleading artifactual results. To prevent this, the marker should be attached or pointed to skin areas that are tightly attached to the underlying connective tissue (Filipe et al., 2006). Considering that soft tissue artifacts are recognized as a major error source in human motion analysis (Andriacchi and Alexander, 2000), skin markers respecting the underlying bone, such as bone pins (Yack et al., 2000), external fixators (Cappozzo et al., 1996) or percutaneous trackers (Manal et al., 2003), have been employed. Paradoxically, due to their invasive nature, they are not applicable for repeated use in laboratory rodents (Filipe et al., 2006). After positioning the markers, the animal is made to walk on a treadmill and recorded using a digital video camera. The rigorous selection and maintenance of the treadmill speed throughout the entire session and in all rats in the experiment is crucial because the timing and the amplitude of excursion of most kinematic variables during each step cycle differ among gaits (Gillis and Biewener, 2001). On the basis of the animal's strain, age and general health status, the speed is usually set between 50 and 400 mm/s. The marker coordinates are used to calculate the knee position and to find the maximum flexion–extension range of each joint. In a spinal cord injured rat, the joint that shows the highest diversity of movements and thus is most suitable for reflecting the recovery of locomotor function is the ankle (Basso, 2000). The flexion–extension range is measured at the initiation of the swing phase, in the middle swing phase, and in the phase in which the paw initially contacts the ground. The middle stance phase is used to determine the weight support. The limb movements of a set number of step cycles, defined as flexion and extension of the limb, are analyzed frame-by-frame and averaged. In order to reduce inter-individual differences, a percentage ratio is calculated from the pre-operative baseline values (Metz et al., 2000). Isolated analysis of foot trajectories from the lift-off phase to the

step-down phase, including horizontal step length and vertical step amplitude, reconstructed in two-dimensional space, is also possible (Cho et al., 1997). Although less important, the technique also allows the experimenter to acquire quantitative data. From the videotapes, the number of hindlimb steps can be obtained (Coumans et al., 2001).

Although time-consuming, this method allows the detection of discrete deficits in the gait and can be utilized for normal and treadmill walking. If the animals show no weight support but do undertake limb movements, a qualitative assessment can be made. Otherwise, this method can quantify foot placement, limb coordination and exact joint angles. This technique adds precise information to other locomotor tests, such as BBB or footprint analysis, and provides a detailed description of step cycle duration and phase relations (Metz et al., 1998, 2000). In order to exclude differences in motivation and motor learning, it is very important to sufficiently train the animals before testing (Filipe et al., 2006). In severely injured animals without weight support and consistent stepping, kinematic analysis is not able to provide any important data and should be replaced by other tests such as the BBB. An alternative to kinematic analysis is cineradiography, introduced by Fischer et al. (2002), in which the displacements of the bones are imaged directly. Although it is precise and non-invasive, it is expensive and potentially dangerous due to radiation emission (Filipe et al., 2006; Fischer et al., 2002; Freeman and Pinskerova, 2005).

4.8. Thoracolumbar height test

This test was developed by van de Meent et al. (1996, 1997) in order to more precisely differentiate between full and partial weight bearings. The idea of this test is based on the knowledge that the height of the thoracolumbal kyphosis depends on a rat's ability to support weight on its hindquarters. Before the beginning of a testing session, the maximal convexity of the thoracolumbar kyphosis is marked on the skin with ink. The animal is put into a transparent walkway corridor, and the position of the marker is monitored during the rat's crossing of the walkway using a video camera connected to a computer equipped with software developed by Frank Hamers's laboratory. The position of the thoracolumbal kyphosis is determined 50 times per second and stored for subsequent off-line analysis (van de Meent et al., 1996, 1997; Van Meeteren et al., 2003). van de Meent et al. (1996) also showed that the thoracolumbar test is more sensitive than Tarlov's open field test for correctly discriminating walking patterns, particularly in the moderate to light severity range of injury. We can speculate that this test would also be able to add unique and important data to that obtained with the BBB test. In mildly or very severely injured animals, its usefulness is very limited.

4.9. Swim tests

Normal rats swim with their body almost parallel to the water surface, with their head, neck and approximately 30% of the dorsal surface of their backs above the surface and the tail at or just below the surface. They use their hindlimbs to provide forward motion by rapid, alternating hindlimb strokes; forelimbs are tucked under their chins and used only occasionally for steering. They exhibit none or only a small degree of rotation along their long axis. By contrast, thoracic spinal cord injured rats prefer forelimbs for their forward movement and exhibit different degrees of posterior body part submersion and trunk instability (Smith et al., 2006a, b). The major advantages of swim tests are the support provided to the animal by the water and the necessity to locomote, due to the lack of sufficient buoyancy to stay afloat without such locomotion, a condition that is not typical in other tests such as open-field based tests.

Originally, the swim test was performed by placing a rat in a pool with a diameter of 125 cm. The movement of the hindlimbs during swimming was evaluated for 45 s, and the rats were scored between 0 and 3, with 3 assigned to rats with a normal performance (Gale et al., 1985; von Euler et al., 1997).

A modification of the swimming test was developed by Arvanian et al. (2006). Rats are gently placed in a 40 cm diameter tub filled with warm water and allowed to swim for 15 s. The sessions are videotaped, and the frequency of rear-leg (hindlimb) kicking is quantified. This test results in significant swimming even in neonatal rats that cannot ambulate on a solid surface (Arvanian et al., 2006).

A recent swim test was developed by Martin Schwab's laboratory (Liebscher et al., 2005). The animal is put into a rectangular Plexiglas basin (150 × 40 × 13 cm). The level of the water (23–25 °C) is high enough to prevent the rat from touching the bottom of the basin. Intact animals swim by paddling with their hindlimbs and their tail, holding their forelimbs immobile under their chin. A total of five runs per rat are monitored using a mirror placed at a 45° angle on the bottom of the pool in order to film the rats from the side and the bottom simultaneously. The swimming performance is analyzed by scoring their movements according to the forelimb usage, hindpaw distance, hindlimb stroke and tail movement. Normal swimming results in a score of 7–8 points, while no locomotion receives a score of 0 (Liebscher et al., 2005).

Scientists from David Magnuson's laboratory in Kentucky recently developed the 18-point Louisville swim scale, based on grading an animal's performance during swimming in a 150 cm long, 18 cm wide and 30 cm deep tank, filled with warm tap water, where an adjustable Plexiglas ramp, covered with soft neoprene, is present at one end of the pool (Smith et al., 2006a). After the trial, the hindlimb movement performance, hindlimb alternation, forelimb dependency, trunk instability and body angle are evaluated from a video camera record and graded in summary from 0 (severe injury) to 17 (normal perfor-mance). Animals with 0–5 points are designated poor swimmers (severe disturbance of all parameters; hardly able or not able to swim), animals with 6–11 points intermediate swimmers (occasional or frequent occurrence of the monitored characteristics; able to swim with visible problems) and animals with 12–17 points as good swimmers (normal or almost normal characteristics; able to swim well). The generation of the scale was based on and evaluated in accordance with the BBB scale. Experiments in the Magnuson laboratory indicate that the scale is very useful for evaluating mild, moderate and moderately severe thoracic SCIs, where it is comparable to the BBB. Conversely, it is not reliable in evaluating severe injuries, in cases where the animals use only their forelimbs for swimming. The hindlimbs of the animals are passively spread, and thus the trunk stability and body angle are not impaired, distorting the results (Smith et al., 2006a).

