CHARLES UNIVERSITY

FACULTY OF PHARMACY

Department of Pharmacology and Toxicology

# Diploma thesis

Salamanca & Hradec Králové 2024 Pavlína Hromadová

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# Role of RFR in developing and recovering from cisplatin-induced renal injury

Diploma thesis

Supervisors: Prof. Rosa Laura Vicente, Ph.D.

Prof. PharmDr. Petr Pávek, Ph.D.

2023/2024

Pavlína Hromadová

I declare I processed this thesis on my own. All bibliographic sources and other materials that I used for this work are listed in the references and cited properly.

Pavlína Hromadová

# Acknowledgement

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# Abbreviations

RFR- Renal functional reserve

AKI- acute kidney injury

CKD- chronic kidney disease

ROS- reactive oxygen species

DAMPS- damage-associated molecular pattern molecules

TLRs- Toll-like receptors

GFR- glomerular filtration rate

RBF- renal blood flow

DM- Diabetes mellitus

# Abstract

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**Supervisor:** Prof. Rosa Laura Vicente, Ph.D.

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**Title of diploma thesis:** Role of RFR in developing and recovering from cisplatin-induced renal injury

The purpose of this study was to find out the role of renal functional reserve (RFR) during development and recovery from cisplatin renal damage. This work focuses on evaluating whether RFR can detect (stages) of kidney damage that are not diagnosed with the clinical reference biomarker: plasma creatinine. To evaluate the RFR, Wistar rats produced in the animal house of the University of Salamanca were used. A protocol was established in which an intravenous perfusion of the amino acid glycine was used as a stressor and the glomerular filtration rate was evaluated through creatinine clearance. The animals received a toxic dose of cisplatin and the RFR was determined in one group two days before the maximum point of toxicity (day 2 of the experiment, evolution of kidney damage), in another one on the day of maximum toxicity (day 4) and in the last group on day 11 (recovery from kidney damage) after drug administration on day 0. The values are compared with those of a control group not treated with cisplatin. While on day 4 (maximum toxicity) the ability to activate RFR has been lost, two days before this mechanism is still active (to a lesser extent than a control) although creatinine is already elevated. On the other hand, on day 10 the animals had not completely recovered, presenting variability in the RFR results. This work lays the foundations to continue addressing the study of the role of RFR in cisplatin damage.

# Abstrakt

**Univerzita v Salamance**

**Fakulta farmacie**

**Katedra fyziologie a farmakologie, Toxikologická oblast**

**Kandidát:** Pavlína Hromadová

**Školitelé:** Prof. Rosa Laura Vicente, Ph.D.

Prof. PharmDr. Petr Pávek, Ph.D.

**Název diplomové práce:** Úloha funkční rezervy ledvin (RFR) při vzniku a zotavení z poškození ledvin způsobeného cisplatinou

Účelem této studie bylo zjistit roli renální funkční rezervy (RFR) při rozvoji a uzdravení z poškození ledvin po aplikaci cisplatiny. Tato práce se zaměřuje na vyhodnocení, zda RFR dokáže zaznamenat (stádia) poškození ledvin, která nejsou diagnostikována klinicky referenčním biomarkerem: plazmatickým kreatininem. K vyhodnocení RFR byly použity potkani kmene Wistar držené ve zvířecím chovu Univerzity v Salamance. Byl vytvořen protokol, ve kterém jako stresor byla použita intravenózní perfúze aminokyseliny glycinu a rychlost glomerulární filtrace byla hodnocena prostřednictvím clearance kreatininu. Zvířata dostala toxickou dávku cisplatiny a RFR byla stanovena v jedné skupině dva dny před maximálním bodem toxicity (2. den experimentu, při vývoji poškození ledvin), v jiné v den maximální toxicity (4. den) a v další skupině 11. den (zotavení z poškození ledvin) po podání léku v 0. den. Hodnoty jsou porovnány s hodnotami kontrolní skupiny potkanů, které nebyly léčené cisplatinou. Zatímco 4. den (maximální toxicita) byla schopnost aktivovat RFR eliminována, dva dny předtím je tento mechanismus stále aktivní (v menší míře než u kontrolních potkanů), ačkoli kreatinin je již zvýšený. Na druhou stranu, 10. den se zvířata úplně nezotavila, což vykozavolo variabilitu ve výsledcích RFR. Tato práce pokládá základy pro pokračování ve studiu role RFR při poškození ledvin cisplatinou.

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# 1. Theoretical part

## 1.1. Renal damage by cisplatin

Cisplatin, also known as cisplatinum or (SP-4-2)-diamminedichloridoplatinum(II) (CDDP) is one of the most effective medicaments and also an option for treating various solid cancers such as testicular, ovarian, bladder, lung, cervical cancer, melanoma, lymphomas, and several other types (Ghosh, 2019).

The chemical compound cis- [Pt $(NH_3)_2Cl_2$] was first discovered by Michele Peyrone in 1845, and henceforward, it was named Peyrone's salt for a long time. This structure was further evolved by Alfred Werner in 1893 and after that cisplatin was researched by other scientists like Barnett Rosenberg, who has exposed in 1969 that cisplatin might have promise in inhibiting sarcoma 180 and leukemia L1210 in mice (Ghosh, 2019).

Cisplatin belongs to a group of drugs that affect cancer cells by damaging DNA, inhibiting DNA synthesis and mitosis, and inducing apoptotic cell death. This also means that cisplatin has many side effects. The most serious side effects are related to nephrotoxicity, hepatotoxicity, hemotoxic disease, neurotoxicity, ototoxicity, cardiotoxicity, and other organ toxicity (Dasari & Tchounwou, 2014).

The primary excretion of cisplatin is through the kidneys (Visacri et al., 2017).

### 1.1.1. Cisplatin and nephrotoxicity

Cisplatin causes multifactorial reactions in the kidneys, which can lead to the loss of renal function and acute kidney injury. A typical sign is an increase in glomerular filtration rate and creatinine concentration in serum (Pabla & Dong, 2008).

The nephrotoxicity of the renal cells may be ascribed to an accumulation of cisplatin in the renal proximal tubule through membrane transport and intracellular change of the medicament into toxic metabolites due to dysregulation of oxidative stress, mitochondrial dysfunction, endoplasmic reticulum stress, inflammatory responses, apoptosis, necrosis, and autophagy (J. Zhang et al., 2021).

Cisplatin is highly accumulated in kidney during the processes like glomerular filtration and tubular secretion. The concentration of cisplatin in the blood is five times lower than the concentration in the proximal tubule, meaning that even sub-toxic cisplatin doses can unfortunately cause toxic concentrations of cisplatin in the kidney (J. Zhang et al., 2021).

The dose of cisplatin, frequency of administration, and cumulative cisplatin dose bring the elevation of renal toxicity same as older age, female sex, smoking, and hypoalbuminemia. The lower possibility of evolving nephrotoxicity occurs in people with some polymorphism in the OCT2 gene (Miller et al., 2010).

The mechanism of nephrotoxicity is summarized in Figure 1.

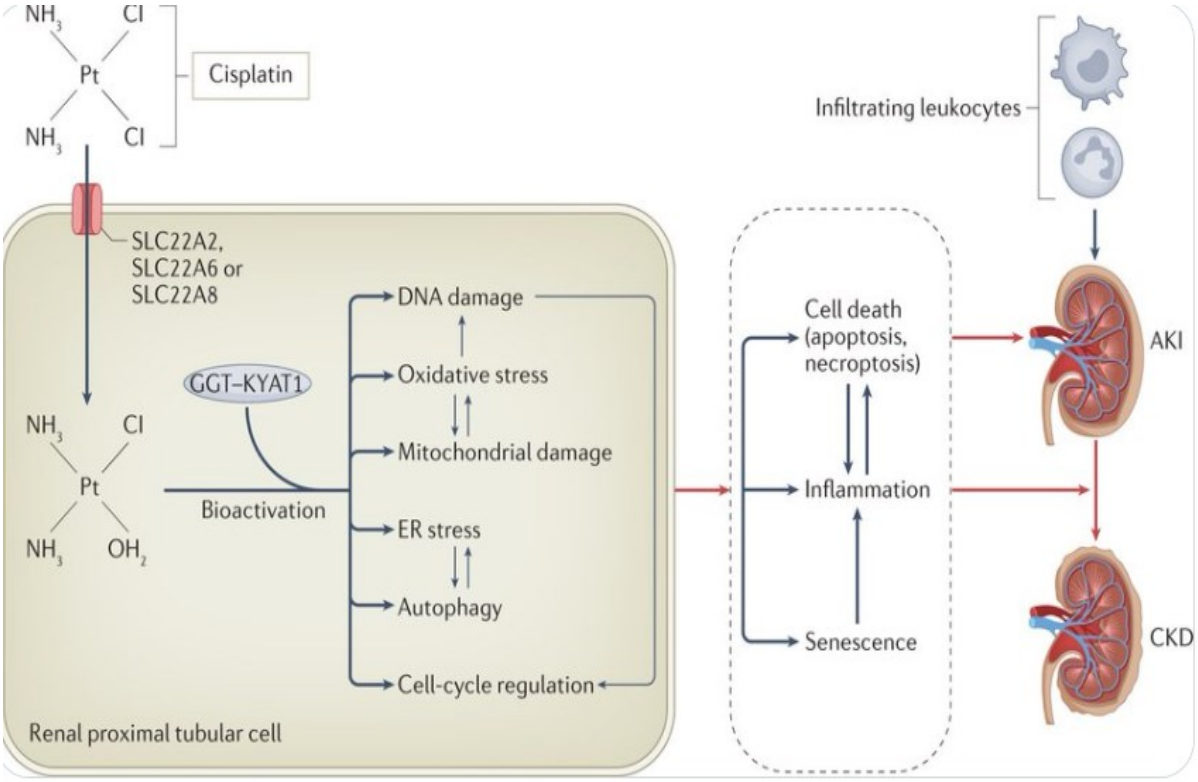


***Fig. 1****- Cisplatin causes kidney damage through multiple mechanisms resulting in acute kidney injury (AKI) or chronic kidney disease (CKD). (Tang et al., 2023)*

## 1.1.2 Cisplatin uptake

Cisplatin enters the renal cells through basolateral influx transporters like organic cation and copper transporters. Following secretion of cisplatin into the urine is facilitated by apical efflux transporters, for example, multidrug resistance-associated proteins (MRPs), multi-

antimicrobial extrusion protein (MATEs), and ATPases. These important transporters are present in both proximal and distal tubules of the kidneys (Holditch et al., 2019).

Cisplatin reduces the level of magnesium in serum, but magnesium plays a significant role in the expression of cisplatin transporters. The declining expression of efflux transporters induces accumulation of cisplatin in tubular cells, unfortunately leading to an increase in AKI severity (Holditch et al., 2019).

### 1.1.3 Oxidative stress

Oxidative stress also plays a role in the development of nephrotoxicity. Elevation of various reactive oxygen species (ROS) is especially obvious in renal tubular cells, kidney slices, and in vivo. Oxidative stress can occur through three mechanisms. First, cisplatin changes into a very reactive form and makes a bond with thiol-group molecules like glutathione, a well-known cellular antioxidant. This utilization or inactivation of the glutathione leads to a change in redox status and causes the accumulation of endogenous ROS and oxidative stress inside the cells. The second hypothesis is that cisplatin can inflict dysfunction of mitochondria and raise the amount of ROS due to the disruption of the respiratory chain. The last mechanism suggests that cisplatin could induce ROS groups in the microsomes through the cytochrome P450 (Pabla & Dong, 2008).

Lipids, proteins, and DNA in the cells can be targeted or modified via ROS. It looks like that ROS should be significant for signalling pathway during cisplatin nephrotoxicity (Pabla & Dong, 2008).

### 1.1.4 Inflammation

Cisplatin has an influence on the inflammatory process inside of kidneys. With hight possibility, cisplatin makes intracellularly injury leading to the release of damage-associated molecular pattern molecules (DAMPS). DMAPS are endogenous molecules released when the tissue is injured. Toll-like receptors (TLRs) make a bond with DAMPS. TLRS are important receptors that have a role in the immune system. They trigger multiple pathways, including chemokines and other cytokines for example TNFα. After a dose of cisplatin, the number of TNFα increases in relation to nuclear factor-kappa B and endothelial adhesion molecules, which helps to attract inflammatory cells (Manohar & Leung, 2018).

### 1.1.5 Fibroproliferative process

The irreversible tubulointerstitial fibrosis visible in the kidneys of experimental animals is the late stage of cisplatin administration. Massive production of matrix proteins via phenotypically altered resident cells is responsible for the development of tubulointerstitial fibrosis. The rise in the deposition of collagen types I, III and IV was spotted in cisplatin-induced tubulointerstitial fibrosis in rats (Taguchi et al., 2005).

