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Effects of obesity on the course of *Trypanosoma Cruzi* infection

Doctoral Dissertation Summary

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Účinky obezity v průběhu infekce *Trypanosoma cruzi*

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Table of Contents

SUMMARY

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Wunnie Brima, MD

Obesity is very widespread and detrimental to health. Obesity brings with it many changes including heightened immune function, and a higher prevalence of major cardiovascular disorders, cancer, diabetes, and Alzheimer disease. Obesity is also associated with shortened lifespan. The detrimental effects of obesity are linked to the "metabolic syndrome", a broad range of changes in metabolic processes and immune function.

As a first approximation, we agree with this formulation but we will then proceed to document some of its weaknesses. (i) Crude mortality rates increase with increasing body mass index (BMI) but as the BMI approaches the normal range, mortality rates reverse (the now classic "J-shaped curve") so that individuals with reduced BMI have elevated mortality. (ii) A multiplicity of medical and surgical conditions have been reported where short term and medium term outcomes are better for overweight patients. These conditions are placed under the heading of "obesity paradox". (iii) The medical community has introduced a binary system for the metabolic syndrome ---- *yes*, patient has it or *no*, the patient does not have it, despite the fact that all of the changes that are considered components of the metabolic syndrome are continuous variables.

Our work is focused on sharpening focus and improving understanding of these three weaknesses in the conceptualization of obesity as a medical problem. Using historical perspective, we have hypothesized that the metabolic syndrome is evolutionarily ancient. In

addition to the well-accepted harm associated with late-in-life disorders, we propose that throughout human history, the so-called syndrome has benefited younger individuals by enhancing host defenses against infectious diseases. We posit that the major benefits of the metabolic syndrome accrue in controlling the widespread potentially ravaging infections that the body cannot self-cure, including tuberculosis and (the subject of the thesis) *Trypanosomiasis cruzi* also known as Chagas disease or American trypanosomiasis. Infection with *Trypanosoma cruzi*, the protozoan parasite that causes Chagas disease, results in chronic infection that leads to cardiomyopathy with increased mortality and morbidity in endemic regions.

In support of our hypothesis that the consequences of the metabolic syndrome may be positive or negative, depending on age and on life events such as infectious diseases, we induced the metabolic syndrome in CD-1 mice by high fat feeding prior to infection with *T. cruzi*. The lethality of *Trypanosoma cruzi* infection was reduced from 55% to 20%.

Účinky obezity v průběhu infekce *Trypanosoma cruzi*

Obezita je velmi rozšířený a škodí zdraví. Obezita s sebou přináší mnoho změn, včetně zvýšené imunitní funkce, a vyšší prevalenci závažných kardiovaskulárních onemocnění, rakovinu, diabetes a Alzheimerovy choroby. Obezita je také spojena se zkrácenou životností. Škodlivé účinky obezity jsou spojeny s "metabolického syndromu", široký rozsah změn v metabolických procesech a imunitní funkce.

Jako první aproximaci, budeme souhlasit s touto formulací, ale pak budeme pokračovat zdokumentovat některé jeho nedostatky. (i) Hrubé úmrtnost zvyšuje se zvyšujícím se ‘body mass index’ (BMI), ale jak se blíží BMI normální rozsah, úmrtnost reverzní (dnes již klasickou "J-křivku ve tvaru písmene") tak, že jedinci se sníženou BMI mají zvýšené úmrtnosti. (ii) Velké množství lékařských a chirurgických případů byly zaznamenány, kde krátkodobé a střednědobé výsledky jsou lepší pro pacienty s nadváhou. Tyto podmínky jsou umístěny pod hlavičkou "obezity paradoxu". (iii) lékařská obec zavedla binární systém pro metabolický syndrom ---- *Ano*, pacient má, nebo *Ne*, pacient nemá ji, a to navzdory skutečnosti, že všechny změny, které jsou považovány za složky metabolického syndromu jsou spojité proměnné.

