Evaluation of Hematological and Biochemical effects of sildenafil in hyperglycemic Rats

Avaliação Hematológica e Bioquímica de Ratos Hiperglicêmicos tratados com Sildenafil

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ABSTRACT
Diabetes mellitus is a pathology related to changes in glucose metabolism. The hormone insulin acts stimulating intracellular glucose metabolic pathways. The increase in cGMP signaling has the potential to increase insulin sensitivity in the muscle and can stimulate the secretion of this hormone by pancreatic beta cells. Sildenafil (SIL) is a potent peripheral vasodilator that acts attenuating the catabolism of cGMP thus increasing the signaling of the pathways involving the protein kinase G. The present study aimed to evaluate the effect of the sildenafil on hematological and biochemical parameters in hyperglycemic rats. 55 male Wistar rats were separated into 4 groups: G1 (control), G2 (streptozotocin), G3 (streptozotocin + 10 mg/kg sildenafil) and G4 (streptozotocin + 15 mg/kg sildenafil). The animals received sildenafil during seven days before the application of streptozotocin, maintaining the treatment for another 3 weeks. After euthanasia, hematological and biochemical analyzes were performed. The group that received the highest dose of SIL, showed a decrease in leukocytes compared to the G2 group (p <0.05). In addition, G2 presented thrombocytopenia. Therefore, sildenafil appears to decrease the number of inflammatory cells in hyperglycemic rats in a dose dependent manner.

Keywords: Diabetes Mellitus, Streptozotocin, Inflammation, Cyclic Guanosine Monophosphate, Erythrogram, Leukogram.

RESUMO
O diabetes mellitus é uma doença que causa alterações no metabolismo de glicose. O hormônio insulina atua na redução dos níveis de açúcares no sangue, estimulando vias metabólicas intracelulares. O aumento na sinalização do cGMP tem potencial para aumentar a sensibilidade à insulina no músculo, além de estimular a secreção desse hormônio pelas células beta pancreáticas. A elevação desse nucleotídeo pode ser conquistada com um inibidor de fosfodiesterase (PDE-5) como o fármaco sildenafil, um potente vasodilatador periférico, que age atenuando o catabolismo do cGMP aumentando assim a sinalização das vias envolvendo a proteína quinase G. O presente estudo teve por objetivo avaliar o efeito do fármaco sildenafil por meio das alterações hematológicas e bioquímicas em ratos hiperiglicêmicos. Foram utilizados 55 ratos machos Wistar separados em 4 grupos: G1 (controle), G2 (estreptozotocina), G3 (estreptozotocina + 10 mg/kg sildenafil) e G4 (estreptozotocina + 15 mg/kg sildenafil). Os animais receberam sildenafil por uma semana antes da aplicação de estreptozotocina, mantendo o tratamento por mais 3 semanas. Após a eutanásia realizou-se análises de hematológicas e bioquímicas. O grupo que recebeu a maior dose de SIL, demonstrou uma diminuição de leucócitos comparados ao grupo G2 (p<0,05). Além disso o G2 apresentou uma trombocitopenia. Portanto, o sildenafil diminui o número de células inflamatórias em ratos hiperiglicêmicos de forma dose dependente.

Palavras Chaves: Diabetes Mellitus, Estreptozotocina, Inflamação, Eritrograma, Leucograma.
1 INTRODUCTION

Diabetes mellitus (DM) is a chronic disease related to glucose metabolism, resulting in chronic hyperglycemia [1]. Hyperglycemia can cause biochemical changes in the metabolism of lipids, carbohydrates and proteins [2], in addition it is considered the main cause of complications in diabetic conditions.

In tissues, insulin acts reducing blood glucose levels, stimulating intracellular metabolic pathways [3]. According to some studies, the acute increases in cyclic guanosine monophosphate (cGMP) signaling increase glucose-stimulated insulin secretion in pancreatic islets, although prolonged increases in cGMP may decrease insulin secretion [4] [5].

Pharmacological strategies to increase cGMP include increasing nitric oxide (NO), activating soluble guanylate cyclase (GCs) or decreasing the phosphodiesterase enzyme (PDE). There are 11 PDE families, with different affinities for cyclic adenosine monophosphate (cAMP) and cGMP. PDE-1, PDE-2, PDE-3, PDE-10 and PDE-11 act on both cyclic nucleotides. PDE-4, PDE-7 and PDE-8 act mainly on cAMP while PDE-5, PDE-6 and PDE-9 are selective for cGMP [6].

Sildenafil (SIL) is a selective inhibitor of PDE-5, an enzyme widely expressed in the organism, responsible for the degradation of cGMP, converting it to guanosine monophosphate (GMP). Therefore, administration of SIL can increase cGMP levels, which could be a alternative to regulate glucose homeostasis, avoiding the detrimental effects of diabetes [7] [8]. In addition, SIL has angiogenic and anti-inflammatory activity [9].