### 1.1.6 Apoptosis

Apoptosis is the active death of cells, used in non-pathological process to keep balance in the internal environment. Cisplatin-induced nephrotoxicity includes three pathways. the mitochondria-mediated intrinsic apoptotic pathway, death receptor-mediated extrinsic apoptotic pathway, and endoplasmic reticulum-mediated apoptotic pathway. From the metabolism of cisplatin origin free radicals which immediately unloose cytochrome C, inducing intrinsic apoptosis (D. Zhang et al., 2023).

### 1.1.7 Autophagy

Cell homeostasis is maintained with the help of autophagy only in eukaryotic cells. Autophagy ensures physiological and pathological operations. The main role of autophagy is to eliminate and demote harmed cellular structures, old organelles, and useless macromolecules in cells. Autophagy is also a part of cisplatin-induced renal injury because treatment with cisplatin initiates an increase in the regulation of microtubule-associated protein light chain 3B, an important autophagy marker. Researchers suppose that autophagy could be an indicator of cisplatin-induced injury (D. Zhang et al., 2023).

## 1.2. Renal functional reserve

**Renal functional reserve (RFR)** is measured with the help of glomerular filtration rate (GFR). RFR is the difference between the basal line of GFR and the maximal capacity of GFR. The increase of GFR is due to some stress factors, which can be oral protein or intravenous amino acid intake. RFR is illustrated in Figure 2. Renal function it is not static, but a dynamic function similar to other human organs, and must react to changes happening in the bodies either physiological or pathological. GFR is very important for suitable doses of medicaments, diagnosing acute kidney injury (AKI) and chronic kidney disease (CKD). It is commonly used in clinical practise as a reliable index of kidney function (Palsson & Waikar, 2018).

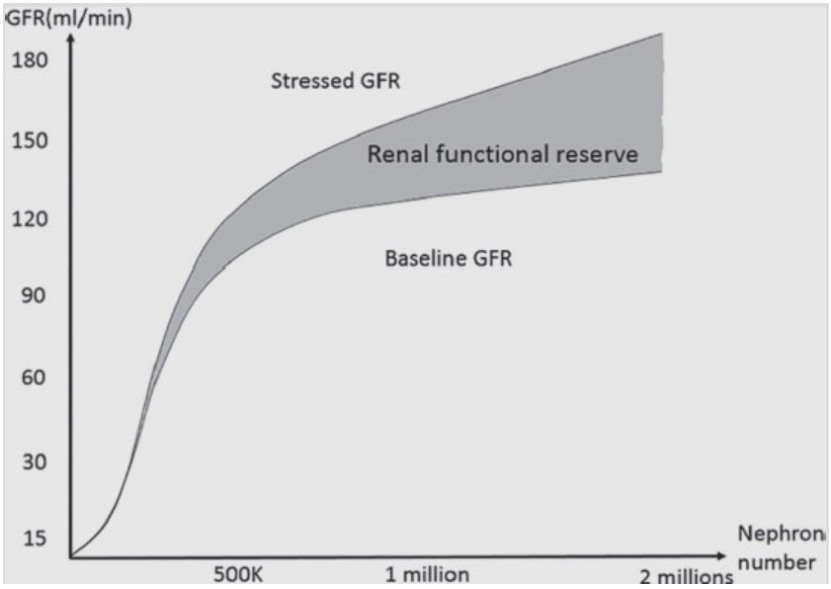


***Fig. 2****- The relationship between baseline glomerular filtration rate (GFR) and stressed GFR is associated with nephron number. The decline in renal functional reserve begins with the loss of nephron number. (Ratanasrimetha et al., 2018)*

Glomerular filtration can be detected using exogenous markers (inulin, iohexol and iothamalate), or endogenous filtration markers (creatinine) collected from blood and urine, using the renal clearance formula (Palsson & Waikar, 2018).

Scientists have an interest in the possibility, that oral intake of proteins or intravenous administration of amino acids, for example, arginine and **glycine**, may be significant for the prognosis of the progression of CKD and for evaluating the risk of AKI. RFR can provide an option for prevention and management of AKI and CKD (Jufar et al., 2020).

Human has an influential diversity in the number of nephrons and renal structure. Genetic and epigenetic predisposition can refer to different RFR and the baseline of GFR (Sharma et al., 2014). GFR relates to age, sex, weight, and nourishment. Via clearance it is possible to compare the function of kidneys regardless of urine flow, the weight of body, and solute concentration in the blood. In the balance stage, clearance presents GFR (Ronco et al., 2017).

Healthy patients show an elevation from 1 to 2 hours after acute protein intake (1-1.2 g proteins/kg body weight). Many ideas about the possible processes happening in our body when the GFR is evaluated after protein intake have been established. The first theory suggests that the elevation of amino acid in plasma causes stimulation of absorption in the proximal tubule, and nitric oxide and prostaglandins are released, leading to vasodilatation and elevation of renal blood flow (RBF). The elevation of GFR seems to be identical to the elevation of RBF, with the same filtration fraction. It is believed that the elevation of RBF is the most significant pathway of RFR activation. Reduction of RFR can raise sensitivity to volume utilization and nephrotoxins (Ronco et al., 2017). But the mechanism of using RFR can change, and it depends on the illness process (Fuhrman, 2021).

It is possible to find 2,357 results on the topic RFR in PubMed dated from 1907-2024. Renal functional reserve has been known for a decade. It has promising potential but must be much more widely explored.

The organ reserve function is normally measured in medicine, in contrast to RFR, which has not been incorporated into standard clinical nephrological care (Mueller & Luyckx, 2024).

### 1.2.1 Knowledge for medicine

1. Patients with the same serum creatinine and the same mass of muscle can have different RFR.

   This information can be profitable for insights into differential responses to injury or physiological stress.
2. Elder people noticed decline of RFR.

   It can help to understand why older people have a higher possibility of progressing AKI.
3. RFR could be rediscovered by administration of proteins.

   This observation can be used to raise urinary volume and enhance GFR, especially by patients in the intensive care unit.

### 1.2.2 Evaluation of Glomerular filtration rate

The glomerular filtration rate is useful for the determination of early kidney disease. GFR has been measured due to inulin urinary or plasma clearance in lots of trials. Inulin is used as an exogenous filtration marker, and it was considered as the golden standard. But in clinical practice endogenous markers, for example, creatinine or cystatin C, are more commonly used, because they are less expensive as well as more reachable (Damianaki et al., 2022).

An appropriate exogenous marker must have these features: soluble in water, not make a bond with proteins, non-metabolized, and can be excreted only by the kidney. Everything must be filtrated through the glomerulus with normal renal function and not be secreted or absorbed by kidney tubules (Speeckaert et al., 2021).

### 1.2.3 Inulin

The protocols of inulin should be practised only by educated and trained staff. The clearance of inulin takes 24 hours, and patients must be prepared with an empty stomach. Oral water drinking is recommended to increase urine flow. The collection of urine and blood must be perfectly timed during the 24 hours. In this article, inulin is considered with concern for analytical inaccuracy. The coefficient of variation for interred inulin concentration measurements is about 7%. There is also a risk of protocol contravention because it is hard to apply and is invasive. Chicory crops as a source of inulin are not easily available in the European Union. The assessment of inulin clearance is complicated, and therefore scientists decided to use another method to replace the inulin protocol (Speeckaert et al., 2021).

Inulin for measuring GFR is applied by i.v. injection. Inulin has been perceived as the golden standard, especially for non-reabsorption and non-secretion by renal tubules. There are two options for estimating GFR via inulin, once by the infusion method and second by single bolus. The best way is the infusion process, which starts with a bolus injection (Huang et al., 2016).

Inulin as a biomarker is costly, invasive, connected with radioactive substances and time-challenging. Also, it is badly soluble in water. In that case, creatinine could be good alternative for measuring the function of kidneys because it is fast and cheaper than the inulin method. Thus, creatinine in blood and in urine is useful for the determination of creatinine clearance or GFR (Shahbaz & Gupta, 2023).

### 1.2.4 Creatinine

Creatinine is a product of meat, and creatinine phosphate occurs in skeletal muscle. Creatinine is also part of our body, and the amount of creatinine depends on amount of muscle mass. The glomerulus filtrates creatinine freely, but creatinine is also secreted by the peritubular capillaries, which is not ideal. Still, creatinine is considered as a sufficient biomarker for measuring GFR (Shahbaz & Gupta, 2023).

Creatinine clearance is influenced by sex and race. Women have lower muscle mass, leading to a lower rate of creatinine production, on the other hand, men have higher muscle mass, thus a higher rate of creatinine production. Black people can produce higher amounts of creatinine clearance, probably because they genetically have larger muscle mass, while the Latino population has lower production of creatinine clearance (Shahbaz & Gupta, 2023).

Vegans, vegetarians, people who take creatine supplements, and people who are underweight, have, of course, different values of creatinine in comparison to the normal healthy population (Shahbaz & Gupta, 2023).

Creatinine in serum appears not to be a perfect and sensitive marker for renal injury or renal function (Ratanasrimetha et al., 2018). The newest experiment affirms that the measurement of serum creatinine is not the best method to detect the loss of renal function. On the other hand, reduced RFR can be an important lead for uncovering the loss of renal function (Taylor et al., 2023).

### 1.2.5 Renal functional reserve as biomarker

Scientists have increasing proof that the response of the kidney to the infusion of amino acids may be useful for patients who are at risk of expanding CKD or AKI, which is visible in Figure 3 and as a therapy aim for AKI (Jufar et al., 2020).

Physiologically, if the kidneys are harmed or the capacity of filtration is fractionally lost, RFR is applied, and the renal function is sustained. The decline of RFR may be obvious before a bigger damage of basal GFR and before CKD or AKI may be determined. Thus, the assessment of RFR can offer an early indicator of beginning renal damage (Jufar et al., 2020).

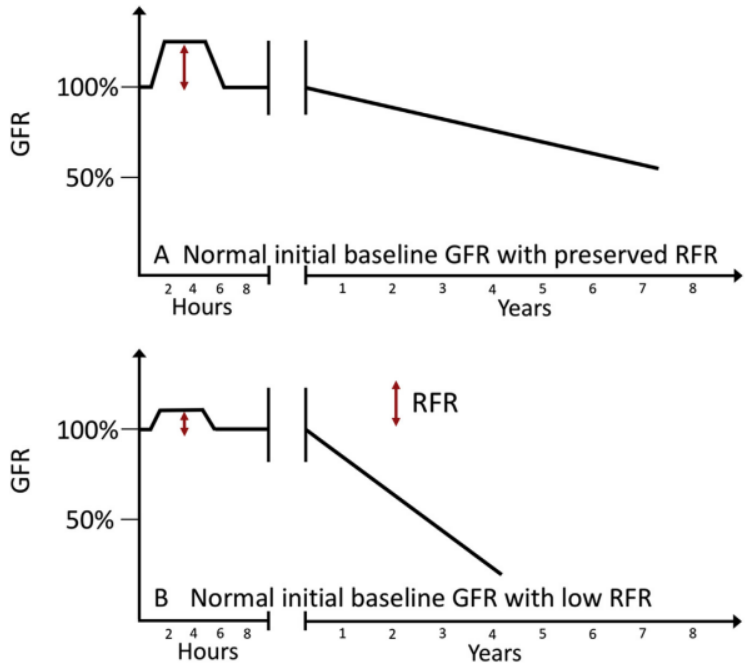


***Fig. 3****- The potential role of renal functional reserve (RFR) as a predictive biomarker is illustrated in this theoretical example. Patients A and B initially exhibit normal baseline glomerular filtration rates (GFR). However, patient B has a low renal functional reserve, leading to a more rapid decline in kidney function and progression of chronic kidney disease (CKD). (Palsson & Waikar, 2018).*

RFR evaluation can be significant information to detect the competence of the kidney to heal completely or partly after AKI. Even if recovery seems to be clinically in order, reduced RFR can be a sign of maladaptive repair or subclinical missing mass of kidney (Chawla & Ronco, 2016).

AKI is a frequent complication after heart surgery. There is not so much data about the recovery of kidneys after heart surgery. RFR can play a key role, especially because even if creatinine serum is stabilized, RFR shows decline and can point out that the kidneys are not fully recovered (Husain-Syed et al., 2019).

Consuming proteins, like cooked beef, tuna, soy, and lactoprotein raise the GFR and RFR. Beef makes a bigger elevation in GFR than soy. Based on this, we known that animal protein causes a bigger elevation of GFR than proteins from plants. It can be an explanation for why vegetarians and vegan populations have lower values of GFR although the value of protein intake is the same (Jufar et al., 2020).

Scientists are using single amino acids or compounds of amino acids to observe RFR.

If the kidney is damaged, GFR still sustains at normal level with the help of RFR by hyperfiltration, until the **50 percent** of the functional nephron is wasted. The baseline GFR may be used neither as a sensible indicator for early uncovering of kidney illness nor for prediction. Nevertheless, detection of RFR can present a solution for discovering subclinical kidney injury even if baseline GFR and concentration of creatinine in serum is at common levels. Current therapeutic methods are expensive, can be risky, and without guaranteed results. The aim for scientists is to find an appropriate way to uncover kidney illness at a subclinical part and prevent its development on a clinical level. Prognosis and diagnosis of CKD and AKI via RFR have key potential (Jufar et al., 2020).