Naše práce je zaměřena na ostření zaměření a lepší pochopení těchto tří slabiny v pojímání obezity jako lékařský problém. Použití historickou perspektivu, jsme předpokládali, že metabolický syndrom je evolučně starobylý. Kromě dobře přijímán újmou v souvislosti s pozdně životni poruchy, navrhujeme, že v celé historii lidstvo , tento syndrom má benefit u mladších jedinců tím, že zvýrazníte přirozenou ochranu proti infekčním nemocem. Jsme předpokládají, že hlavní benefit metabolického syndromu narůstat při kontrole rozšířené potenciálně devastující infekce, které tělo nedokáže samo se ovládat, včetně tuberkulózy a (předmět práce) trypanosomiázu cruzi také známý jako Chagasova choroba nebo amerického trypanosomiáze. Infekce *Trypanosoma cruzi*, parazitickým prvokem, který způsobuje,

Chagasova choroba, vede k chronické infekce, která vede ke kardiomyopatii s zvýšené úmrtnosti a nemocnosti v endemických oblastech.

Na podporu naší hypotézy, že důsledky metabolického syndromu může být pozitivní nebo negativní, v závislosti na věku a na životními událostmi, jako infekčních nemocí, indukovali jsme metabolicky syndrom u CD-1 myší krmeni vysokým obsahem tuku před infekcí *T. cruzi*. Úmrtnost z infekce *Trypanosoma cruzi* se snížil ze 55% na 20%.

1. INTRODUCTION

Obesity is a result of chronic positive energy balance and is associated with chronic low-grade inflammation and insulin resistance. The metabolic syndrome, defined by a constellation of interconnected physiological, biochemical, clinical, and metabolic factors, produces immune activation and metabolic alterations that promote complications of obesity and diseases of later life, including myocardial infarction, diabetes, Alzheimer's disease and cancer. Increasing prevalence of the metabolic syndrome parallels the rise in the prevalence of obesity. Worldwide prevalence of the metabolic syndrome ranges between 10% to as much as 84%, depending on the region, composition (sex, age, race, and ethnicity) of the population studied, and the criteria used to define the metabolic syndrome[1, 2].

The metabolic syndrome is a state of chronic low grade inflammation. This has been linked to the release of cytokines by adipose tissue [3-5]. Adipose tissue is a heterogeneous mix of adipocytes, stromal preadipocytes, immune cells, and endothelium, and can respond to alterations in nutrient excess through adipocytes hyperplasia and hypertrophy [6]. Adipose tissues, in addition to being a major organ for energy storage is also considered to be an endocrine and paracrine organ. It plays an integral role in energy metabolism, neuroendocrine function, and immune function. Adipose tissue is sub-classified into white adipose tissue (WAT), brown adipose tissue (BAT) and beige adipocytes. Fat in some depots promotes inflammation more than fat in other sites. The term visceral fat, as currently used by the scientific community, is a misnomer as it refers to mesenteric or omental fat. Fat deposits around visceral organs, such as, epicardial fat and perinephric fat are currently referred to as ectopic fat or metastatic fat by the scientific community. Subcutaneous fat is more energy efficient, wasting fewer calories on immune related processes. Visceral or ectopic fats

deposits are highly inflammatory fat deposits when compared to subcutaneous fat deposits [7].

Several authors have noted a linkage between adipose tissue and immune function; the immune system is a major user of energy (it consumes about 15% of resting metabolic rate) and extremely malnourished individuals have been shown to have impaired immune function. Adipose tissue secretes into the general circulation interleukins and adipokines (such as IL-6, adipsin, TNF alpha, leptin, monocyte chemoattractant protein-1 etc) that are predominantly involved with immune functions [8].

Emerging positive outcomes in studies on obesity and the metabolic syndrome are grouped by the biomedical community under the rubric of "obesity paradox". The obesity paradox refers to the inverse relationship between body fat composition, (defined by the BMI), and all-cause mortality [9]. Several studies have observed that obesity, despite it being a major risk factor in the development of cardiovascular and other diseases, can be protective and is associated with greater survival in individuals with certain diseases including heart failure, infections, sepsis, thromboembolism, end stage renal disease (ESRD), and sterile injury. To date, there are over 800 articles on the obesity paradox in pubmed.org.