4.10. Eshkol–Wachmann notation

Eshkol–Wachmann movement notation (analysis) was originally created for recording dance movements in humans by Eshkol and Wachmann (1958). It was designed to enable choreographers to write a dance down on paper that dancers could later reconstruct in its entirety, in a manner analogous to a musical score (Eshkol and Wachmann, 1958). It views the body as a set of limbs connected with joints; the body is treated as a system of articulated axes and a limb as any part of the body that either lies between two joints or has a joint and a free extremity. The notation system has been used to describe the details of rat forelimb movements during precision reaching (Farr and Whishaw, 2002; Whishaw and Pellis, 1990). Through single-frame analysis of videotaped record-ings, each behavior is subdivided into separate movements of the component limbs and limb segments, and a rating scale is applied to each component. The strength of this approach is that the individual movements by which an animal accomplishes a task can be identified in detail. The Eshkol–Wachmann notation of reaching movements in animals with and without spinal cord lesions reveals differences that are neither noted from cursory visual examination of videotapes nor can these differences be detected using endpoint measures of reaching success. Although Eshkol–Wachmann notation is not a quantita-tive method, it has proven useful as a screening device to identify movement differences, which can subsequently be quantified with other tests (Muir and Webb, 2000; Whishaw and Pellis, 1990; Whishaw et al., 1993). It has been also used for evaluating exploratory behavior in rats during the cylinder test (Gharbawie et al., 2004).

5. Motor tests

In this section, behavioral tests that test skeletal muscle function not primarily involved in locomotion are dis-cussed.

1. Inclined plane

The inclined plane is a 28×30 cm floor covered with a grooved, 1 mm thick rubber surface and 20×30 cm walls, 10 cm high on three sides. This task evaluates the animal's ability to maintain its body position on a board that is incrementally raised to increasing angles. The rat is placed on the inclined plane with its head down, up, to the right or to the left. Alternatively, it can be placed in such a position that its body is perpendicular to the axis of the plane. Usually, testing in two upright directions, called bi-directional inclined plane testing, is sufficient (Pearse et al., 2005). The angle of inclination is then gradually increased towards the vertical position until the rat can no longer remain in place at the starting position. The greatest angle at which the rat can maintain a stable position for 5 s is recorded. Performance on the inclined plane correlates with the integrity of the rubrospinal tract and other non-pyramidal pathways after SCI (Fehlings and Tator, 1995). In addition, this test can be used as an index of animal strength (Gale et al., 1985; von Euler et al., 1997). The inclined plane has been shown to be a sensitive and reliable test for clip compression injury (Fehlings and Tator, 1995; Rivlin and Tator, 1977).

2. Limb hanging test

This test, introduced by Diener and Bregman (1998), utilizes the natural grasping function of the paw. Although it is able to evaluate both forelimb and hindlimb function, it is mainly employed for testing forelimb muscle function in animals with cervical spinal cord lesions (Pearse et al., 2005). The stimulation is provided using a 12-cm long and 1.8 mm wide rounded metal rod, which is applied to the volar surface of the forepaw, and the presence or absence of grasping and the release time in seconds are evaluated. In addition, the testing of animal's forelimb muscle strength is also possible, when the rod is elevated above the surface and suspended. The contact of the body, hindlimb or tail with the ground or parts of the equipment on the sides should be prevented. The time the rat holds onto the suspended rod is measured and recorded. The testing should be done repeatedly, typically five times, and the mean values calculated (Pearse et al., 2005). In severely injured animals, the forelimb hanging test provides only dichotomous yes/no data; it should thus be employed in combination with other tests or replaced by other tests. It should be noted that a normal or mildly impaired animal might pull itself on top of the hanging device, rendering such a trial immeasurable (Pearse et al., 2005). We believe that this can be prevented by using a rotating rod.

3. Limb grip strength test

The limb grip strength test was developed on the basis of the previous test in order to assess neuromuscular function, in particular strength, by sensing the peak amount of force

an animal applies in grasping a specially designed pull bar assembly. This test can be used for evaluating both forelimb and hindlimb function. It requires a special device called a grip strength meter (San Diego Instruments, CA or Columbus Instruments, OH), which is constructed on the basis of the Meyer Method (Meyer et al., 1979). The animal is gently held and permitted to grasp with its limbs a mesh grip attachment that has been placed on a digital force gauge. The animal is then drawn along a straight line leading away from the sensor until the animal releases the grip mesh. At this point, the maximum grip strength in Newtons is attained and displayed. The values can be recorded manually or the whole device can be connected to a computer. Usually, three consecutive trials are undertaken (Pearse et al., 2005). The weakness of this test is similar to that of the previous test—it is not able to evaluate severely injured animals. In addition, special equipment is needed. However, the obtained data are precise and unique—not many tests are able to measure limb muscle strength using such a simple method.

5.4. Forelimb asymmetry test

The forelimb asymmetry test (paw preference test, limb-use asymmetry test, cylinder test) is sensitive to asymmetries produced by a variety of central nervous system insults, including SCI at the cervical level. It is a natural feature of a rat to explore vertical surfaces by rearing up on its hindlimbs and exploring the surface with its front paws and vibrissae (Gharbawie et al., 2004). The number of times an animal in a clear upright plastic cylinder independently places its left, right or both forepaws against the side of the so-called Schallert's cylinder during weight-supported movements is recorded using a video camera, counted and analyzed. Limb use is scored as the percentage of left, right, or both-limb wall placements relative to the total number of placements observed. It is also possible to obtain a single limb-use asymmetry score by subtracting the percentage of independent use of the impaired limb from the percentage use of the unimpaired limb. Higher numbers then indicate a greater bias for the use of the unimpaired limb. It is important to prevent the habituation of the rat to the cylinder by testing during the dark cycle and by dividing long trials into shorter segments separated by several minutes, during which the rat is placed back in its home cage. A notable feature of this test is its high degree of sensitivity to chronic deficits that might be masked by post-lesion compensatory behaviors. In addition, it is able to detect chronic sensorimotor deficits that many tests fail to detect (Gensel et al., 2006; Schallert et al., 1986, 2000; Schallert and Woodlee, 2005).

5.5. Rearing test

The equipment required for the rearing test is similar to that for the forelimb asymmetry test, but the idea behind the test is different. The rats are tested individually in an

open field for a period of 10 min by an investigator blinded to the treatment condition. The sessions are videotaped and the number of rearings quantified from the videotape. This test is a sensitive measure of the selective use of the hindlimbs defined by the simultaneous lifting of both front paws off the floor and then adopting either a free standing posture or leaning up in a standing position against a side wall for balance with most weight still supported on the rear paws. The smooth plastic surface of the cage prohibits the rat from compensating for rear limb weakness by using the front paws to pull itself up to a rearing stance (Arvanian et al., 2006).

Animals with moderate and moderate-severe SCI rarely develop weight supported rearing. Rather, they explore with their forelimbs from a crouched position and their rearing action is then called attempted rears. One possibility is to analyze the number of such rears during a 3 min observation period and then to analyze them separately (Yoshihara et al., 2006).