### 1.2.6 Chronic kidney disease

10 % of the human population on Earth suffer from CKD, particularly in poorer states (Noel & Parikh, 2023) Patients with CKD have a lower curve of RFR depending on severity, stage 1: 19.1%; stage 2: 15.4%; stage 3: 8.9%; stage 4: 6.7%. compared with control patients who have 23.4%. The primary goal is to identify individuals at risk of evolving CKD. RFR can be also used to predict the fast decrease of renal function in CKD patients or serve as a marker for the subclinical stage of CKD. It is not that simple to reveal CKD, because lots of pathological processes, with connection to CKD, happening in our body can amp up the common baseline GFR curve and like that recruit the RFR ratio. Diabetes mellitus (DM) can be a factor in developing CKD. Glomerular hyperfiltration is the first sign of DM before an apparent decrease in GFR during the progression of diabetic nephropathy. It was discovered that the lowest value of RFR was significant in patients, who had the highest baseline GFR (Jufar et al., 2020).

### 1.2.7 Acute kidney injury

RFR can function as a marker for the risk of AKI before heart operation or administration of a radiocontrast compound. It may be influential in assessing the recovery level after an AKI attack or after exposure to a stressor that can initiate subclinical kidney harm (Jufar et al., 2020).

### 1.2.8 Renal stress testing

The theory of RFR was first described by Bosch and associates in 1983. The outcome of their work was that only protein, not fat or carbohydrates administration, raises the GFR in healthy kidneys (Chawla & Ronco, 2016).

The possibility of testing of the reserve of an organ system offers an interesting indicator of illness. Two types of renal stress testing are glomerular and tubular. If the kidney is healthy the glomerular and tubular system cooperate in harmony. If the kidney is harmed, the glomerular and tubular systems can be influenced equally, leading to changes in renal capacity or renal form (Chawla & Ronco, 2016).

Stress tests can be an option for determining the physiologic limits of nephron function (Armenta et al., 2022).

### 1.2.9 Renal recovery

Full recovery and good health outcomes depend on the previous health condition, the severity of disease, and the healthcare approach (Mehta, 2020).

The Formula for how our kidneys recover after AKI is a widely discussed topic in the field of medical science. The reason is that reachable preventative tests and therapeutic treatments have some limits. The goal is to support renal recovery as fast as possible and prevent AKI progression into CKD (Göcze et al., 2017).

In some cases, stricter limits have been set, like a return to baseline or reference creatinine or a return to a creatinine level within 0.3 mg/dl of that at baseline. With this definition, the full renal recovery rate was decrease due to the increasing number of patients with incomplete recovery. Non-recovery form of AKI related to unfavourable output (Göcze et al., 2017).

The definition of AKI is an injury lasting 7 days or more. Acute kidney disease lasting approximately 90 days is marked as CKD (Forni et al., 2017). The recovery phase from AKI can have various pathways. Even after full recovery, patients still have a raised chance of progressing to CKD. So, prophylaxis of AKI in high-risk patients is a crucial part of AKI control (Göcze et al., 2017).

# 2. Aim of the diploma thesis

The purpose of this diploma thesis was to evaluate the role of RFR in two key points of kidney damage caused by cisplatin: during its evolution and in the recovery phase.

# 3. Experimental part

## CISPLATIN – 6 mg/kg

The dose was chosen based on the laboratory's previous experience, where a toxic dose for rats was established to be between 4 to 7 mg/kg.

11 days of experiment: day 0 – day 10

On days 0, 2, 4, 6 and 8, blood samples were collected, and day 10 was designated as the day of surgery.

| | |
|---|---|
| Laboratory Animals: | Rats |
| Origin: | Salamanca animal house |
| Species: | Wistar |
| Sex: | Males |
| Age: | 2 months |
| Weight: | Approximately 250 g |

Conditions at the Vivarium:

| | |
|---|---|
| Temperature: | 20 degrees of Celsius |
| Humidity: | approximately 60 % |
| Daily light time (photoperiod): | 12 hours |

Continuous activity of the renewal and air and odour filtration.

Schedule:

Day 0: application of cisplatin only on day 0, blood collecting without cisplatin, measuring weight

Day 1: measuring weight

Day 2: measuring weight, blood collecting with cisplatin

Day 3: measuring weight

Day 4: measuring weight, blood collecting with cisplatin

Day 5: measuring weight

Day 6: measuring weight, blood collecting with cisplatin

Day 7: measuring weight

Day 8: measuring weight, blood collecting with cisplatin

Day 9: measuring weight

Day 10: day of surgery, collecting urine and blood

**Day 0 – Day of Application of cisplatin**

Equipment: 12 capillaries (with solid heparin), plasticine box injection, scissors, weight, box, gloves, laboratory coat, plasticines box, marker, heating lamp, Eppendorf tubes, pipette, tips for pipette

1. The animal house is entered, and the room containing the rats is opened.

   Three **male** rats with approximately the same weight (250 g) are chosen.

   The weights of the three rats are recorded after weighing.

   The rats are placed together in a single cage.

   The rats are marked with numbers 1, 2, and 3 on their tails.

2. All equipment is gathered, and the laboratory is entered.

   Capillaries are prepared and marked accordingly. Beginning with rat number 1, it is placed into a slipper.

The red lamp is turned on to facilitate better blood flow (heat widens the blood vessels).

A small piece is cut from the end of the tail, and the blood is collected into four capillaries.

The capillaries are placed into a plasticine box to seal the bottoms.

3. The rat is caught by the neck, and occasionally, placing a blanket on the rat beforehand can help to calm it.

   Cisplatin is administered intraperitoneally, with the dose calculated based on weight. The rat is then returned to its cage.

   The collection of blood and administration of cisplatin is repeated three times (for three rats).

   The table is cleaned first with water and then with ethanol.

   The rats are then returned to the animal house, with the experiment number labelled on their cages.

4. Samples of blood are obtained. The capillaries are then placed in the fridge to allow the plasteline to solidify for 20 minutes.

5. A centrifuge is used to separate plasma from the rest of the blood.

   It must be balanced, and plasteline plugs are placed at the edge of the centrifuge's wheel. Program 2, which runs at 11,000 rpm for 3 minutes, is selected.

6. Following centrifugation, the capillaries are removed.

   The capillaries are then broken, focusing on the yellow plasma part. A pipette with a tip is utilized to extract the plasma from the capillaries, which is then collected into three Eppendorf tubes labelled RFR 1, 2, 3, along with the date and cisplatin dose.

7. The samples are stored in the freezer for future concentration measurement. Alternatively, if the samples are to be measured the next day, they are placed in the fridge.

**Day 1, day 3, day 5, day 7, day 9**

Equipment: weights, gloves, coat, box, blanket, notebook

The weight of the rats is measured.

**Day 2, day 4, day 6, day 8**

Equipment: weights, gloves, box, blanket, notebook, capillaries, scissors, plasteline box, slipper, coat, heating lamp, Eppendorf tubes, pipette, tips for pipette

The weight of the rats is measured.

Blood is collected from the rat's tail into four capillaries. In total, there are 12 capillaries, with four from each rat.

The capillaries are placed into the centrifuge. After separation, they are broken, and the plasma is collected into Eppendorf tubes.

**Day 10- day of surgery**

All equipment is prepared, including tweezers, scissors, threads, two warming tables, a heating lamp, a pump, glycine solution, pentobarbital dilution for anaesthesia, physiological solution, injections, tissues, a beaker, rack, box of plasteline, capillaries, Eppendorf cannulas for bladder and jugular vein filled with physiological solution to prevent precipitation, Eppendorf tubes, pipette, and pipette tips.

a. Preparation:

   The injection of pentobarbital is administered peritoneally into the rat.

   Waiting for the rat to fall asleep typically takes around 15 minutes, depending on its weight.

   The mouth is checked to ensure there is no sawdust from the bottom of the cage, which could cause choking.

   Using tweezers, the tongue is gently lifted.

   The rat is then placed on the surgery warming table under the heating lamps.

   Its four legs are secured with pasteboard.

   The rat's movement is observed when its foot is touched.

   If the rat is still not asleep, more pentobarbital is administered.

Once it is confirmed that the heart is still beating and the rat does not react to touch, the surgery can commence.

If any movement is observed during the procedure, additional pentobarbital is administered.

***Fig. 4*** *- Demonstration of animal surgery - after a rat felt asleep, the legs are fixed with pasteboard to a surgery desk and surgery can start.*

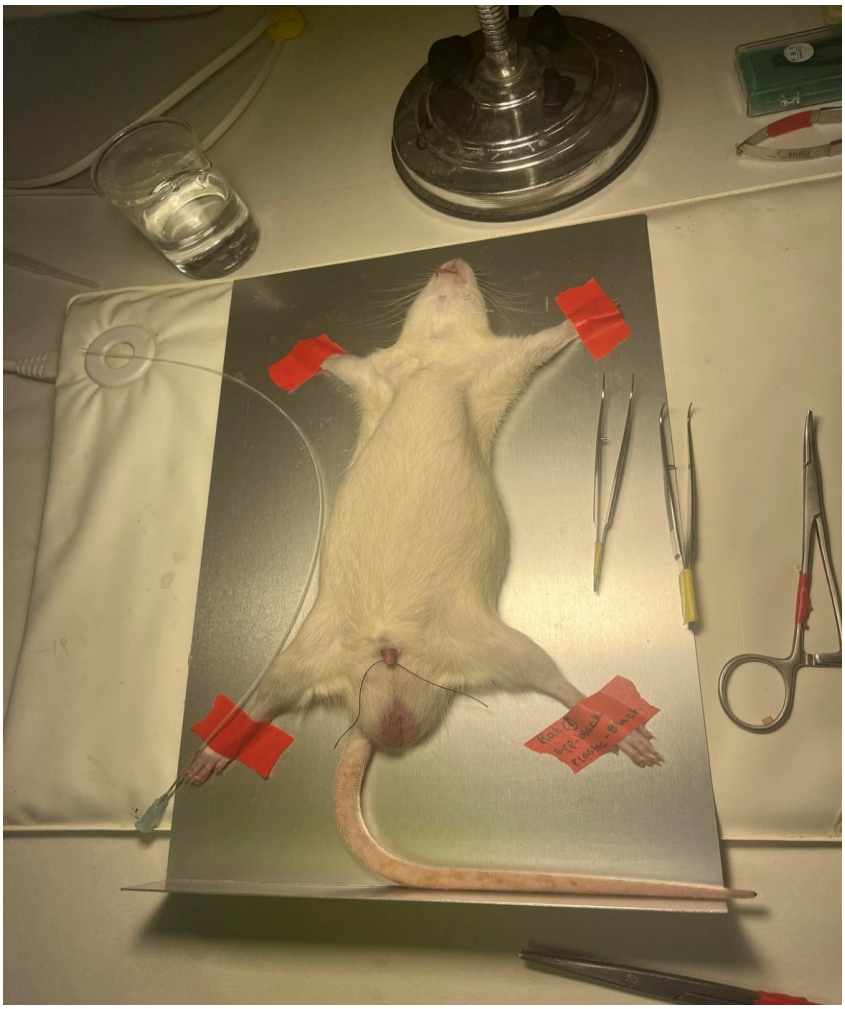

***Fig. 5****- Demonstration of animal surgery - The penis is tied to prevent loss of urine, and constant monitoring is necessary to ensure the effectiveness of anaesthesia and that the rat remains deeply asleep.*

b. <u>First part of surgery – bladder:</u>

The penis is tailed to prevent the escape of urine.

This procedure can be harmful to the rats; if they are anesthetized, they won't feel anything. Otherwise, it is necessary to wait longer until the pentobarbital takes effect.

Following this, the skin in the area of the bladder is removed using scissors, located in the middle lower part of the belly.

An abdominal incision is made, and the muscle layers are separated with tweezers until the bladder is visible.

The lower part of the bladder is prepared for tying.

Once the cannula is inserted into the bladder incision, it is tied and placed into an Eppendorf tube.

Finally, the surgical site is cleaned with physiological solution, and the open area is covered with a blanket during the operation.

To maintain moisture in the operated area, drops of physiological solution are applied.

If any movement is observed, pentobarbital is administered.