The obesity paradox is observed in several medical conditions, non-infectious and infectious. It has been observed that overweight and obese patients with coronary artery disease undergoing percutaneous coronary intervention have better outcome compared with their normal-weight counterparts [10]. The obesity paradox has also been observed in other conditions such as in patients with end stage renal disease undergoing dialysis, patients who underwent cardiac and non-cardiac surgeries, critically ill patients in intensive care units, community acquired pneumonia, sepsis and osteoporosis [9-11]. These suggest that adiposity provides an "immuno-metabolic shield" that benefits individuals in their struggles with infectious and non-infectious diseases.

American trypanosomiasis or Chagas disease, caused by the protozoan parasite, Trypanosoma cruzi, is classified by the World Health Organization as one of the major infectious diseases of the world. It is responsible for substantial morbidity and mortality amongst individuals in endemic regions of Latin America and those that have immigrated to nonendemic areas of the world such as the United States and Europe [12, 13]. Chronic infection results in cardiomyopathy and/or megasyndromes with significant associated morbidity and mortality.

We demonstrate that the lethality of acute *Trypanosoma cruzi* infection in mice can be markedly attenuated by pre-feeding with a high-fat diet (HFD) to mimic the metabolic syndrome. The findings presented in this study and in a companion piece [14] demonstrate that mice fed with a high fat diet to mimic the human metabolic syndrome are significantly protected against the lethality of Trypanosoma cruzi infection. These data are consistent with human epidemiologic observations that increased body weight positively correlates with improved outcomes in Chagas disease [15] (as well as with tuberculosis, sepsis and other infectious diseases [16-18]. Exceptions occurs when (i) hyperglycemia supervenes or (ii) when the metabolic syndrome is severe or (iii) of long duration.

2. AIMS OF RESEARCH

Obesity and the metabolic syndrome have a negative reputation as a major detriment to health and longevity. The metabolic syndrome, as presently construed, produces immune activation and metabolic alterations that promote complications of obesity and diseases of later life, such as myocardial infarction, stroke, diabetes, Alzheimer's disease and cancer. Using an evolutionary approach, we hypothesized that for millions of years, the channelling of host resources into immune defenses starting early in life ameliorated the effects of infectious diseases, such as Chagas disease. Throughout human history, the so-called syndrome has benefited individuals by enhancing host defenses against infectious diseases. In addition to support during acute self-limited infections (e.g. community acquired pneumonia), we raise the possibility that the major benefits of the processes associated with the metabolic syndrome accrue in controlling the widespread potentially ravaging infections that the body cannot self-cure, including American trypanosomiasis, the subject of the thesis.
The specific aims are:

1. To assess the effect of obesity and the metabolic syndrome (induced by a high fat diet) on the course of *T. cruzi* infection.

2. To investigate the relationship between high fat feeding and parasitemia and parasite burden.

3. To assess the effect of metformin treatment [which is known to ameliorate the metabolic syndrome] on the course of *T. cruzi* infection

3. EXPERIMENT

3.1. MATERIALS AND METHODS

3.1.1. Study design

As stated earlier, there are several animal models of the metabolic syndrome. However, no animal model can be expected to mimic all the features of the metabolic syndrome as seen in humans. Other common models like the streptozotocin model, and the genetic models including ob/ob and db/db mouse model, can be useful in evaluating specific molecular mechanisms in development of obesity in mice and the metabolic syndrome in humans is not a monogenic disorder.

We opted for a dietary model as it mimics, albeit imperfectly, the features of the metabolic syndrome in humans: obesity, dyslipidemia and insulin resistance. CD-1 inbred mice were fed a high fat diet to induce obesity and the metabolic syndrome. CD-1 inbred mice are robust, inexpensive, and come without any genetic manipulation. CD-1 mice were subsequently infected with Trypanosoma cruzi, a protozoa causing Chagas disease. Trypanosoma cruzi infection is widely studied by a world-renowned research group at the Albert Einstein College of Medicine, in New York. Our study was conducted in close collaboration with the group at Albert Einstein College of Medicine and the Feinstein Institute for Medical Research.