5.6. Food pellet reaching test

This examines the ability of the forelimbs to reach, touch, grasp and retrieve a food pellet. For SCI experiments, it can thus be used only for lesions at the cervical level. The objective performance of the test requires pretraining the rats to reach a 190 mg food pellet through a 1–2 cm wide opening in the wall of a clear Plexiglas box (30 × 36 × 30 cm). During the test, the retrieval of each food pellet is documented using one or two video cameras for further analysis. To ensure that the rat approaches the opening with a new stance on every reach, a 45 mg pellet is dropped into the back of the box each time the rat retrieves the pellet, so the rat is encouraged to leave the reaching area. The "success rate" of food grasping is calculated as the number of times the rat successfully grasps and retrieves the food pellet, divided by the number of attempts. For evaluation, a 10-point scale developed by Whishaw (2000) is used, which scores the successful execution of the sequential aspects of the reach, i.e. reaching, grasping and retrieval of the pellet. In addition, the amount of time needed to grasp 10 pellets can be measured (Chan et al., 2005; Whishaw et al., 1993, Z'Graggen et al., 1998). A modification of the foot pellet reaching test is the staircase test (paw reaching test), used mainly for the behavioral testing of rats after stroke (Montoya et al., 1991; Grabowski et al., 1993).

6. Sensory tests

Here are included the behavioral testing methods able to detect sensory system disorders in terms of hyperactivity (hypersensitivity, allodynia) or hypoactivity (decrease or loss of sensoric functions) of the sense of touch, cold, heat, or pain. To locate the borderline between a sensory-motor response involving both the sensory ascending and descending motor pathways and a pain-based withdrawal

reflex-response is not always possible. For example, in hot plate-based tests, the limb can be withdrawn due to either the sensing of heat or the stimulation of nociceptive endings on the basis of skin damage. Although a cut-off time is utilized to prevent the latter, one cannot always be sure that all responses are based on proper temperature sensing.

6.1. Hot plate-based tests

Several different variations of the hot plate withdrawal test equipment and arrangement exist. For example, Gale et al. (1985) used a hotplate pre-heated to 50 °C for a period of 60 s and measured the time that transpired before the rat licked each hindpaw. Animals which showed no reaction were removed after 60 s to avoid paw injury. A standardized and commercially available example of such a test is the Plantar Heater Test (Ugo Basil, Comerio, Italy), in which three rats are put into three separate plastic cages and each animal is tested three times (sequence 1, 2, 3, 1, 2, 3, 1, 2, 3) (Fig. 1). It is crucial to always test the same part of the limb during the entire experiment, usually the center of the rat's hindpaw sole (Hargreaves et al., 1988). The cut-off time of a movable infrared generator, located under the Plexiglas floor of the cages, is usually set to 35 s, and its intensity is set between 50 and 60 units, which correspond to the midpoint of the emission range. This testing arrangement precludes any injury to the animal's paw (Hargreaves et al., 1988; Syková and Jendelová, 2005; Syková et al., 2006a; Urdzíková et al., 2006). A modification of the plantar hot-plate test is the tail-flick test. The testing procedure is the same with the exception that the base of the tail is heated (Merkler et al., 2001).

6.2. Cold sensitivity-based tests

In the cold-spray test, ethyl chloride is sprayed on shaved skin and the response of the animal is recorded and graded as: 0—no response, 1—localized response, i.e. transient skin twitch, 2—transient vocalization, 3—sustained vocalization (Yu et al., 1998; Vaquero et al., 2006). Another option for testing cold sensitivity is the application of 100 µl of acetone onto the plantar hindpaw. The response to five applications of acetone is recorded and converted to
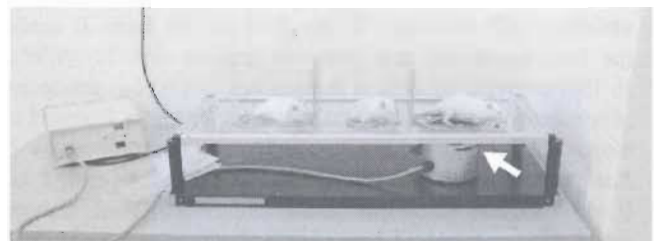

Fig. 1. A plantar apparatus (Ugo Basile, Comerio, Italy) which measures the hind paw withdrawal latency from a radiant heat source (arrow). Note that three rats can be tested in one session.

a percentage. At least 3 min should elapse between each session (Choi et al., 1994).

6.3. Von Frey filaments

Von Frey filaments (Von Frey hairs, Semmes-Weinstein monofilaments) are often used to evaluate the degree of mechanical allodynia—one type of neuropathic pain resulting in an increased sensitivity to innocuous stimuli, i.e. stimuli that are not painful for an uninjured animal or person (Gris et al., 2004). The calibrated filaments of ascending target forces (0.008–300 N or 2.5–125 g) are sequentially applied to the plantar surface of the forelimb or hindpaw, with a pressure that causes a slight bend of the filament, until a withdrawal response is elicited. Usually, a Plexiglas box with a fine-grid bottom is used (Liebscher et al., 2005). If a Plexiglas box is not available, testing might be done in the tester's hand, but a food reward throughout testing should be given to prevent visual recognition of the application of the filament. A positive response occurs when the paw is briskly withdrawn from the filament. This response might be accompanied by flinching, escape, licking, vocalization or abnormal aggressive behaviors (Gris et al., 2004). After a brief 3–5 min rest period, the paw should be re-tested with the same filament. Since a change in the absolute number of withdrawals to a stimulus may indicate an increase in the spinal reflex and not the development of allodynia, only withdrawals accompanied by supraspinal behaviors, such as head turning to attend to the stimulus or biting the Von Frey filament, are counted as a response (Chan et al., 2005; Hutchinson et al., 2004; Levin et al., 1978; Mills et al., 2001).

6.4. Paw compression test

This test, first described by Randall and Selitto (1957), enables the tester to measure hindpaw hyperalgesia. For the procedure, a commercially available analgesia meter can be used (Ugo Basile, Comerio, Italy), in which a plastic device is in contact with the third interdigital space of the hindlimbs, which are lying on a plane surface. A progressive weight is then applied and the test stopped when the animal performs any movement of the paw or when the weight reaches a maximum, cut-off level. The force at which the rat withdraws its hindpaw is noted, multiplied by 10, and the withdrawal force in grams is thus obtained (Randall and Selitto, 1957; Giglio et al., 2006).

6.5. Withdrawal reflexes

Withdrawal reflexes are a group of stimulus-based reflex response reactions. They are evaluated as to both the speed and the force with which the hindlimb is withdrawn when stimulated by extension, pain, or pressure. This is done by pulling the hindlimb backward with 2 fingers (extension),

pricking the sole of the foot with a needle (pain), or pressing the foot between the tester's thumb and index finger (pressure). The reflex response is considered absent, normal or abnormal (Gale et al., 1985; von Euler et al., 1997). Although their performance does not need any special device and is thus quite inexpensive, such tests are not very sensitive and can be only used for evaluating the integrity of spinal segmental reflex circuits. However, in many SCI models, mainly the ascending and descending pathways are disrupted, so the use of withdrawal reflexes in such models is very limited.

7. Sensory–motor tests

An accurate response in such behavioral tests requires functional sensory and motor systems and, most importantly, their proper connection.

7.1. Rope walk testing

Experimental animals have to cross a 125 cm long horizontally oriented rope, 4 cm in diameter, three times between two platforms. During each run the number of slips and falls is counted. Successful completion of this task requires hindlimb weight support, precise paw placement, posture and coordinated balance of the body. This test is very sensitive to unilateral lesions, such as hemisections. Trained unlesioned rats cross the rope quickly, seldom slip and do not fall. Usually, the "total error/step" ratio is counted (no. of slips + 2 × [no. of falls]/ [total no. of steps]) (Kim et al., 2001) or a 0–4° scale is used to evaluate the locomotor performance. Each rat is tested three times. A limitation of this test might be the required training of the rats, which can take five weeks before the experiments (Kim et al., 2001; Ruitenberg et al., 2003).