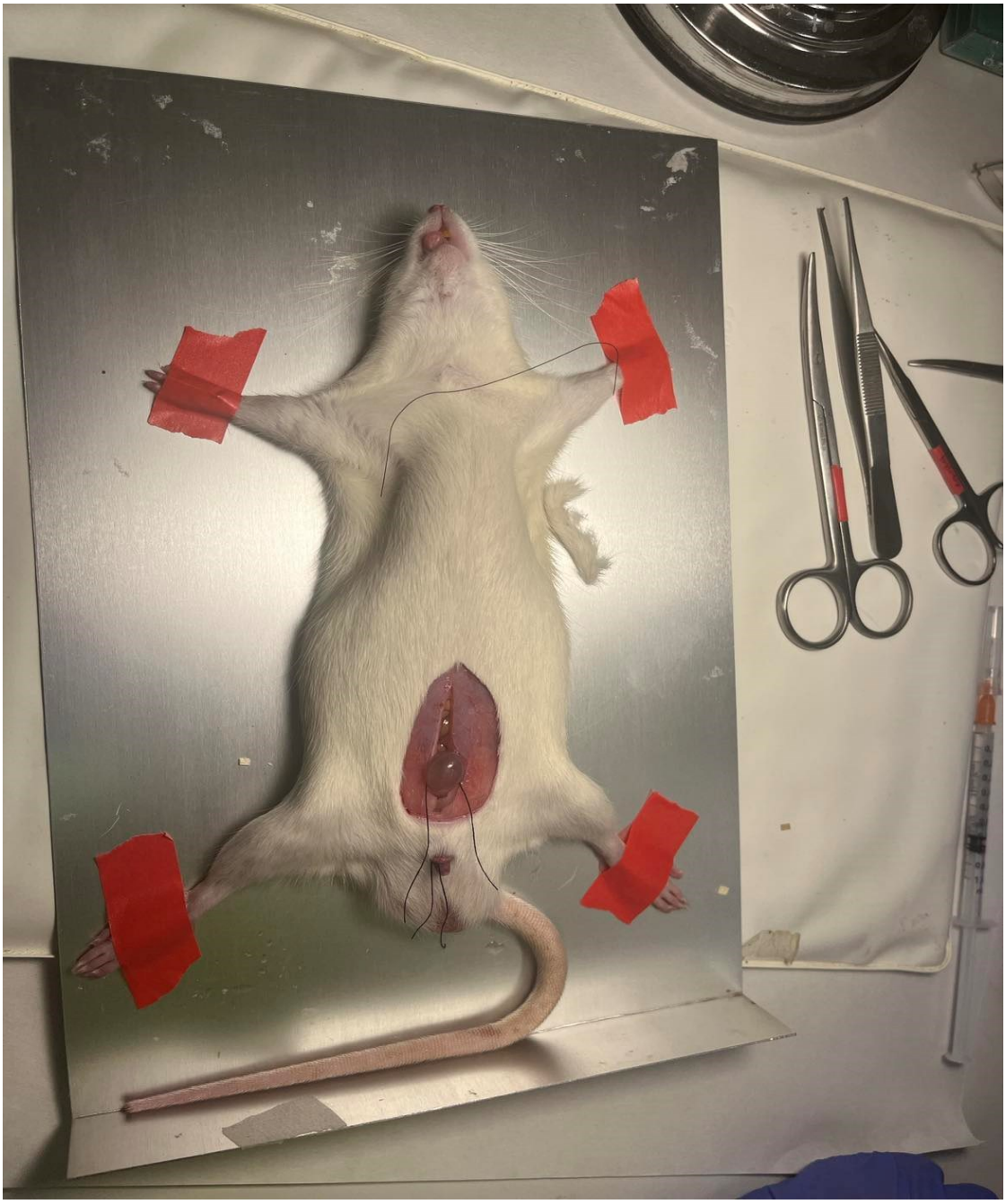

***Fig. 6-*** *Demonstration of animal surgery - after a lower skin cut, loops is prepared to tie the bladder.*

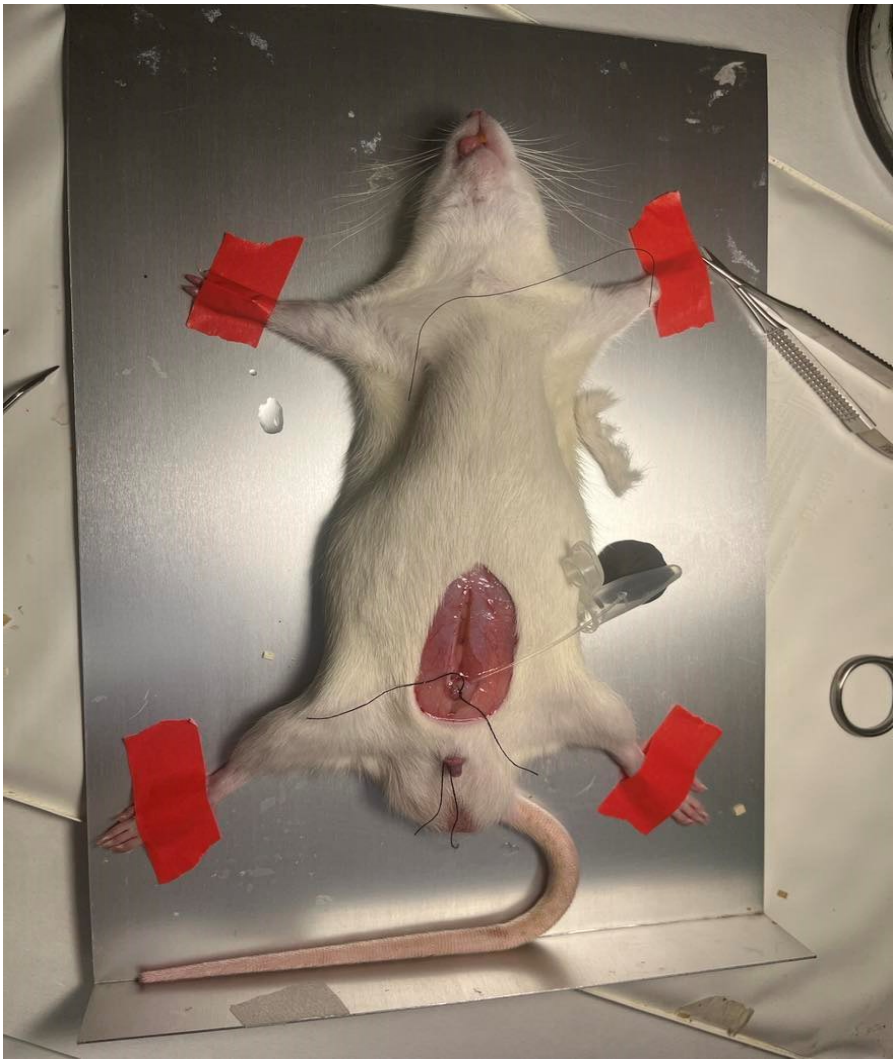

***Fig.****7- Demonstration of animal surgery - very small cut in bladder is done to insert cannula inside of it for collecting urine.*

c. <u>second part of operation – jugular vein:</u>

The skin in the neck area is cut with scissors.

An incision is then made in the neck area, and tweezers are used to separate the salivary gland, allowing access to the jugular vein.

The surrounding area is cleaned.

Careful incisions are made with micro scissors to avoid damaging the vein.

After inserting the cannula, it is secured with two ties.

Continuous monitoring ensures the rat is deeply asleep. With the operation complete, samples are now collected.

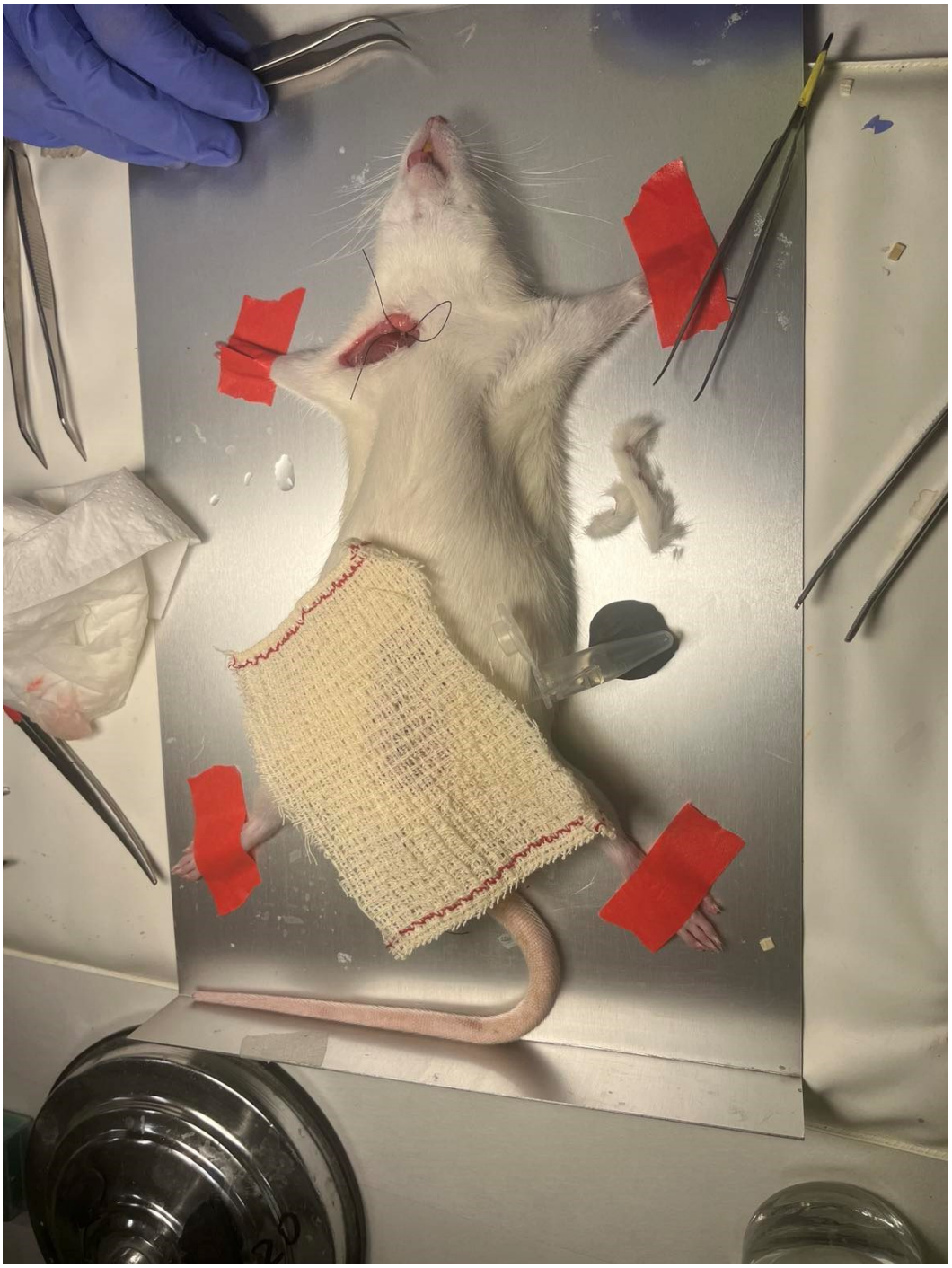

***Fig.8****- Demonstration of animal surgery - a very accurate cut is made in jugular vein and the cannula is inserted to start pumping glycine through it.*

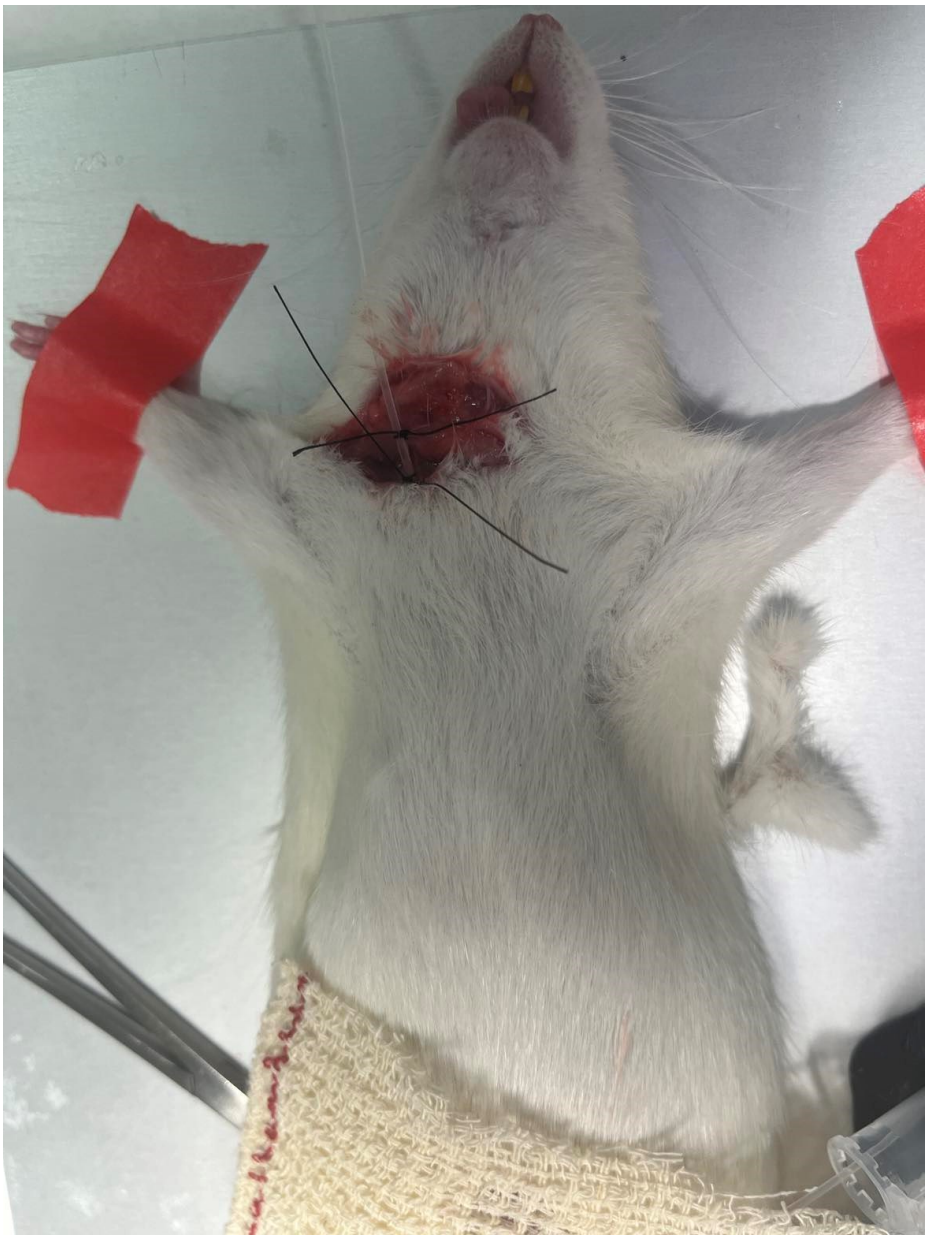

***Fig. 9-*** *Demonstration of animal surgery – closer focus on the implementation of cannula into jugular vein.*

d. A 20-minute wait period is observed before commencing the collection of blood and urine.