Experimental Design

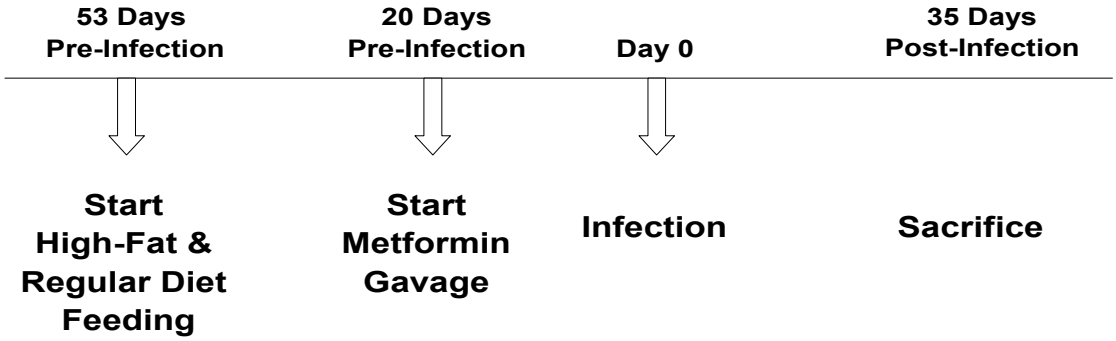

Table 1: Basic experimental design

Five-week-old CD-1-inbred mice (n=220), weighing on average 28 g (Charles River Laboratories, Wilmington, MA, United States), were maintained on a 12-h light–dark cycle in a temperature and humidity controlled room. Animals were housed in groups of five per cage with free access to water and food. Body weights were recorded every 2 weeks, and cages were changed three times per week.

Mice were randomly divided into a high fat diet (HFD) group (n=100) or a regular diet (RD) group (n=120). Mice fed a regular diet included 20 uninfected mice, 20 uninfected mice treated with metformin, 40 infected mice and 40 infected mice treated with metformin. Mice fed a high fat diet included 20 uninfected mice, 20 uninfected mice treated with metformin, 30 infected mice and 30 infected mice treated with metformin. The high fat diet consisted of (by kilocalories) 60% fat with added cholesterol, 20% protein and 20% carbohydrate; the regular diet consisted of 10% fat, 20% protein and 70% carbohydrate (Research Diets Inc., New Brunswick, NJ). Diets were matched for sucrose. All mice were fed the assigned diets for the duration of the experiment.

20 days before infection, a subset of mice were started on metformin (Research Grade, Sigma-Aldrich, St. Louis, MO). The metformin was administered daily by gavage (after weighing and blood draws) by using a bulb tipped gastric gavage needle attached to a syringe.

	Regular Diet	**High Fat Diet**
Uninfected	**20**	**20**
Uninfected + Metformin	**20**	**20**
Infected	**40**	**30**
Infected + Metformin	**40**	**30**

Table 2: Experimental groups based on diet

Mice were grouped in based on whether they were fed a regular diet vs a high fat diet, infected vs uninfected and whether they were treated with daily metformin

On the day of infection (designated as postinfection day 0), subsets of mice in both high fat diet and regular diet groups were infected intraperitoneally with 5×10^4 trypomastigotes Brazil strain of T. cruzi, maintained by passage in C3H/Hej mice (Jackson Laboratories, Bar Harbor, ME), as described by Tanowitz et al.[19].

Parasitemia was determined at 10, 15, 20, 24, 27, 30, 33, 35 and 38 days post infection (dpi) by microscopy using a Neubauer haemocytometer[20, 21].

On day 35 post-infection, mice (n=54) were sacrificed by cervical dislocation. Liver, heart and white adipose tissues were harvested; liver and heart were weighed. All harvested tissues were stored at -80 °C and/or fixed in formalin for future studies.