Taken these effects together, the hypothesis was that the SIL administration could be protective against ischemic and inflammatory changes caused by hyperglycemia. Thus, the present study proposed to analyze some hematological and biochemical markers in rats previously treated with SIL and diabetized with the streptozotocin toxin.

2 MATERIAL AND METHODS

2.1 ETHICAL CONSIDERATIONS AND ANIMAL HANDLING

This experiment was approved by the Animal Experimentation Ethics Committee of State University of Ponta Grossa (protocol number 19.000004698-3). Male adult Wistar rats (Rattus norvegicus), normoglycemic, from the Advanced Nucleus of Life Studies (NAEVI) of the State University of Ponta Grossa (UEPG) (Ponta Grossa / Paraná-
Brazil) were used. The weight of the animals varied between 208 to 340 g, and they were healthy on clinical examination.

Animal handling was supported by Law 11794, of October 8, 2008. The animals were packaged in polypropylene cages. The cages were kept on vertical shelves in the UEPG vivarium, at 12 / 12h (light and dark), with a controlled temperature of 25˚C. The feeding was carried out with a normoproteic commercial diet and natural water (ad libitum), throughout the experiment period, following the guidelines of the Brazilian College of Animal Experimentation (COBEA) and ARRIVE (Animal Research: Reporting of In Vivo Experiments).

2.2 SAMPLE PLANNING

The sample calculation was based on preliminary data from the research group, Lipinski et al. [10]. A significance level of p = 0.05 was adopted. Calculations were performed based on the mean and standard deviation, establishing a test power of 0.80 and an effect size of 0.4 (www.3Rs-Reduction.co.uk). The result obtained was n = 13. Predicting possible losses and exclusions during the 10% experiment, a “n” sample of 14 animals per group was considered. All calculations were performed using the G * Power 3.1.9.4 program.

2.3 EXPERIMENTAL DESIGN AND DIABETIZATION PROTOCOL

Fifty-five animals were randomly separated into 4 experimental groups. The experiment was started with the administration, for one week, of sildenafil (SIL) orally in groups G3 and G4 at a dose of 10 and 15 mg/kg respectively. At the same time, groups G1 and G2 were drawn with 0.50 ml of saline (SS) daily.

In week 2, groups G2, G3 and G4 were submitted to streptozotocin (STZ) (Sigma) via intraperitoneal (i.p), while group G1 received SS 0.9% via i.p in order to undergo the same stress. Before application, under a 6-hour water and food fast, the measurement of serum glucose was performed in peripheral blood obtained by sectioning the final portion of the tail. The automated and calibrated glucometer (Accu-chek® Active) was used. The tip of the tail was sectioned and a drop of blood required to read the device was removed.

From the weight of animals G2, G3, G4, STZ was weighed and solubilized in 0.9% saline. The animals in the aforementioned groups received a dose of 40 mg/kg of STZ i.p., while the control group received 1 ml of 0.9% SS i.p. Then the animals returned to their cages, with water and feed.
After 24 hours these groups were submitted to a new blood glucose analysis, rats with blood glucose below 200 mg/ml received 40 mg/kg of STZ, rats with blood glucose between 200 - 250 mg/ml received 20 mg/kg of STZ and the with blood glucose above 250 mg/ml did not receive a new dose of the toxin. 48 hours after the second application of STZ, the blood glucose of these groups was again measured, rats with blood glucose below 250 mg/dl were excluded from the study. Rats with blood glucose above 250 mg/dl were considered diabetic. The animals continued receiving gavage with SS and SIL until the end of the 4 weeks.

2.4 EUTHANASIA AND SAMPLES

Euthanasia was performed by anesthetic overdose. 140 mg / kg of Ketamine (Cetamin®; Laboratório Syntec, Santana de Parnaíba, SP, Brazil) and 20 mg / kg of Xylazine (Xilasin®; Laboratory Syntec, Santana de Parnaíba, SP) were used intraperitoneally.

After administration of anesthetics, cardiac puncture was performed from the opening of the hemithorax of each rat with a 5 mL syringe and needle (1.20 x 40 mm) and the samples were separated for analysis. To perform hematological analysis, blood was collected in an ETDA tube. For biochemical analyzes, blood collected in a tube with separator gel was subjected to centrifugation at 3000 rpm (rotations per minute) for 10 minutes to obtain the serum. The collected serum was stored in eppendorfs. Simultaneously, blood smear was performed with the remaining blood from the syringes.