   The rats are then transferred to the next table to begin the operation on another rat.

   Urine collection begins, with the Eppendorf tubes changed every 20 minutes.

   After the initial 10 minutes, blood is collected from the tail into three capillaries.

Glycine solution (200 mg/ml, 20 ml) is pumped into the jugular vein starting after the initial 20 minutes.

The pumping of glycine solution ceases after 60 minutes, and the remainder of the collection is conducted without glycine.

The entire collection process lasts 150 minutes.

A total of 8 Eppendorf tubes of urine and 8 sets of capillaries containing blood are collected.

Time schedule for collecting samples:

| |
|---|
| 20 mins free → 1.urine (0 mins, without glycine) → 1.blood (10 mins, without glycine) → 2.urine (20 mins) → start pumping glycine → 2.blood (30 mins) → 3.urine (40 mins) → 3.blood (50 mins) → 4.urine (60 mins) → stop pumping glycine → 4.blood (70 mins) → 5.urine (80 mins) → 5.blood (90 mins) → 6.urine (100 mins) → 6.blood (110 mins) → 7.urine (120 mins) → 7.blood (130 mins) → 8.urine (140 mins) → 8.blood (150 mins) |

e. Following this procedure, the rat is swiftly euthanized by cutting the heart, ensuring a humane and rapid end to its life.

f. The table and tools are cleaned once surgery and sample collection are completed.

g. Dead rats are transported to the animal house and placed in a freezer until they can be taken to a designated incinerator for experimental animals.

h. Urine samples are stored in the fridge.

i. Blood samples are centrifuged to separate the plasma, which is then stored in Eppendorf tubes in either the fridge or freezer.

## Protocols for determination of creatinine:

CREATININE URINE DETERMINATION WITH KIT “QUANTICHROM”

Preparation of the patron line:

Prepare 100 ml of the creatinine solutions at 5 mg/dl, 2.5 mg/dl, 1 mg/dl, 0.5 mg/dl and 0 mg/dl from the standard of 50 mg/dl according to the following indications:

50 mg/dl: directly from the standard

10 mg/dl: 20 µl standard + 80 µl distilled water

5 mg/dl: 10 µl standard + 90 µl distilled water

2.5 mg/dl: 5 µl standard + 95 µl distilled water

1 mg/dl: 2 µl standard + 98 µl distilled water

0,5 mg/dl: 1 µl standard + 99 µl distilled water

0 mg/dl: 100 µl distilled water

PROCEDURE:

Keep reagents A and B at room temperature for 30 minutes.

Make dilutions 1:10 of the urine samples with distilled water (10 µl sample and 90 µl Water).

Pipette 5 µl of the sample (including the standard straight) into a 96-well plate.

Calculate the amount of total reagent that will be necessary to add 200 µl in each well.

Mix in proportion (1:1:2) the reagent a + 50 ml reagent b + 100 ml of water.

Add over each well 200 µl of the Mixed reagent (it´s only for 1 wale but we have 50 wells so x 50= 10000 µl together)

Put immediately the plate into the lector plates, shake 10 seconds and measure the absorbance between 490-530 nm (first measuring of absorbance at on wave 492 nm)

Read the absorbance when it passes 5 minutes (5 minutes of waiting and then second measuring of absorbance)

Use the values of the increase of the absorbance in 5 minutes.

Program do the change between two measuring of absorbance and show the graph, absorbance2 (higher concentration=more reaction) - absorbance1(lower concentration).

DETERMINATION OF CREATININ IN PLASMA WITH KIT “QUANTICHROM”

Preparation of the patron line:

Prepare 100 ml of the creatinine solutions at 5 mg/dl, 2.5 mg/dl, 1 mg/dl, 0.5 mg/dl and 0 mg/dl from the standard of 50 mg/dl according to the following indications:

50 mg/dl: directly from the standard

10 mg/dl: 20 µl standard + 80 µl distilled water

5 mg/dl: 10 µl standard + 90 µl distilled water

2.5 mg/dl: 5 µl standard + 95 µl distilled water

1 mg/dl: 2 µl standard + 98 µl distilled water

0,5 mg/dl: 1 µl standard + 99 µl distilled water

0 mg/dl: 100 µl distilled water

PROCEDURE:

Keep reagents A and B at room temperature for 30 minutes.

Make dilutions 1:10 of the urine samples with distilled water (10 µl sample and 90 µl Water)

Pipette 30 µl of the sample (including the standard straight) into a 96-well plate.

Calculate the amount of total reagent that will be necessary to add 200 µl in each well. (We have 50 wells so x 50=10000 µl)

Mix in proportion (1:1) the reagent A+ reagent B just before adding it to the wells (100 µl A and 100 µl B).

Put immediately the plate into the lector plates, shake 10 seconds and measure the absorbance between 492 nm (first measuring of absorbance)

Read the absorbance when it passes 5 minutes (5 minutes of waiting and then second measuring of absorbance)

Use the values of the increase of the absorbance in 5 minutes.

Program do the change between two measuring of absorbance and show the graph, absorbance2 (higher concentration=more reaction) - absorbance1(lower concentration).

# 4. Statistical analysis

Profile of serum creatinine over 10 days of the experiment. Six Rats (n=6) have been used in the experiment and serum creatinine profile indication cisplatin-induced nephrotoxicity have been analysed using ANOVA assays with Bonferroni post hoc test. Data are presented as individual measurements (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$). GraphPad Prism software ver 10 has been used.

# 5. Results and discussion

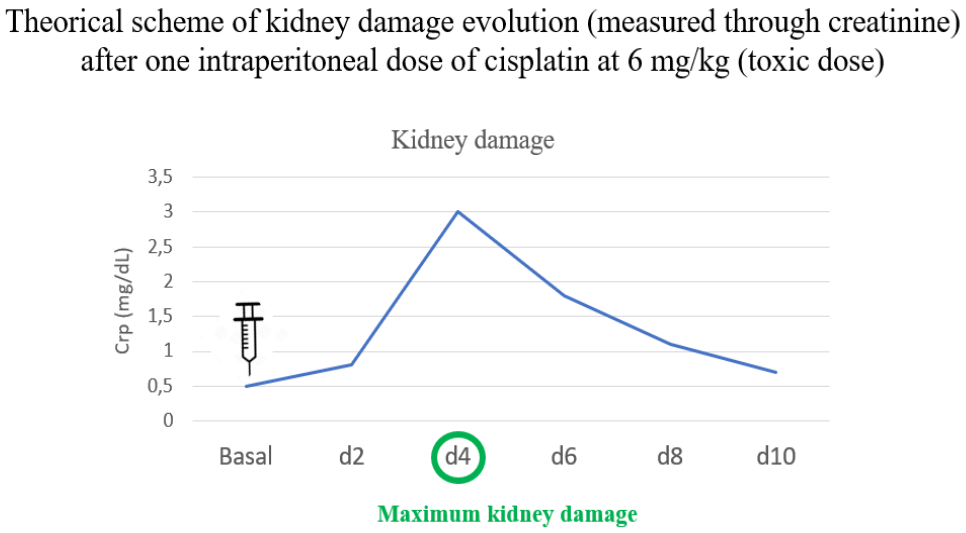


***Fig. 10*** *- The evolution of kidney damage that theoretically should occur after the administration of a toxic dose of cisplatin (6 mg/kg i.p.). Cisplatin is administered once on day 0. A slight increase in toxicity is observed on day 2, with day 4 indicating the peak of toxicity. Days 6 and day 8 demonstrate a noticeable decline. Values on day 10 are expected to return to those observed on day 0.*

# CONTROL RATS

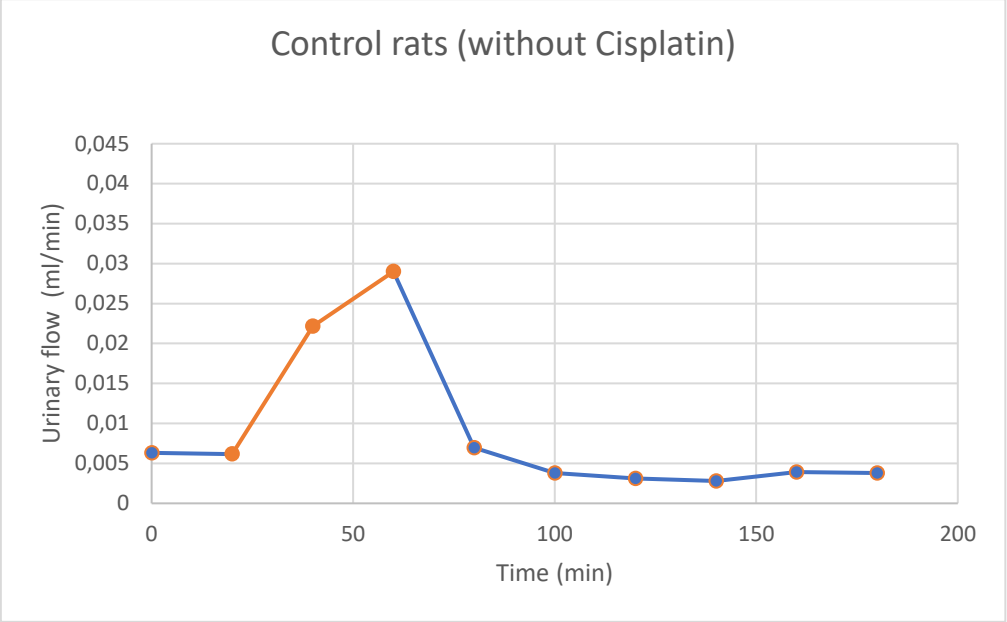


***Fig. 11*** *– The urinary flow of control healthy rats without cisplatin treatment over time. Orange lines represent the glycine infusion.*

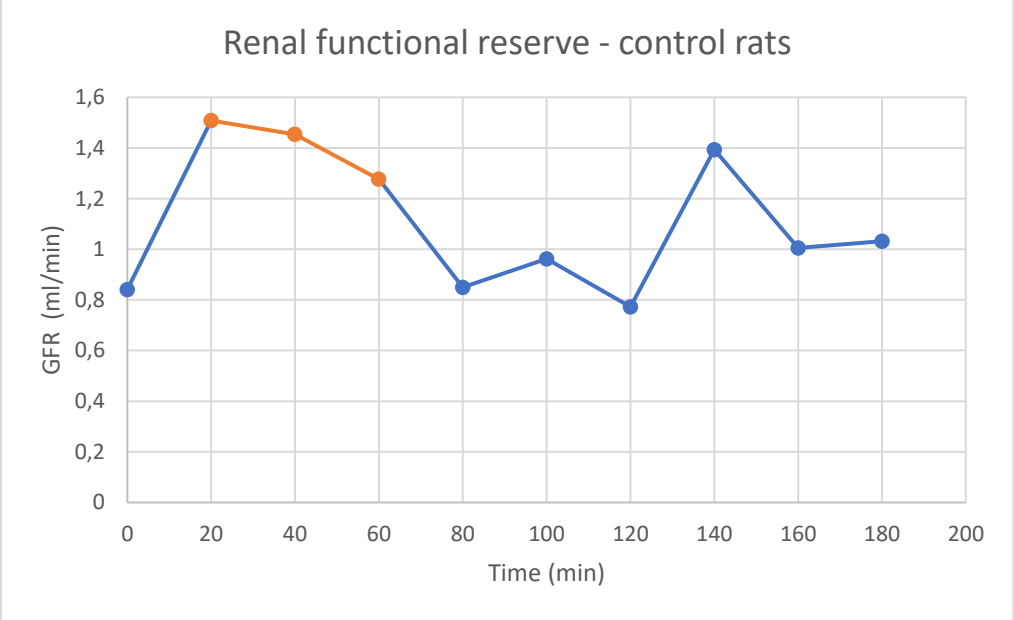


***Fig. 12*** *– The curve of control rats represents the basal GFR over time, illustrating how the RFR appears without the administration of cisplatin by healthy control rats. The value of RFR is calculated to be 0.7 at 20 minutes. Orange lines represent the glycine infusion. Abbreviations: GFR=glomerular filtration rate; RFR= renal functional reserve*