3.2. Cell culture experiment to determine the effect of metformin on cultured cells

3.2.1. Mammalian cell culture

Human foreskin fibroblasts (ATCC CRL 1475) were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum and 1% penicillin–streptomycin at 37 °C in 5% CO_2. The fibroblasts were plated in 24-well plates (Falcon™ Tissue Culture Plates) and Nunc™ four-well dishes (Sigma-Aldrich) at a density of 2.5×10^5 per millilitre 24 h prior to the start of the experiment to achieve 80–90% confluence.

Eight hours prior to infection, half of the wells in each plate were pre-treated/incubated with selected concentrations of metformin (0, 5, 10, and 50 μg/mL) (Sigma-Aldrich).

3.2.2. Infection of Human Foreskin Fibroblasts

Tissue culture-derived trypomastigotes were generated by weekly passage in confluent monolayers of fibroblast cells in DMEM containing 10% foetal bovine serum. Trypomastigotes, harvested from cell culture supernatants, were washed two times and re-suspended in DMEM. Confluent monolayers of fibroblasts were infected with parasite at a density of 5×10^5 and incubated with 0–50 μg/mL of metformin at 37 °C in 5% CO_2. Infection was allowed to proceed for 24h. The number of live parasites/trypomastigotes and infected host cells in each well was determined with a hemocytometer at 24h post-infection. Plates were washed two times with 10% phosphate-buffered saline at 37 °C, fixed with 4% paraformaldehyde and stained with May–Grünwald–Giemsa according to a standard protocol [22]. Next, two concentrations of metformin (0, and 10 μg/mL) were used to pre-treat wells in 24-well plates (Falcon™ Tissue Culture Plates) plated with fibroblast as described above. Confluent monolayers of fibroblasts were infected with parasite at a density of 5×10^5 and incubated with 0–10 μg/mL of metformin at 37 °C in 5% CO_2. Infection was allowed to proceed for 24, 48 and 72 h. The number of live parasites/trypomastigotes and infected host

cells in each well was determined with a hemocytometer at 24, 48 and 72 h post-infection. Plates were washed, fixed and stained with May–Grünwald–Giemsa as described above.

4. RESULTS

4.1. Mortality of CD-1 mice during acute T. cruzi infection

As expected, when infected with the Brazil strain of *T. cruzi*, CD-1 mice maintained on a regular diet (RD) displayed a high mortality rate (Figures 1A and 2). The first death in this group occurred on day 26 post-infection (Figures 1A and 2). Deaths were recorded until day 42 post-infection, by which time mortality had reached 55%. CD-1 mice fed a high fat diet (HFD), which was begun 53 days prior to infection and continued throughout the study, displayed a markedly attenuated mortality (Figures 1B and 2). Overall, the mice on the high fat diet had a death rate of 20% as compared with the mice on the regular diet (55%).

The co-administration of metformin resulted in a marked drop in mortality in both diet groups (Figures 1C and D and 2). In regular diet mice, there was a reduction in mortality from 55% to 25%. In high fat diet mice, the metformin reduced mortality from 20% to 3% (Figures 1B and D and 2).