7.2. Narrow beam test

In the narrow beam test (beam walking test), originally described by Hicks and D'Amato (1975), three types of beams are used as narrow pathways: a rectangular 2.3-cm wide beam, a rectangular 1.2-cm wide beam and a round dowel of 2.5-cm diameter. All beams are 1 m long and elevated 30 cm from the ground. The pre-training of rats is required, and the rats must be able to transverse the horizontal beams with less than 3 footfalls. A scoring system is used for each beam: 0 indicates the complete inability of the animal to walk on the beam and an immediate fall, 0.5 if the animal is able to traverse half of the beam, 1 point is given for traversing the whole length, 1.5 when stepping with the hindlimbs is partially possible and 2 points are awarded for normal weight support and accurate foot placement. Thus, the scale ranges from 0 (0 + 0 + 0) to 6 (2 + 2 + 2) (Hicks and D'Amato, 1975; Metz et al., 2000). Quantitative assessment of narrow beam performance is a very sensitive tool to monitor even discrete deficits in foot placement and body balance,

including tail movements. Another advantage of this paradigm is that the difficulty of this task can be varied via the narrowness of and the shape of the beams. The ability of rats to cross a narrow beam is dependent upon the function of spinal networks as well as on supraspinal motor control from the cortico-, rubro-, and possibly the vestibulospinal tracts (Metz et al., 2000). In paralyzed animals, such a scoring system can be useful since the rats can be trained to traverse the beam without the use of the hindlimbs by crawling with the forelimbs only. In the scoring system, these observations can be taken into account (Metz et al., 2000). Modifications of this test have also been developed, for example, von Euler et al. (1997) used seven different planks of different widths, on which the rats are tested in sequence from the widest to the narrowest. The narrowest plank that a rat can cross without slipping is recorded.

The use of a tapered and/or ledged beam is also possible, although its use predominates in the behavioral testing of rats following stroke. In this test, the rats are first trained and then allowed to traverse an elevated beam that is tapered along its extent and has an underhanging ledge that the rat can use as a crutch if it slips. The difficulty of this test increases as the rat moves along the narrowing beam, leading to more foot faults. Hindlimb foot faults are measured as an index of hindlimb function. The strength of this test is that it does not allow the rat to mask its motor deficits by compensatory mechanisms (Ohlsson and Johansson, 1995; Sutton and Feeney, 1992; Schallert and Woodlee, 2005; Schallert et al., 2002).

7.3. Grooming test

This test was originally developed by Bertelli and Mira (1993) for testing recovery in a brachial plexus injury model. Now, it is often used for testing following SCI in the cervical region (Gensel et al., 2006). Cool tap water is applied to the rat's head and back with soft gauze, then the animal is returned to its home cage. Grooming activity is then recorded by a video camera and evaluated. The scale is set between 0 and 5. Zero reflects the inability of the animal to contact any part of its head while 5 represents a normal animal, able to contact the area above the ears with its forepaws (Bertelli and Mira, 1993; Gensel et al., 2006).

7.4. Foot slip test

In the foot slip test (horizontal ladder walking test), animals walk along a horizontal ladder with variable rung spacing. The ladder consists of side rails and metal rungs, with a platform on each side. To prevent the animals from learning the pattern and anticipating the position of the rungs, the distances between metal rungs are irregular and the pattern differs depending on whether the animal is walking from the left or the right side. Crossing the
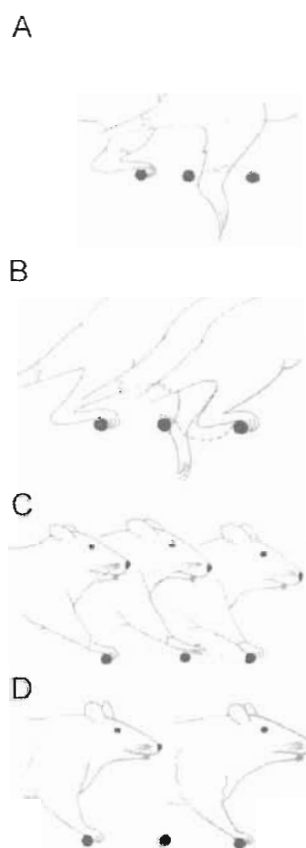

Fig. 2. Representation of the four horizontal ladder movement categories. (A) Miss. (B) Slip. (C) Touch. (D) Step.

horizontal ladder requires that animals accurately place their limbs on the bars (Fig. 2). Walking over the ladder is recorded with a video camera and analyzed in slow motion. The foot slip (missteps, errors) frequency is calculated as the number of foot slips by each limb divided by the total number of steps (Chan et al., 2005; Metz and Whishaw, 2002). In addition, qualitative evaluation of forelimb and hindlimb placement can be performed using a foot fault scoring system (Table 7), developed by Metz and Whishaw (2002).

7.5. Grid walking test

The grid walking test (grid walk test, foot fault test) is a very sensitive test for evaluating the sensory–motor coordination of the forelimb and hindlimbs and the descending motor control of the limb motor pathways. Animals are allowed to cross a 1–1.2 m long grid with irregularly assigned gaps (0.5–5 cm in diameter) between round metal bars while the number of limb displacements, described as both foot falls or foot faults, is counted and averaged. In addition, plastic garden fencing (3 ft × 3 ft) stretched over a metal frame can also be used. Crossing the grid requires that the animals accurately place their limbs on the bars. If an animal is not able to move its hindlimbs,

Table 7
Foot fault scoring system

Category	Type of foot misplacement	Characteristics
1	Total miss	Deep fall after limb missed the rung
	Deep slip	Deep fall after limb slipped off the rung
2	Slight slip	Slight fall after limb slipped off the rung
3	Replacement	Limb replaced from one rung to another
4	Correction	Limb aimed for one rung but was placed on another
		Or: limb position on same rung was corrected
5	Partial placement	Limb placed on rung with either digits/toes or wrist/heel
6	Correct placement	Mid-portion of limb placed on rung

Reprinted from the Journal of Neuroscience Methods, 115, Metz GAS and Whishaw IQ. Cortical and subcortical lesions impair skilled walking in the ladder rung walking test: a new task to evaluate forelimb and hindlimb stepping, placing, and co-ordination, pp. 169–179, copyright (2002), with permission from Elsevier.

a maximum score of 20 is given. The numbers of errors counted can be also classified as a non-parametric grid walk score: 0–1 error is scored as 3 points, 2–5 as 2 points, 6–9 as 1 point and 10–20 footfalls as 0 points. Every animal has to cross the grid at least three times (Behrmann et al., 1992; Metz et al., 2000).

To successfully cross the grid, the animal requires normal forelimb–hindlimb coordination, which is mediated by ventrolateral tracts, a functioning reticulospinal system to initiate the stepping rhythm, as well as voluntary movement control, which is predominantly mediated by the corticospinal and rubrospinal systems in rats (Metz et al., 2000). Therefore, complex tasks such as the grid walk paradigm can reveal deficits that are not apparent during normal locomotion. In order to improve the effectiveness of the training and reduce the training effect of repeated trials, a variably spaced grid should be used. However, gait velocity and stress can influence the outcome of this task—more foot faults may occur when animals are crossing the grid faster or inattentively. By using a 4-point rating scale and respecting the animal's individuality, this interference can be filtered out (Metz et al., 2000, 2001). The most beneficial aspect of this test is the possibility to focus on the isolated function of the hindlimbs in thoracic spinal cord injured rats, whereas these injuries do not produce noticeable deficits in overground locomotion in quadrupedal rats in comparison with humans, in whom they are enough to produce a complete loss of walking ability (Norrie et al., 2005). This test is, however, useful mainly for low or moderate SCI, after the rats are able to accomplish weight-supported hindlimb plantar placement and some degree of coordinated stepping; otherwise they simply drag their hindquarters across the runaway. Thus, some authors start to use it after the time at which the rats gain such locomotor skills (Gris et al., 2004).