RFR 1,5-0,8=**0,7**

# DAY 4

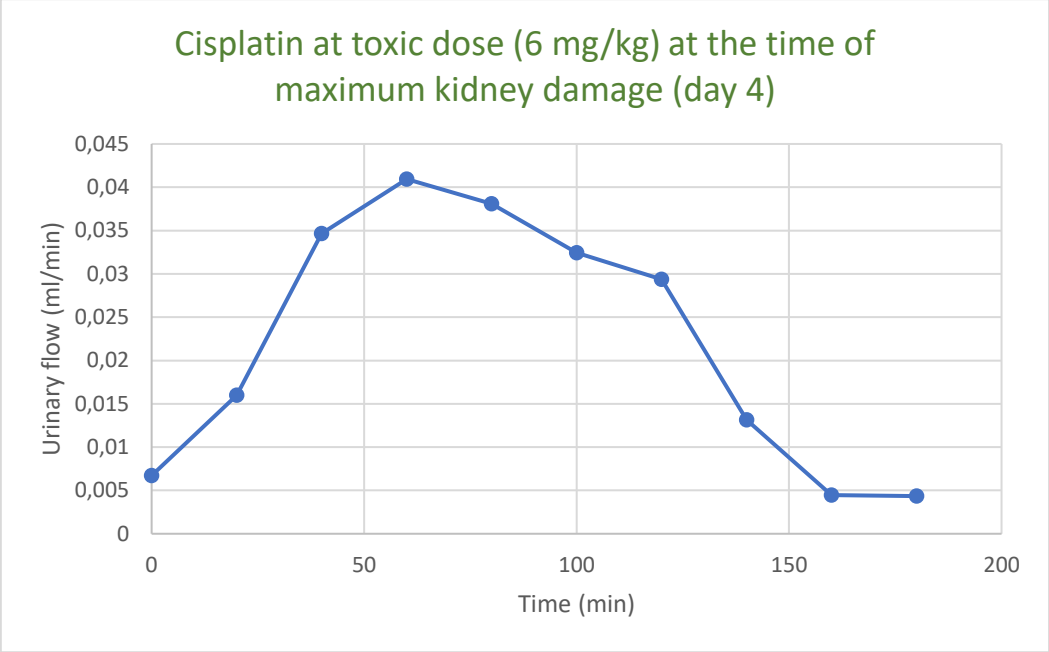


***Fig. 13*** *– The graph illustrates the urinary flow pattern over time on day 4 following the administration of a peritoneal injection of cisplatin (6 mg/kg) into rats on day 0. Day 4 represents the maximum peak of toxicity for rats treated with cisplatin, displaying the urinary flow over time during the period of highest nephrotoxicity.*

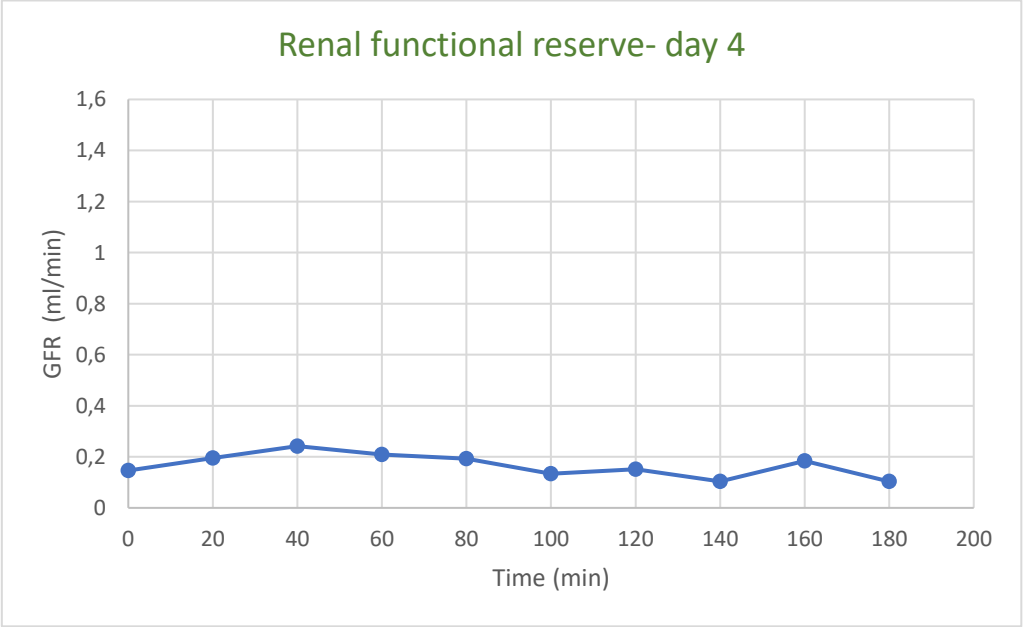


***Fig. 14****– Renal functional reserve on the day 4. On day 4 at this point the RFR does not work, because it is the day of maximum toxicity and the RFR loses its functionality, as indicated by a horizontal line without a peak. Abbreviations: RFR= renal functional reserve.*

Theorical scheme of kidney damage evolution (measured through creatinine) after one intraperitoneal dose of cisplatin at 6 mg/kg (toxic dose)

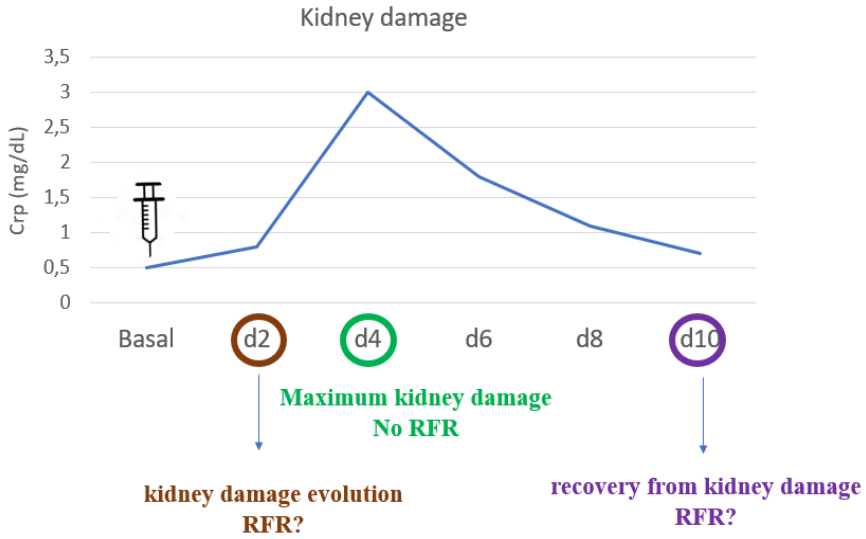


***Fig. 15****- Theoretical scheme of kidney damage evolution. We have established day 4 as the day of maximum toxicity for the rats treated with a toxic dose of cisplatin (6 mg/kg), now we want to focus on what is happening on day 2 and day 10, as elucidated in the following graphs.*

***Tab. 1*** *displays concentration of serum creatinine measured by spectrophotometry, values of 6 rats measured from day 0 to day 10 every second day.*

| ID rat | day 0 | day 2 | day 4 | day 6 | day 8 | day 10 |
|---|---|---|---|---|---|---|
| 1 | 0.487 | 0.693 | 5.008 | 3.251 | 1.953 | 0.664 |
| 2 | 0.511 | 0.651 | 4.957 | 3.984 | 1.864 | 0.673 |
| 3 | 0.547 | 0.597 | 4.567 | 2.943 | 0.997 | 0.522 |
| 4 | 0.499 | 0.598 | 3.999 | 2.014 | 0.98 | 0.436 |
| 5 | 0.528 | 0.712 | 5.721 | 3.658 | 1.666 | 0.574 |
| 6 | 0.489 | 0.601 | 4.653 | 2.954 | 0.993 | 0.551 |

*The biomarker evaluated is serum creatinine (mg/dL)*

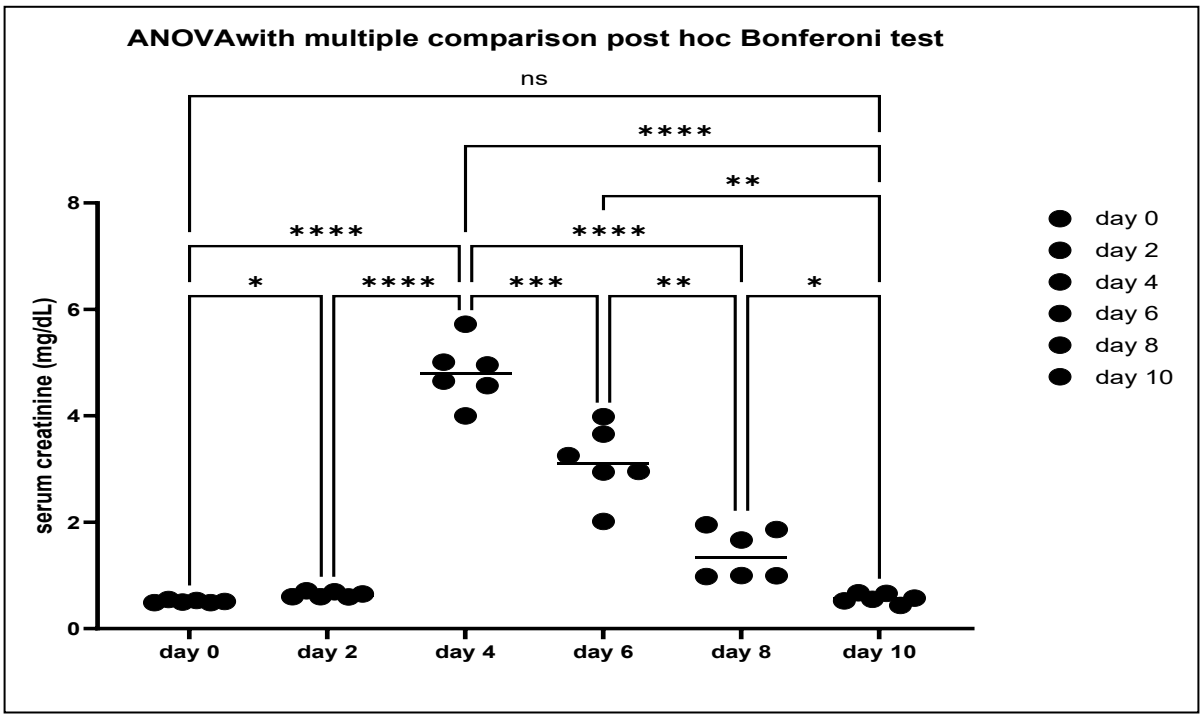


***Fig. 16****- The toxicity becomes evident by day 2 as suggested by statistically significant increase in creatinine serum level ($P < 0.05$). The levels on days 0 and 10 remain the same, while there is a significant increase on day 4 ($P < 0.0001$) compared to days 0 and 2. Conversely, from day 4 to day 10, there is a noticeable statistically significant decline in toxicity levels.*

# DAY 2

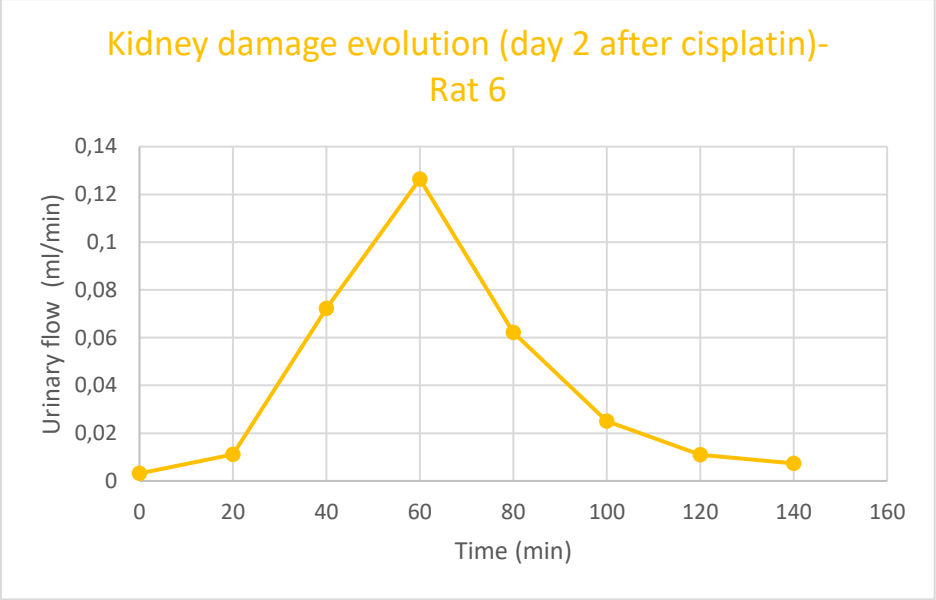


***Fig. 17****- Urinary flow in rat 6 on day 2, shows urinary flow on day 2 over time after administration of cisplatin on day 0, capturing the evolution of renal damage.*