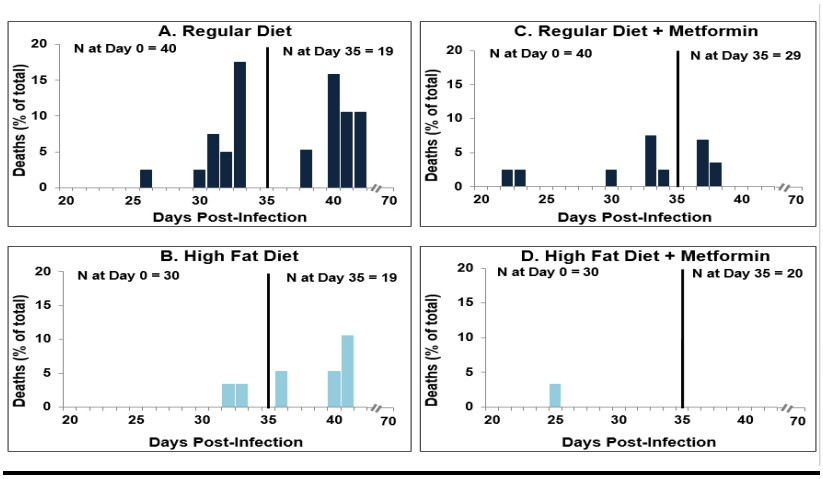

Figure 1: The impact of diet on the course of acute *T. cruzi* infection

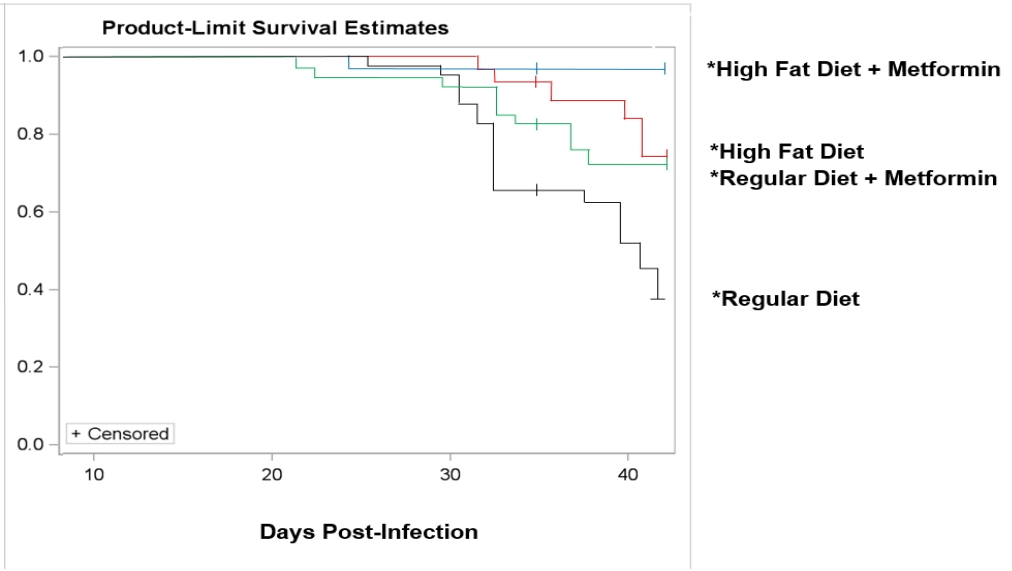

Figure 2: Kaplan-Meier survival plot: The same data that were presented in figure 1A-D are re-expressed here in the form of a Kaplan-Meier survival curve. The different Kaplan-Meier curves for each group were found to be statistically significant (using the log-rank test) for diet (p=0.0003) and metformin treatment (p=0.002).

4.2. Body weight of CD-1 mice

Pre-infection, mice fed the high fat diet (HFD) revealed a much greater weight gain than those on regular diet (RD) (Figure 3A and B). Post-infection, the regular diet mice displayed an attenuation of weight gain and then a modest weight loss (Figure 3B) in comparison with the uninfected mice (Figure 3A). High fat diet mice on day 5 post-infection began a steep weight loss so that their weights approached those of the infected regular diet mice (Figure 3B).

This is consistent with the effect of *T. cruzi* infection on adipocytes [23, 24] . Pre-infection, mice treated with metformin had an initial fall in weight and persistently lower weights than their respective non-metformin-treated mice, both with the high fat diet and regular diet (Figure 3A and B). After infection, both the high fat diet (HFD) and regular diet (RD) mice treated with metformin showed a gradual modest weight loss, resulting in somewhat lower weights throughout the course of the experiment in comparison with their respective non-metformin-treated mice (Figure 3B).