8. Reflex response-based tests

8.1. Toe spread reflex

This reflex is studied by lifting the rat by its tail with its legs hanging free and observing the spread of the toes. The reflex response is considered absent, normal or abnormal. An abnormal response presents as hyperextension and/or shaking of the toes or feet (Gale et al., 1985; von Euler et al., 1997). For several days after injury, there is no response, followed by an abnormal response. A hyperactive hindlimb response seems to reflect decreased control of the locomotor system from the upper motor neurons. Subsequently, the reflex response normalizes, first as a minimal spread of the toes, then the full normal response develops (Seki et al., 2002).

Toe spread is caused by the contraction of the musculi interossei and the abductor hallucis muscle, which are innervated by the common peroneal nerve, which is a branch of the sciatic nerve. For this reason, the reflex is also used for evaluating regeneration after experimental sciatic nerve damage (Pockett and Philip, 1987; Renno et al., 2006).

8.2. Contact placing response test

The contact placing response test (tactile placing test, hindlimb placing test) is based on the fact that contact placing of the hindpaw can be elicited by lightly touching the skin of the dorsal side of the foot without any joint displacement—the animal responds by lifting the hindlimb and placing it upon the obstacle (Kunkel et al., 1993; Metz et al., 2000). The animal is held, supported by the upper body, with the hindlimbs hanging free. The dorsum of each foot is touched with the edge of a piece of paper. The total number of placing responses of 10 trials per limb is noted, and the placing rate for the individual animal is determined from baseline data taken as 100% (Kunkel et al., 1993; Metz et al., 2000). The old version of this test is called the placing reflex test, in which the lateral and dorsal aspects of each foot are rubbed against a table to elicit the reflex response. The speed and accuracy in placing the foot on the table are evaluated. The reflex response is considered absent, normal or abnormal (Gale et al., 1985; von Euler et al., 1997).

Unfortunately, the placing reactions of injured and even normal rats are variable, so this test might provide distorted results when not used in combination with other tests (Metz et al., 2000). This finding was confirmed in spinal cats (Forssberg et al., 1974). The placing response might also depend on muscle tone—after acute SCI, paralyzed animals often show a clearer placing reaction than do normal animals. On way to reduce intra-individual variability is to calculate the ratio of post-operative to pre-operative values.

The development of placing responses temporally correlates with the post-natal growth of corticospinal

axons into the gray matter of the spinal cord. In normal animals, a tactile hindlimb response first appears on postnatal day 13 and slowly declines with age (Donatelle, 1977). In a study by Marshall (1982), the reflex response was present in 90% of rats 6–12 months old but in only 24% of animals older than 24 months. Proper contact placing thus depends on the integrity of the corticospinal tract and cortical control. In cats and rats, this reflex has been shown to be dependent upon spinal circuitry that normally is under supraspinal control, but which remains elicitable in decerebrate animals (Woolf, 1984). Another study indicates that the tracts running in the ventrolateral part of the spinal cord may play a role in mediating this reflex response (Metz et al., 2000).

8.3. Righting reflex

The righting reflex (static reflex) is usually defined as any one of a number of various reflexes that tend to bring the body into a normal position in space and resist forces acting to displace it from its normal position. In experimental SCI, righting reflex testing usually means the time in seconds spent by an animal to assume a normal ventral position after being placed on its back. It is elicited by holding a rat in one hand, turning it over on its back, 7–8 cm above a cushioned table surface and then dropping the animal. The reflex response is considered absent, normal or abnormal and its length might be measured. In addition, the reflex response might also be rated as 0 (no righting reflex), 1 (attempt to right itself), 2 (rights itself during the drop) or 3 (rights itself immediately after the drop) (Gale et al., 1985; von Euler et al., 1997). The righting reflex response might be affected by previous sleep deprivation (Tung et al., 2005).

9. Autonomic tests

Autonomic system disturbances, such as disruption of urinary bladder function or sexual reflexes, are important complications of SCI in both animals and humans. In the majority of animal studies, the testing of autonomic functions is unfortunately largely neglected, although urogenital system disturbances represent a very important clinical problem in human medicine (for review, see Karlsson, 2006).

9.1. Urinary bladder function

The most common autonomic dysfunction in experimental rats with SCI is detrusor–sphincter dyssynergia of the urinary bladder during the first 1–2 weeks. This affection manifests as urine retention and requires daily manual evacuation of the urine—the Credé maneuver. Emptying the bladder is crucial for avoiding urinary retention, which may cause damage to the bladder wall, with bleeding and renal damage by urine reflux. The increased chance of acquiring a urinary infection is clear.

The bladder function scoring system was developed by Martin Schwab's lab (Liebscher et al., 2005) as follows: (i) dysfunction is defined as a full bladder, medium to high pressure required for manual voiding of the bladder; (ii) normal function is an empty to half-full bladder, voiding after a slight touch. Following SCI at the cervical level, detrusor–sphincter dyssynergia does not occur, so the animals do not need this kind of intensive care (Soblosky et al., 2001).

For the quantitative study of the various phases of the urinary bladder voiding cycle, a cystometric procedure, described by Maggi et al. (1986) that involves a non-stop transvesical infusion of warm saline in anesthetized rats, might be used. The saline is infused into the bladder through a needle inserted into the bladder's dome. The recording is performed by a transurethral bladder catheter. During the bladder detrusor contractions, fluid is released by flowing around the catheter in the urethra. The signal is amplified, sampled, acquired and analyzed by a special device connected to a computer (Maggi et al., 1986). The main disadvantages of this method, i.e. the necessity of anesthesia and the quite complex nature of the monitoring device, are compensated for by the acquisition of unique and precise data (Maggi et al., 1986; Pikov and Wrathall, 2001, 2002; Yoshihama and de Groat, 2002).

9.2. Erection-based tests

The most frequently used erection-based tests are the ex copula reflex erection test, the non-contact erection test and the mating test. In the ex copula reflex erection test, the preputial sheath of a conscious animal is retracted to elicit a reflex erection and maintained in this position for 20 min by placing the glans of the penis through a hole in a small piece of tape fastened to the abdomen. Events are visually scored from 1 (weak glans engorgement) to 5 (intense flaring or cup of the distal glans together with dorsiflexion or flip of the penile body greater than 90° with respect to the body of the rat) (Holmes et al., 1988; Schmidt et al., 1995; Nout et al., 2007). In the non-contact erection test, a male rat is put into one half of a cage, separated from the other half by a sheet of wire mesh, and left for 5 min to adjust to his new environment. After that, an estrous female rat, whose rut is ensured by the administration of estradiol and progesterone and a preliminary approximation to a healthy male rat, is placed into the second half of the cage and events are observed for 30 min. They are scored using a 3 point scale (1—visible erections, 2—grooming of the body parts, 3—grooming of the genital area) (Sachs et al., 1994; Nout et al., 2007). The mating test has a similar arrangement as the one above, except the male and female rats are put together and the scale is 6-pointed. The first 3 points of the scale are the same and the other 3 points describe the following mating behaviors: 4—mounts, 5—intromissions, 6—ejaculations (Nout et al., 2007).