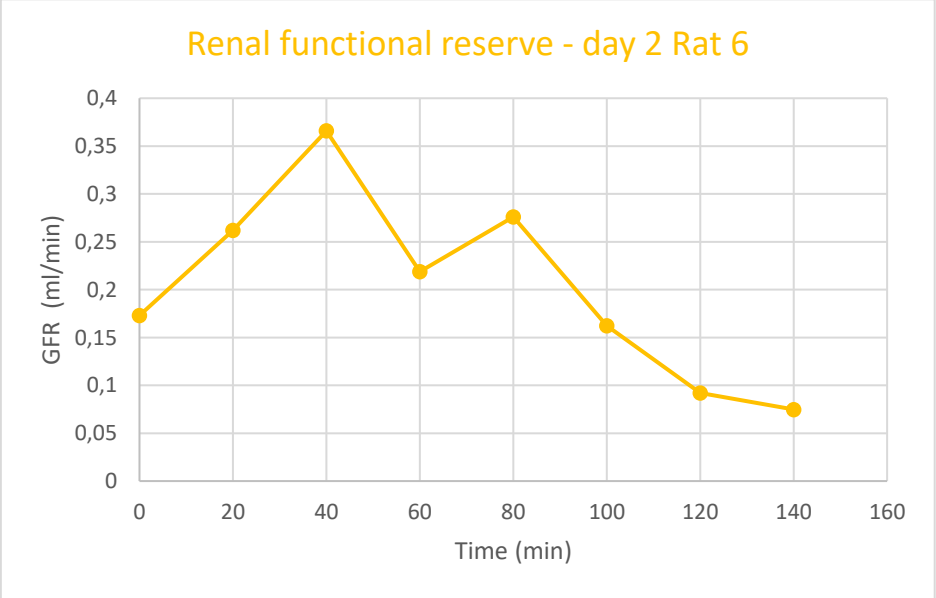


***Fig. 18****- RFR in rat 6 on day 2, the graph illustrates the GFR pattern over time on day 2 following the administration of a peritoneal injection into rats on day 0. RFR during the evolution of the renal damage, GFR loses its function. The value of RFR is calculated as 0.2. Abbreviations: GFR=glomerular filtration rate; RFR= renal functional reserve*

RFR= 0.37 – 0.17 = **0.2**

***Tab. 2-*** *Results obtained in rat 6 displaying an increase in the basal plasma creatinine level on day 2, with a value of* ***1.13*** *mg/dl.*

| RAT 6 | Time (min) | Crp (mg/dl) | Cru (mg/dl) | Volume of urine (ml) | Fu (ml/min) | GFR (ml/min) |
|---|---|---|---|---|---|---|
| 1 (Basal) | 0 | **1.13** | 62.61 | 0.06 | 0.00 | 0.17 |
| 2 | 20 | 0.97 | 22.79 | 0.22 | 0.01 | 0.26 |
| 3 | 40 | 1.02 | 5.19 | 1.44 | 0.07 | 0.36 |
| 4 | 60 | 1.09 | 1.89 | 2.53 | 0.13 | 0.22 |
| 5 | 80 | 1.02 | 4.53 | 1.24 | 0.06 | 0.28 |
| 6 | 100 | 1.01 | 6.51 | 0.50 | 0.03 | 0.16 |
| 7 | 120 | 1.04 | 8.71 | 0.22 | 0.01 | 0.09 |
| 8 | 140 | 1.06 | 10.91 | 0.15 | 0.01 | 0.07 |

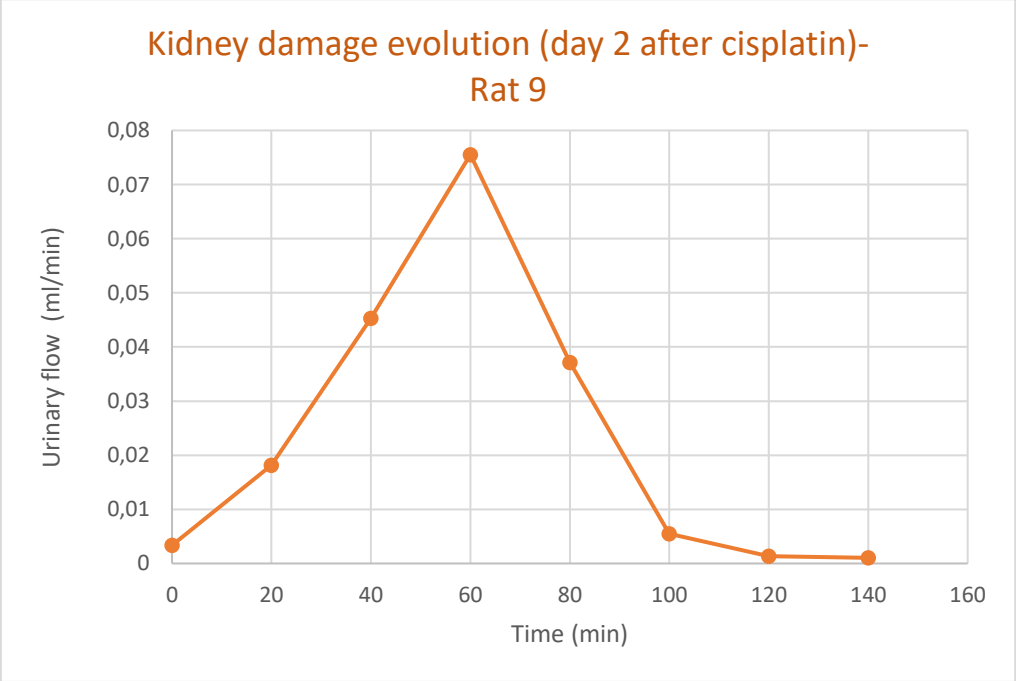


***Fig. 19-*** *Kidney damage evolution in rat 9 on day 2, shows urinary flow on day 2 over time after administration of cisplatin on day 0, capturing the evolution of renal damage.*

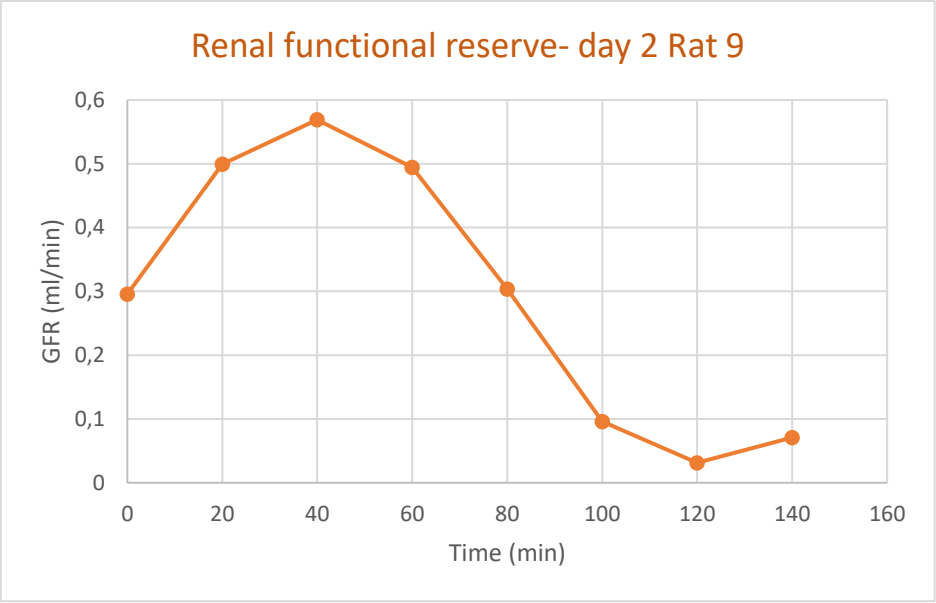


***Fig. 20-*** *Renal functional reserve in rat 9 on day 2, the graph illustrates the GFR pattern over time on day 2 following the administration of a peritoneal injection into rats on day 0. RFR during the evolution of the renal damage, GFR loses its function. The value of RFR is calculated as 0.27. Abbreviations: GFR=glomerular filtration rate; RFR= renal functional reserve.*

RFR= 0.57 – 0.30 = **0.27**

***Tab. 3-*** *Table presents data obtained in rat 9 displaying an increase in the basal plasma creatinine level on day 2, with a value of* ***0.93*** *mg/dl.*

| RAT 9 | Time (min) | Crp (mg/dl) | Cru (mg/dl) | Volume of urine (ml) | Fu (ml/min) | GFR (mL/min) |
|---|---|---|---|---|---|---|
| 1 (Basal) | 0 | **0.93** | 81.98 | 0.07 | 0.00 | 0.30 |
| 2 | 20 | 0.92 | 25.43 | 0.36 | 0.02 | 0.50 |
| 3 | 40 | 0.89 | 11.13 | 0.91 | 0.05 | 0.57 |
| 4 | 60 | 0.79 | 5.19 | 1.51 | 0.08 | 0.49 |
| 5 | 80 | 0.77 | 6.29 | 0.74 | 0.04 | 0.30 |
| 6 | 100 | 0.84 | 14.87 | 0.11 | 0.01 | 0.10 |
| 7 | 120 | 0.95 | 22.57 | 0.03 | 0.00 | 0.03 |
| 8 | 140 | 0.32 | 21.90 | 0.02 | 0.00 | 0.07 |

# DAY 10

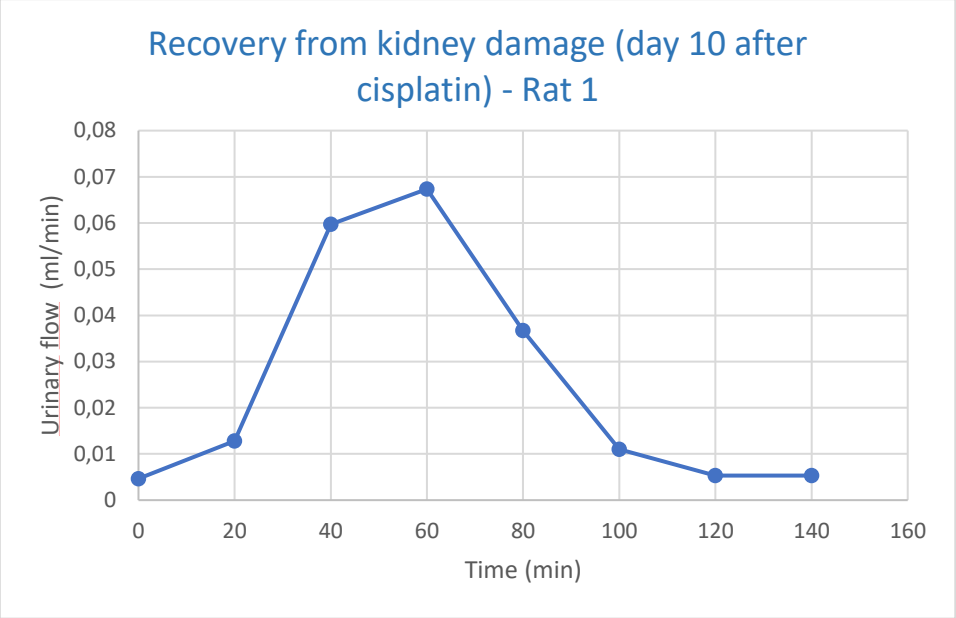


***Fig. 21-*** *Recovery from kidney damage in rat 1 on day 10. Figure shows urinary flow on day 10 over time after administration of cisplatin on day 0, capturing the recovery phase.*