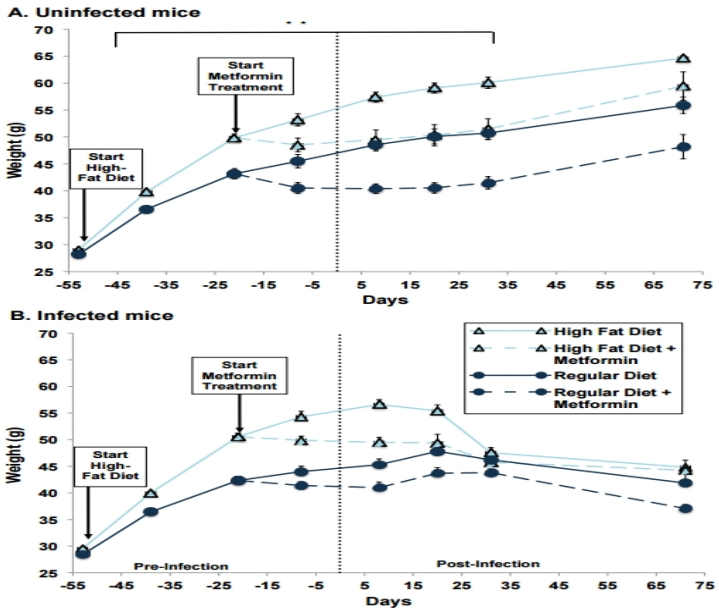

Figure 3: Body weights of CD-1 mice.

3A- Uninfected Mice: Body weights between regular diet-fed mice and high fat diet-fed mice differed significantly at day -53 ($p=0.0052$), day -39 ($p=0.0009$), and day -20 ($p=0.0011$). Following metformin treatment, body weight between RD, RD + Metformin, HFD, and HFD + Metformin groups continued to differ significantly at days -8 ($p=0.0061$), day 8 ($p=0.0353$), day 20 ($p=0.0353$), and day 30 ($p=0.0323$), but not on day 70 ($p=0.0638$).

3B- Infected mice: Body weights between regular diet-fed mice and high fat diet-fed mice differed significantly at day -53 ($p<0.0001$), day -39 ($p<0.0001$), and day -20 ($p<0.0001$). Following metformin treatment, body weight between RD, RD + Metformin, HFD, and HFD + Metformin groups continued to differ significantly at days -8 ($p<0.0001$), day 8 ($p<0.0001$), day 20 ($p<0.0001$), day 30 ($p=0.0293$), and day 70 ($p=0.0347$).

4.3. Parasitemia

In regular diet mice, parasite levels in blood began to increase 15 days post-infection, reached a peak at day 25 and then fell slowly to undetectable levels by day 33 (Figure 4). In high fat diet mice, parasitemia reached its peak later (at day 30 post-infection) and cleared the blood rapidly, becoming undetectable by day 38 post-infection. A nonparametric comparison Kruskal–Wallis test demonstrated statistical significance ($p<0.01$) amongst all four groups on

days 27, 30, 33 and 35 post-infection. The high fat diet group had a greater parasitemia, demonstrated by the larger area under the curve (Figure 4).

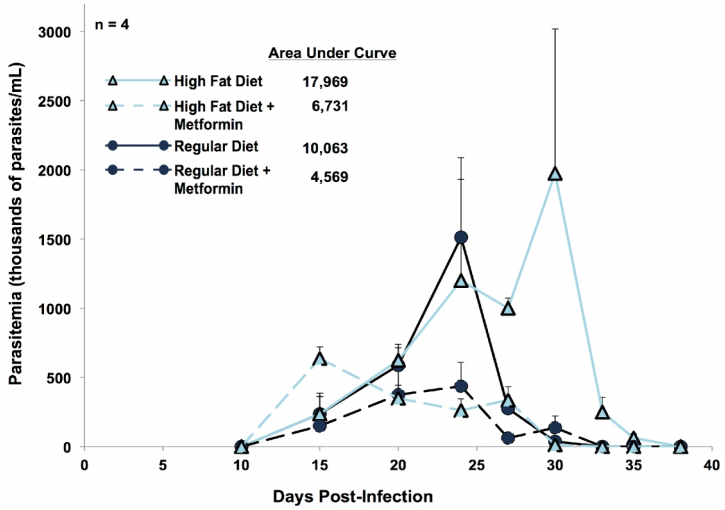

Figure 4: Parasitemia: Blood was drawn from 4 mice per group at intervals from post-infection day 10 through day 38 for measurements of parasites. Each point represents the mean (+ SEM). The numbers in the key represent the relative areas under each curve. A non-parametric version of ANOVA, the Kruskal-Wallis test, demonstrated that there was a statistically significant difference in parasitemia levels amongst all groups on day 27 ($p=0.0001$), day 30 ($p=0.0035$), day 33 ($p=0.0286$), and day 35 ($p=0.0286$) post-infection.