9.3. Telemetric monitoring

To simultaneously evaluate micturition parameters and describe erectile events in rats with SCI, a new method of telemetric monitoring of corpus spongiosum penis pressure has been recently developed (Nout et al., 2005, 2007). The development of this technique was based on previous experiments by Schmidt et al. (1995). After the minimally invasive implantation of a telemetric pressure transducer catheter, an analysis of the pressure waveforms of micturition, full erectile and partial erectile events together with the video recording of micturition events and the performance of a reflex erection test—ex copulatory reflex erection test (Schmidt et al., 1995)—is possible in conscious, freely moving rats. In the first 3–4 weeks following moderate SCI, continuous dribbling of urine, i.e. a typical sign of overflow incontinence, can be seen on video recordings, which results in no detectable changes of corpus spongiosum penis pressure. Measurable characteristics first appear following the beginning of the return of autonomic–somatic motor function (Nout et al., 2005, 2007).

9.4. Autonomic dysreflexia testing

Autonomic dysreflexia, also known as hyperreflexia, is a massive sympathetic discharge that occurs in both spinal cord injured animals and human patients. It is manifested by often debilitating hypertension accompanied by bradycardia, sweating, skin flushing and pounding headaches. It is triggered by a variety of noxious stimuli, including bladder distention, irritation of the urinary tract, bowel distention or impaction, skin ulcers, fractures, abdominal emergencies or uterine contractions. Autonomic dysreflexia most likely develops on the basis of an injury-induced loss of descending tonic and baroreceptor-related control of sympathetic pre-ganglionic neurons in the intermediolateral cell column of the thoracolumbal spinal cord (Cameron et al., 2006; Gris et al., 2004).

The testing method, also called noxious colorectal distention, is based on monitoring blood pressure during and after bowel distention induced by balloon inflation inside the colon lumen. Before the testing session, the carotid or femoral artery of the animal is cannulated under general anesthesia, and the animal is put into a special cage to prevent damage to the cannula. After 3–4 days, the cannula is connected to a monitoring device, and the animal left to stabilize its blood pressure to obtain a baseline value. This usually does not take more than 10 min. Then, a balloon-tipped catheter is inserted into the animal's colon, slowly inflated with 2 ml of air over 15 s and maintained for 1 min (Ditor et al., 2006). The inflation of the balloon with 2 ml of air generates a colon distension similar to that during the passing of a large fecal bolus, thus mimicking the clinical manifestation of fecal impaction (Cameron et al., 2006; Marsh and Weaver, 2004). An animal is regarded as dysreflectic if colorectal distention produces a rise in blood pressure and a decrease in heart rate for as long as the period of colorectal distention. More than one trial might be conducted. As autonomic dysreflexia occurs only in spinal injuries above the Th6 level, the use of the testing method would thus be suitable only for cervical and upper thoracic spinal cord lesions (Cameron et al., 2006; Weaver et al., 2001). The main advantage of this test is its ability to uncover the presence of autonomic dysreflexia and thus obtain unique data, reflecting the presence or absence of this pathologic condition. In addition, it provides the experimenter with objective, reliable and precise data. However, this test is relatively invasive and requires a special device for monitoring blood pressure and heart rate. More importantly, it can hardly be performed repeatedly. This can be overcome by performing the test only in one session, most frequently two weeks post-injury (Cameron et al., 2006; Weaver et al., 2001). In case the severity of autonomic dysreflexia is not the only studied parameter, this test should certainly be combined with other tests.

In the future, other types of autonomic tests might be developed based, for example, on changes in the vasoconstriction/vasodilatation of skin vessels due to sympathetic system function changes, including autonomic dysreflexia. Also, the frequent monitoring of temperature might be useful. In some animals, the presence of bowel constipation due to SCI-induced spasticity of the anal sphincter and the decreased activity of the descending colon might be observed and its presence or absence included in the observation protocol. However, constipation does not occur in SCI rats (Santos-Benito et al., 2006).

10. Increasing the skills of the animals

Intensive daily training improves functional locomotor recovery after SCI in both animals and humans (for review see Barbeau et al., 2002), and the benefits are retained for some time after training (Norrie et al., 2005). The recovery of sensorimotor functions in behavioral enrichment procedures is based mainly on the facilitation of neuronal plasticity, including neural or astrocytic growth factor expression, axonal sprouting, synapse remodeling, receptor density changes, neural-glial interactions and cell mitotic activity, differentiation and migration (for review, see Ding et al., 2005). Studies in rodents and humans suggest that the timing of the training onset may be important because a long delay between injury and the commencement of training appears to reduce the beneficial effects of training regimens (Norrie et al., 2005; Wernig, 2006). Thus, a delay period makes SCI victims less responsive to rehabilitative training (Norrie et al., 2005; Wernig, 2006). On the other hand, several authors have shown that post-injury training is task specific; animals that are trained to walk show improvements in walking, training to stand will improve standing but not walking, swimming will improve mainly swimming skills, etc. (De Leon et al., 1998a, b; Hutchinson et al., 2004; Smith et al., 2006a, b). In the future, it should

thus be reasonable to combine rehabilitative techniques, as is being done in humans suffering from SCI. Several behavioral tests can also be used as training methods, for example, the use of a horizontal ladder (Norrie et al., 2005).

10.1. Enriched environment

Although they have no agreed-upon definition, enriched environments are housing conditions that go beyond meeting the fundamental requirements of animal welfare by offering complex and stimulating conditions that are more conducive to natural behavior than what animals experience in standard housing (Döbrössy and Dunnet, 2004). Even the most sedentary of people does not experience as impoverished an environment as a rat living in an isolated home cage (Schallert and Woodlee, 2005). The simplest methods of enriching the environment are increasing the number of cage-mates, introducing additional objects such as wooden blocks or sticks and enriching the food with, for example, forage grains or pellets. Enrichment should also be defined as the presence of sufficient space for hiding or escaping from conspecifics in the cage (Tatlisumak and Fisher, 2006). In contrast to standard housing conditions, animals in an enriched environment have greater opportunities for sensory and motor stimulation, activity, social interaction and exploration of the environment (Fig. 3). It has been shown that an enriched environment promotes neurogenesis within the population of resting stem cells and enhances the recovery from central nervous system injury at both the structural and functional levels (Döbrössy and Dunnet, 2004; Rose et al., 1993; Young et al., 1999). To increase the activity of animals, dairy food might be placed inside the toys of an enriched environment (Santos-Benito et al., 2006).

10.2. Treadmill and running wheel training

Locomotor training has been shown to accelerate locomotor recovery in cats and humans (Barbeau and Rossignol, 1987, 1994), indicating that training can be a valuable tool during rehabilitation. For example, the introduction into clinical practice of weight-supported training of patients with partial SCI on a treadmill was firmly based on animal research (Barbeau and Rossignol, 1987; Fouad and Pearson, 2004; Harkema, 2001).

Generally, rats do not spontaneously develop rhythmic locomotor movements of the hindlimbs following complete spinal cord transection (Weber and Stelzner, 1977). In quadrupeds with SCI, there is overwhelming evidence that neuronal networks, referred to as central pattern generators, can generate a variety of rhythmic patterns, depending on the manner in which they are activated, the chemical environment, and the extent of isolation from other neural tissue (for review, see Fouad and Pearson, 2004). We know the central pattern generator for each hind leg is distributed within the lumbar region of the spinal cord in rodents (Kiehn and Kjaerulff, 1998). Undoubtedly the greatest uncertainty is whether central pattern generators exist in the human spinal cord (Fouad and Pearson, 2004). Rhythmic stepping movements are not common in patients with complete SCI, but have been frequently observed in patients with severe incomplete injury, in both cases following locomotor training (Dimitrijevic et al., 1998; Wernig et al., 1999).