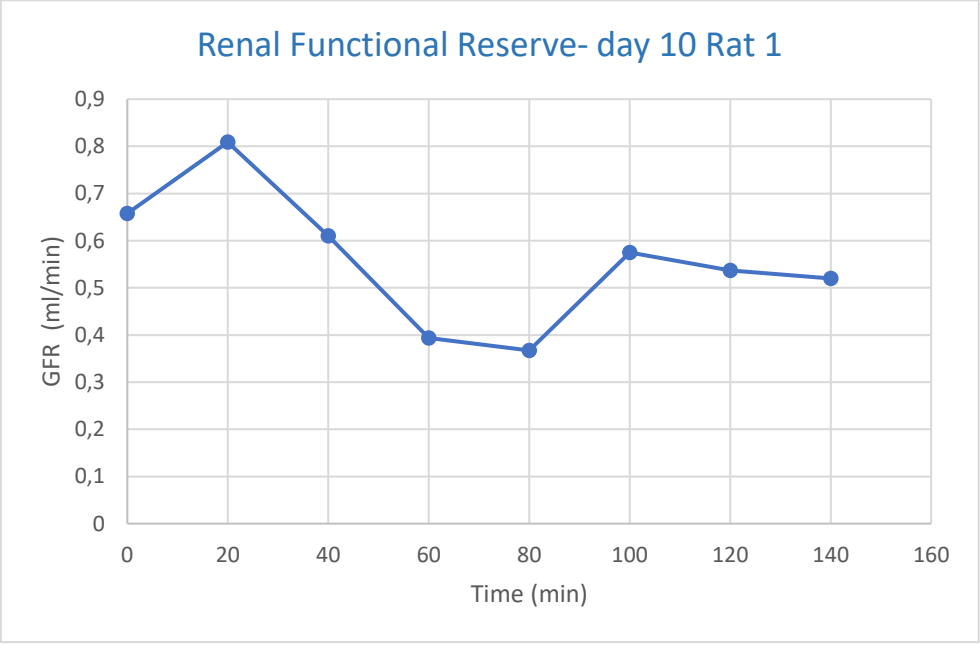


***Fig. 22-*** *Renal functional reserve in rat 1 on day 10. The graph illustrates the GFR pattern over time on day 10 following the administration of a peritoneal injection into rats on day 0. RFR during recovery phase, indicating that kidney function is not fully restored, it is not even close to the normal basal RFR function of control rats, as shown in Figure 12, with a value of 0.7. The value of RFR in this case is calculated as 0.30. Abbreviations: GFR=glomerular filtration rate; RFR= renal functional reserve.*

RFR= 0.80 – 0.50 = **0.3**

***Tab. 4*** *– Results obtained in rat 1. The basal plasma creatinine level remains elevated on day 10, with a value of* ***0.80*** *mg/dl.*

| RAT 1 | Time (min) | Crp (mg/dl) | Cru (mg/dl) | Volume of urine (ml) | Fu (ml/min) | GFR (ml/min) |
|---|---|---|---|---|---|---|
| 1 (Basal) | 0 | 0.80 | 84.29 | 0.09 | 0.00 | 0.49 |
| 2 | 20 | 0.79 | 49.63 | 0.26 | 0.01 | 0.81 |
| 3 | 40 | 0.86 | 8.76 | 1.19 | 0.06 | 0.61 |
| 4 | 60 | 0.75 | 4.39 | 1.35 | 0.07 | 0.39 |
| 5 | 80 | 0.69 | 6.94 | 0.73 | 0.04 | 0.37 |
| 6 | 100 | 0.78 | 40.51 | 0.22 | 0.01 | 0.58 |
| 7 | 120 | 0.81 | 82.10 | 0.11 | 0.01 | 0.54 |
| 8 | 140 | 0.90 | 87.94 | 0.10 | 0.01 | 0.52 |

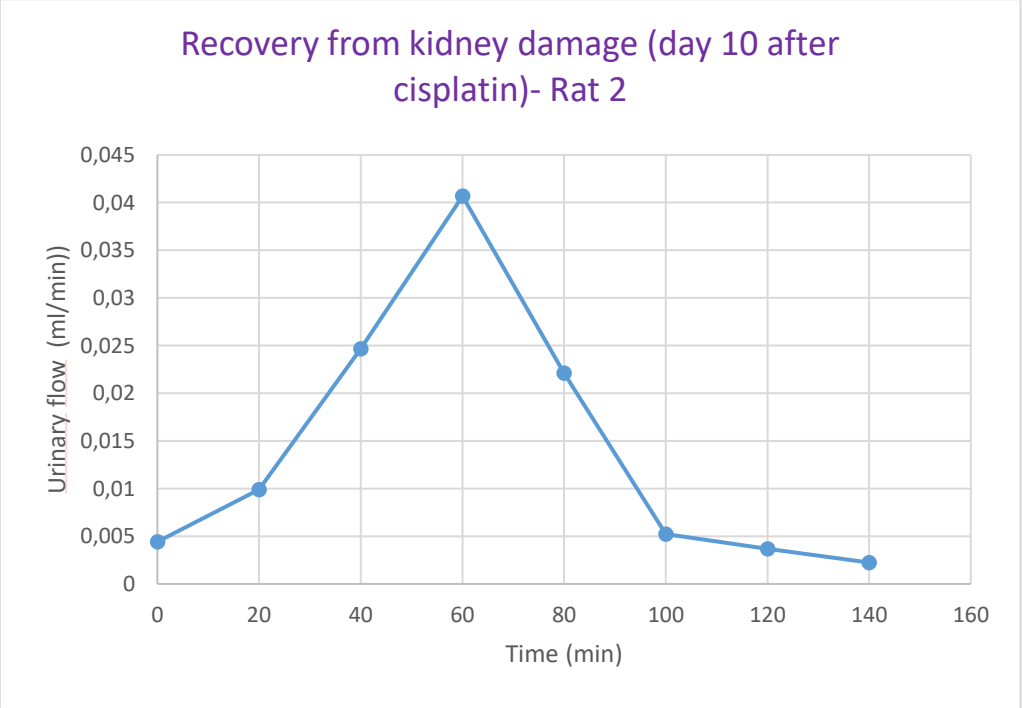


***Fig. 23-*** *Recovery from kidney damage in rat 2 on day 10. Figure shows urinary flow on day 10 over time after administration of cisplatin on day 0, capturing the recovery phase.*

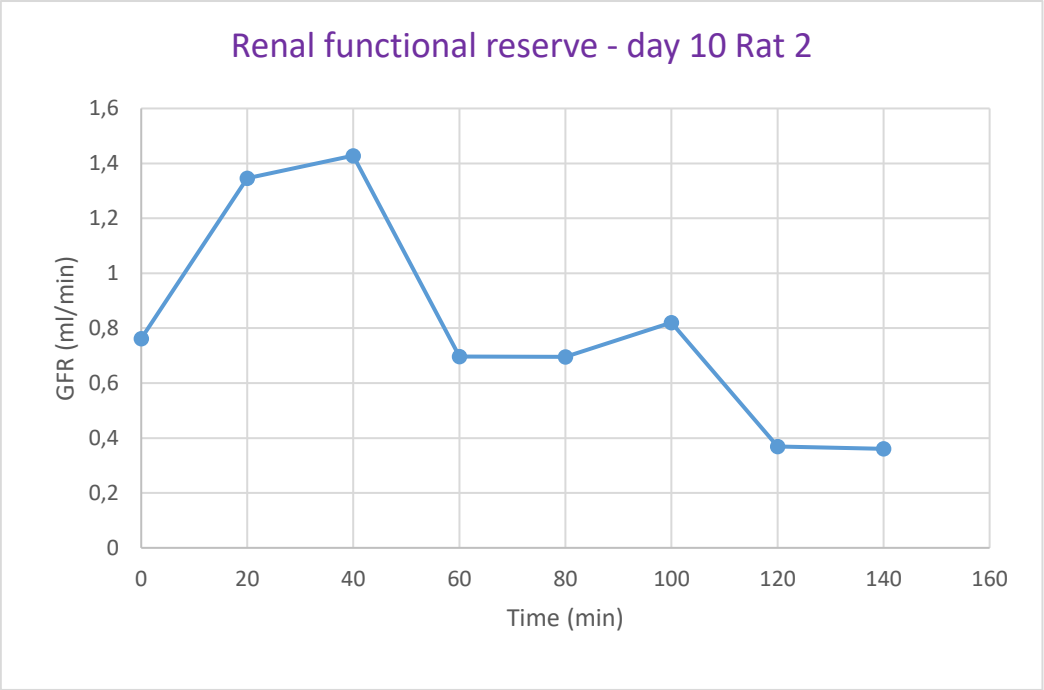


***Fig. 24-*** *RFR in rat 2 on day 10. The graph illustrates the GFR pattern over time on day 10 following the administration of a peritoneal injection into rats on day 0. During the recovery phase, the RFR indicates that kidney function is not fully restored but is closer to the normal basal RFR function of control rats, as shown in Figure 12, with a value of 0.7. The value of RFR in this case is calculated as 0.64. Abbreviations: GFR=glomerular filtration rate; RFR= renal functional reserve*

RFR = 1.4 - 0.76 = **0.64**

***Tab. 5-*** T*he basal plasma creatinine level remains elevated on day 10 in rat 2, with a value of* ***0.81*** *mg/dl.*

| RAT 2 | Time (min) | Crp (mg/dl) | Cru (mg/dl) | Volume of urine (ml) | Fu (ml/min) | GFR (ml/min) |
|---|---|---|---|---|---|---|
| 1 (Basal) | 0 | 0.81 | 139.38 | 0.09 | 0.00 | 0.76 |
| 2 | 20 | 0.79 | 107.64 | 0.20 | 0.01 | 1.35 |
| 3 | 40 | 0.60 | 34.67 | 0.49 | 0.02 | 1.43 |
| 4 | 60 | 0.96 | 16.43 | 0.81 | 0.04 | 0.70 |
| 5 | 80 | 0.73 | 23.00 | 0.44 | 0.02 | 0.70 |
| 6 | 100 | 0.67 | 105.45 | 0.10 | 0.01 | 0.82 |
| 7 | 120 | 1.29 | 128.80 | 0.07 | 0.01 | 0.37 |
| 8 | 140 | 1.08 | 173.68 | 0.04 | 0.00 | 0.36 |

The Figure 10 represents the evolution of kidney damage (measured through plasma creatinine) that theoretically should occur after the administration of a toxic dose of cisplatin (6 mg/kg i.p). Figures 11 and 12 show us the results from healthy control rats. The RFR value of control rats is possible to see in Figure 12. The RFR is calculated as the maximum point minus the basal point and the result is 0.7.

On day 4 we know from previous experience as the day of maximum nephrotoxicity at this dose, the RFR does not act. The kidneys do not filter correctly as is shown by the low initial filtration rate as is depicted in Figure 14. and the fact that after a stimulus with glycine, this filtration does not increase, there is no renal functional reserve.

The investigators in experimental studies say, the renal functional reserve (RFR), the increase in glomerular filtration rate (GFR) induced by a protein load, seems to be diminished or even lost in renal failure. (Laouari et al., 1990)

Our question is what happened on day 2, during the evolution of the damage, and on day 10 when the kidney is supposedly recovering from kidney damage?

On day 2 is noticeable that the kidneys start to be damaged, because the value of creatinine in plasma is 1.13 mg/dL for rat 6 (Tab. 2) and 0.93 mg/dL for rat 9 (Tab. 3), normal basal amount of creatinine in plasma is 0.5 mg/dL. (day 0, Tab. 1) Glomerular filtration loses its function. If we compare the RFR of rat 6, who has 0.2 (Fig. 18) and rat 9, who has 0.27 (Fig. 20) with normal control rats while their amount of RFR is 0.7. (Fig. 12) It means if the kidneys are damaged, the RFR decreases as well.

On day 10 we suppose that kidneys should be recovered after the first past experiments. But in our experiment, the recovery is not total, that is, the values are lower than those observed on day 4 (around 5 mg/dL) but still do not correspond to the values of a healthy animal (less than or equal to 0.5 mg/dL). The amount of creatinine in blood is 0.80 mg/dL (Tab. 4) for rat number 1 and 0.81 mg/dL (Tab. 5) for rat number 2. The RFR is quite different between rat 1 and rat 2. The RFR of the rat n.1 is 0.30 (Fig. 22) and the RFR of the rat n. 2 is 0.64 (Fig. 24), which is closer to the control rats RFR, which is 0.7 (Fig. 12). The values are variable and show how each animal recovers differently, indicating that although their creatinine is similar between these two, the RFR is not. This variability is frequently observed in animals which would lead us to propose new experiments by following strategy: increase the n of each group to confirm what happens at each point and establish another time as the recovery day, further away from

the point of nephrotoxicity. For example, on day 12 would be great to evaluate if when the creatinine values show normal data the RFR is already acting like a in control animal or if, on the contrary, it is still altered.

This information is very relevant since it has been observed how the repeated administration of doses of cisplatin that produce mild kidney damage can eventually lead to chronic damage (Yamashita et al., 2021).

The study of the RFR could give us information on whether the administration of cycles of cisplatin alters in the long run the ability of the kidneys to activate their RFR in such a way that this capacity is lost little by little and therefore leads to an inability to filter. Establishing a preclinical protocol to evaluate RFR would be very useful to answer this question, with this and other nephrotoxic drugs.

# 6. Conclusion

During the evolution of kidney damage (day 2), RFR is decreased and may be one of the most affected mechanisms after damage with cisplatin. After a toxic administration of cisplatin, the RFR is recovering even though it had been lost at the point of greatest nephrotoxicity. With the foundations laid in this study, work protocols can be established that allow for a more in-depth study of the role of RFR in the evolution and repair of damage caused by cisplatin and other nephrotoxic agents.

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