4.4. *Effects of metformin on Trypanosoma cruzi*

In both diet groups, parasite levels in blood (expressed as areas under the curve) were about 40% as high with metformin as in its absence (as shown in figure 4). Metformin in vitro had no effect on parasites growing on human fibroblasts when cells were exposed to metformin (up to 50 µg/L) for up to 72 h (Figure 5).

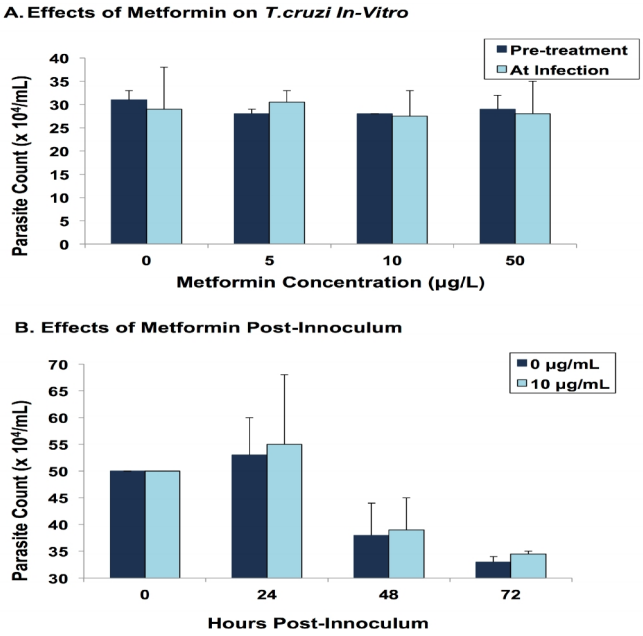

Figure 5: In Vitro Effects of Metformin

5. DISCUSSION AND CONCLUSION

The last decades of the 20th century and the first part of the 21st has been characterized by an epidemic of obesity and an increase in the recognition of the negative health consequences of obesity, including the metabolic syndrome. In our study we reviewed epidemiologic studies where obesity brought with it better outcomes for a clutch of major infectious diseases including acute illnesses such as pneumonia, and sepsis and protracted illnesses such as TB and Chagas disease. To test whether the relationship was cause and effect related, we experimentally reproduced the benefit of obesity in our studies of mice with Chagas diseases; short term overfeeding of mice with a high fat diet enhanced survival. We showed that 8 weeks of overfeeding, in the form of a high fat diet, diminished the lethality of *T. cruzi* in mice from 55% to 20%. This result is consistent with the recently published article from our group; high fat diet mice starting on the day of infection reduced mortality from 60% to 15% [14]. High fat diet started 30 days before infection dropped mortality from 60% to 8% [14]. The benefits of overfeeding were clear, despite the moderate hyperglycemia that accompanied the high fat diet. In our mice, metformin, a medication widely used to control hyperglycemia (and weight gain) in patients with diabetes, further reduced mortality.

Our finding that a high fat diet producing the metabolic syndrome markedly reduced mortality amongst mice infected with *T. cruzi* is consistent with our broadly based hypothesis that immune-metabolic interactions and the associated metabolic syndrome appear to protect against the consequences of many serious conditions such as sepsis, pneumonia, TB and Chagas disease.

Introducing an evolutionary lens, we raise the possibility that extra nutrition with immune activation were highly beneficial in protecting the populace, especially the young, from the ravages of infectious illnesses that typically decimated young humans. Only within the last

century in wealthy countries has clean water, immunizations, antibiotics and other public health advances turned the tide. Now with rampant obesity, longer life-spans, and infection control, the benefits of the added weight have diminished while the deficits have increased.

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