From studies on experimental animals it is known that a motor pattern for locomotion can be expressed following the application of serotonin (Feraboli-Lohnherr et al., 1999; Gimenez y Ribotta et al., 1998). The challenge, therefore, is to facilitate activity in these networks. It has been shown that treadmill-training techniques (Fig. 4) are effective in improving the locomotor performance of rats with incomplete SCI, most probably on the basis of such activation of the central pattern generator network (Multon et al., 2003; Thota et al., 2001). However, some studies found no effect of such treatment (Fouad et al., 2000).

Similarly, a majority of reports indicates that both combined pre- and post-injury or isolated post-injury running wheel training has beneficial effects on the recovery of neurological functions in spinal cord injured rats (Engesser-Cesar et al., 2007; Hutchinson et al., 2004;
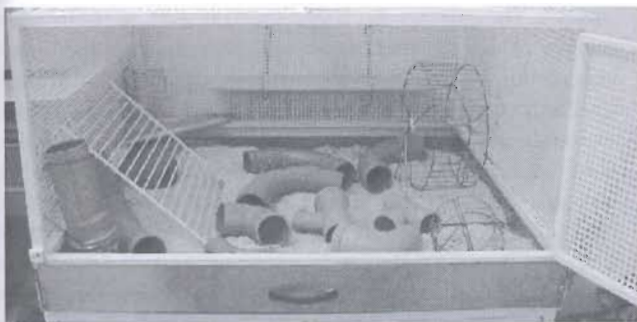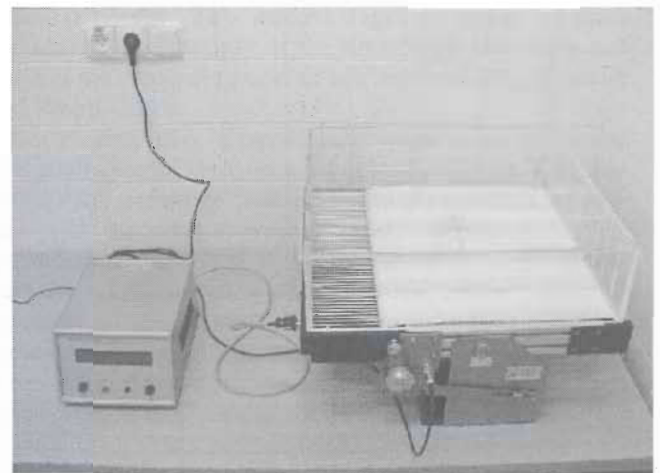

Fig. 3. Enriched environment.

Fig. 4. Treadmill.

Van Meeteren et al., 2003), but some authors have found no effect of such training (Erschbamer et al., 2006). Engesser-Cesar et al. (2007) recently found an increase in serotonin fiber length caudal to the lesion in the running vs. non-running groups, indicating the above-mentioned role of spinal nervous plasticity in terms of the development of the central pattern generator. Interestingly, they did not find a significant difference in behavioral outcome in rats which ran 3 days/week compared to 7 days/week, but both groups had significantly different behavioral outcomes from non-runners (Engesser-Cesar et al., 2007). Taken together, both treadmill and running wheel training might have an important role in the locomotor training of rats suffering from SCI. In addition, it is generally believed that treadmill (Fig. 4) or running wheel-based exercise has mood-enhancing and anxiety-reducing effects (Burghardt et al., 2004).

10.3. Swimming

As stated earlier in regards to swimming tests, swimming is a natural form of locomotion for rodents, including rats, which involves repetitive stepping-like movements of unloaded limbs. In comparison with a treadmill, swimming involves a higher number of step cycles being produced by the central pattern generator circuitry (Smith et al., 2006b). However, during swimming, both cutaneous feedback and loading of the limbs are significantly reduced (Muir and Steeves, 1995). It has been recently shown that swimming improves the functional recovery of spinal cord injured rats, especially sensory function (Hutchinson et al., 2004). If artificial cutaneous feedback is provided by adding buoyant centrifuge tubes attached to the bottom of the pool that touch the feet of the animals during swimming, locomotion is improved, but only to a limited extent (Smith et al., 2006a, b). This method was originally described in experiments with chicks by Muir and Steeves (1995).

10.4. Robot-assisted hindlimb extension

The development of robot-assisted hindlimb extension was based on the hypothesis that the training of hindlimb locomotion would be more efficient if an appropriate swing motion could be reliably elicited. The robotic "rat stepper" device used in this training method consists of a body weight support mechanism that can provide precise amounts of upward force to the torso, two lightweight robotic arms that can measure and manipulate hindlimb motion in the para-sagittal planes, and a miniature treadmill (Fig. 5). Beside its use in training spinal cord injured animals to step, this device can also be used for quantifying the body weight that spinal cord injured rats can support during stepping, for performing a detailed analysis of weight-supported, bipedal stepping and for examining the response of the spinal locomotor controller to small changes in the load on the hindlimbs (Nessler
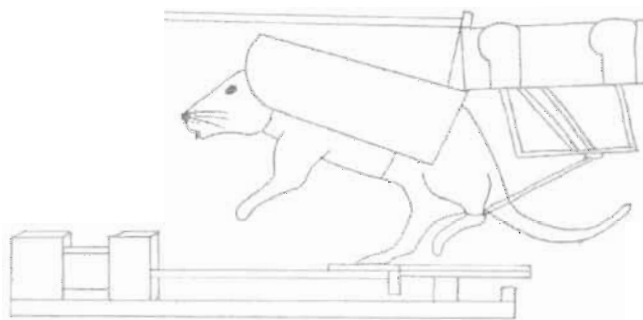

Fig. 5. Robot-assisted hindlimb extension device. The rat steps bipedally in the device, while placed in a cloth harness and attached to the end of the body weight support lever.

et al., 2005, 2007; Timoszyk et al., 2002). The value of robotic training is limited in complete spinal cord transection. In addition, and unfortunately, the majority of rodents used as models are injured as neonates (Timoszyk et al., 2005), which is in contrast to the usual clinical situation (Bracken et al., 1985; Syková et al., 2006b). The robotic stepper is also limited in its use because most studies involve bipedal stepping (Nessler et al., 2005, 2007; Timoszyk et al., 2002, 2005).

11. Electrophysiology and fMRI

11.1. Evoked potentials—transcranial

Animals are anesthetized and motor evoked potentials are elicited by transcranial electrical stimulation of the motor cortex using percutaneously placed stainless steel stimulating electrodes. Responses are recorded from a peripheral skeletal hindlimb muscle, usually the gastrocnemius, using needle electrodes. Conversely, brain activity can be recorded in response to sensory stimulation, eliciting somato-sensory evoked potentials (Maršala et al., 2004; Metz et al., 2000). The advantages of electrophysiological techniques lie in their direct and precise measurement of muscle activation, reflex latency and the relative strength of reflex responses. The disadvantages of these methods include the implantation of the stimulation electrodes and the fact that recording devices are required (Fig. 6) (Muir and Webb, 2000).

For the recording of transcranial magnetic motor evoked potentials, needle electrodes are introduced into a peripheral muscle, reference electrodes into the muscle's tendon, and over the skull a magnetic coil, responsible for the activation of subcortical structures, is placed. Subsequently, magnetic pulses are generated; action potentials descend in the ventral spinal cord and synapse on motoneuron pools. Electromyograms are recorded from the peripheral muscle, usually the gastrocnemius. The main advantages of this technique is its minimal invasiveness, the possibility of repeated measurements and the consistency of the results (Cruz-Orengo et al., 2007; Linden et al., 1999; Loy et al., 2002; Magnuson et al., 1999).
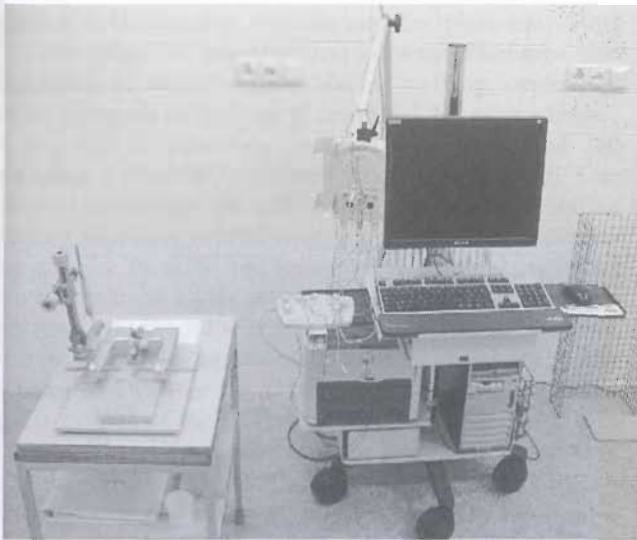
Fig. 6. Electrophysiological device connected to a computer (right) with a stereotactic holder (left).

11.2. Motor evoked potentials—intracranial–intraspinal

In this test, the animals are usually anesthetized and intubated. Atropine is injected to reduce tracheal secretions and pancuronium bromide for muscle relaxation. Body temperature is monitored. The animal is placed in a stereotactic holder (Fig. 6), the skull surgically opened and the motor cortex is directly stimulated by an electrode positioned by a micromanipulator. Recording of the impulse is done by epidural recording electrodes, positioned after laminectomy at the lumbar level. The motor evoked potentials are transmitted to and analyzed by special software (Fehlings et al., 1987; Lee et al., 2005). The disadvantages of this method are its invasiveness and the need for specialized devices and operators. In addition, testing cannot be repeated daily or weekly.

The above-mentioned data studies indicate that evoked potential monitoring is generally performed in anesthetized animals to eliminate stress and pain. For transcranial evoked potentials, intubation of the animals and supplementation with other drugs is usually not necessary, although for the more invasive evoked potential techniques, such procedures might be of benefit (Fehlings et al., 1987; Lee et al., 2005).

11.3. Contact electrode recording

This testing method combines the advantages of a horizontal runway and electrophysiological monitoring. First, the animals are pre-trained to cross a horizontal ladder, then they are fitted with small contact electrodes; eventually, the electromyography electrodes are also implanted. The contact electrodes, i.e. thin copper wires twisted into a spindle shape, are attached to the palmar or plantar surface of the paws by a thin rubber band. After some time, the rats tolerate the electrodes quite well and do

not try to remove them. The runway is covered with conductive material connected to a low voltage supply that enables recording (Gorska et al., 1998). The main advantage of this method is its accuracy. In addition, no anesthesia is needed during the testing session (Majczynski et al., 2007).

11.4. Functional MRI

Functional magnetic resonance imaging (fMRI) is a precise but challenging method, demanding a special MR-spectrometer. It is usually performed in anesthetized and mechanically ventilated rats. Bipolar stimulation electrodes must be implanted into the forelimbs and hindlimbs. Muscle relaxation with pancuronium bromide and temperature monitoring are also needed. After the stimulation of the limb electrodes, a signal in the somatosensory cortex and/or subcortical sensory areas can be recorded. This method makes it possible to distinguish between the recovery of sensory and motor function (Hofstetter et al., 2003).

12. Systematic protocols

The use of more than one test in a single experiment decreases the bias level and reduces the variability in animal performance caused by stress, handling, circadian rhythms, habituation or post-operative pain. Also, the degree to which treatment effects are mimicked or masked by spontaneous recovery and compensatory mechanisms is reduced. An important issue in combining tests is a potential partial overlap in outcomes between parameters due to a common neural basis. For many of these tests, the pathway involved in mediating the respective behavior is not completely known. Therefore, tests have to be selected carefully to cover a broad spectrum of parameters and to yield reproducible data over days or weeks. The effect of training must also be considered. Because most tests are differentially sensitive to the degree of injury, a combination of tests allows for a more complete and precise evaluation of the overall deficit than any individual test alone. Muir and Webb (2000) recommend that the testing battery should include: (i) a measure of motor abilities during spontaneous locomotor activity; (ii) a measure of abilities during one or more trained behavioral tasks; and (iii) an assessment of reflex function. Several methods and hints how to correctly choose the right behavioral tests and how to design an SCI study have been proposed (Basso, 2004; Goldberger et al., 1990; Kunkel et al., 1993; Metz et al., 2000).

12.1. Combined behavioral score

The combined behavioral score (CBS) was originally developed by Gale et al. (1985) (see also Kerasidis et al., 1987). To calculate the CBS, a battery of tests is used, including toe spread reflex, placing reflex, withdrawal in

response to stimulation, righting and hot plate tests. Rats are also tested for coordination between forelimbs and hindlimbs and weight support during walking, swimming, and standing on an inclined plane. The CBS ranges from 0 to 100, with 0 indicating no functional deficit and 100 indicating abnormal responses in all of the tests. Our analysis showed that the CBS is used mainly for evaluating recovery following contusion injury, followed by compression models. In both, the lesions were incomplete. It has been shown to be highly sensitive, therefore optimal for use with small spinal cord lesions (von Euler et al., 1997). Thus, for transection, evaluation methods other than CBS should be employed (Table 5). One problem with the CBS is similar to that of the BBB: it is not a linear test and thus points in the score represent more or less discrete aspects of behavior. Most importantly, because the final value is computed using a battery of different tests, the same value in two animals might reflect two different levels of recovery. For example, the first animal exhibits rapid locomotor recovery (weight support, swimming), the second one recovers sensory and sensory–motor pathways (reflexes, hot plate), and both achieve the same score. In extreme cases, the first animal is actively moving around, exploring the open field, but would easily hurt itself if it contacts some sharp or hot object. The second one would react to all sensory stimulation, but it is not able to move itself for even a few centimeters. Which one recovered more rapidly?

13. Conclusions

For the evaluation of functional recovery after SCI in the rat, several sensory, motor or autonomic behavioral testing methods as well as electrophysiology and fMRI can be used. Fundamentally, the more general the test, the broader its scope and the lower its precision. In addition, the greater the test's applicability, the less sensitive it tends to be. Before beginning a study, the proper selection of previously crosschecked behavioral testing methods is crucial for the success of the study.

Acknowledgments

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12. Přehled publikací autora

12.1. *Publikace s IF, které jsou podkladem disertace*

1. Šedý J, Urdziková L, Hejčl A, Burian M, Likavčanová K, Jendelová P, Zicha J, Kuneš J, Syková E. Low concentration of isoflurane promotes the development of neurogenic pulmonary edema in spinal cord injured rats. J Neurotrauma 2007, 24:1487-1501. **IF(2006)=3.453**

2. Šedý J, Zicha J, Kuneš J, Jendelová P, Syková E. Rapid but not slow spinal cord compression elicits neurogenic pulmonary edema in the rat. Physiol Res. In press. **IF(2006)=2.093**

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