2.5 DATA ANALYSIS

2.5.1 Hematological Analysis

The blood samples of the animals were processed in a hematological analyzer (Hematoclin 2.8 VET, Belo Horizonte, MG, Brazil), the parameters investigated were: erythrocyte count, hemoglobin, hematocrit, mean corpuscular hemoglobin (HCM), mean corpuscular volume (VCM), mean corpuscular hemoglobin (CHCM) concentration, leukocytes, platelets, and differential leukocyte count.

The differential leukocyte count was performed by reading the slides stained by the May-Grünwald-Giemsa method under an optical microscope.
2.5.2 Biochemical Analysis

The biochemical parameters (cholesterol, triglycerides and total protein) were analyzed using commercial kits (InterKit, Katal, Belo Horizonte, MG, Brazil). The determination of total cholesterol was performed by enzymatic method, while total proteins by colorimetric method and enzymatic colorimetric triglycerides, in semi-automatic biochemical analysis (BIO PLUS 200, Barueri, SP, Brazil).

2.6 STATISTICAL ANALYSIS

The results were presented in mean and standard deviation (EP), run by the software of the company GraphPad, Prism 7 (University of California, San Diego, USA). Analytical statistics were conducted with parametric (ANOVA with one or two criteria and ANOVA with repetition) and non-parametric (Kruskal-Wallis and Dunn's post-test) models according to the data distribution. The level of significance adopted was 5% (α = 0.05).

3 RESULTS

3.1 EVALUATION OF GLUCEMIA AND BODY MASS

At the beginning of the study, all groups were normoglycemic (Figure 1) and with similar mean weights (Figure 2A). After the application of streptozotocin (STZ), groups G2, G3 and G4 demonstrated a significant increase in the mean blood glucose value, maintaining hyperglycemia until the end of the experimental period (Figure 1). At the end of the four weeks, the G1 group showed weight gain, while in the other groups there was a weight loss (Figure 2B).

![Figure 1: Graph of blood glucose variation at the beginning and end of the study. The results are expressed as mean and standard deviation (SD) of blood glucose values (n = 10). A ≠ B indicates statistical difference between groups (ANOVA, Dunn’s p <0.05.).](image-url)
Figure 2: A. Graph of the animals’ initial weight before the diabetic process. B. Graph of the final weight of the animals before euthanasia. The results are expressed as mean and standard deviation (SE) (n = 10). A ≠ B indicates statistical difference between groups (ANOVA, Turkey, p < 0.05).

3.2 AVALIAÇÃO HEMATOLÓGICA

The erythrocyte lineage did not showed significant differences between the groups studied (Table 1). In the leukogram (Table 2), the G2 group presented the highest leukocyte value, obtaining a statistical difference in relation to the other groups (p = 0.05), while the G4 demonstrated a value similar to G1 (control). The platelet count showed a decrease in the hyperglycemic groups compared to the control group, in this parameter, G2 group presented a more accentuated thrombocytopenia.

<table>
<thead>
<tr>
<th></th>
<th>G1</th>
<th>G2</th>
<th>G3</th>
<th>G4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Erythrocytes (mm³)</td>
<td>8.88</td>
<td>9.13</td>
<td>8.9</td>
<td>9.26</td>
</tr>
<tr>
<td>Hemoglobin (g/dL)</td>
<td>14,5</td>
<td>14,8</td>
<td>14,8</td>
<td>14,5</td>
</tr>
<tr>
<td>Hematocrit (%)</td>
<td>53,88</td>
<td>53,81</td>
<td>53,14</td>
<td>54,88</td>
</tr>
<tr>
<td>HCM (pg)</td>
<td>16,3</td>
<td>16,1</td>
<td>16,0</td>
<td>15,6</td>
</tr>
<tr>
<td>VCM (fL)</td>
<td>60,8</td>
<td>59,0</td>
<td>59,9</td>
<td>59,3</td>
</tr>
<tr>
<td>CHCM (g/dL)</td>
<td>26,8</td>
<td>27,5</td>
<td>26,8</td>
<td>26,3</td>
</tr>
<tr>
<td>RDW (%)</td>
<td>11,55</td>
<td>11,3</td>
<td>11,58</td>
<td>11,63</td>
</tr>
</tbody>
</table>
Table 2: Leukogram and Plaquetogram of the hyperglycemic groups compared to the reference control group. A ≠ B ≠ C indicates statistical difference between groups (ANOVA, Turkey, p <0.05)

<table>
<thead>
<tr>
<th></th>
<th>G1</th>
<th>G2</th>
<th>G3</th>
<th>G4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leukocytes (10³/μL)</td>
<td>4.04A</td>
<td>7.04B</td>
<td>6.19AB</td>
<td>4.63A</td>
</tr>
<tr>
<td>Lymphocytes (10³/μL)</td>
<td>2.18A</td>
<td>3.36A</td>
<td>2.9A</td>
<td>2.16A</td>
</tr>
<tr>
<td>Neutrophils (10³/μL)</td>
<td>1.54A</td>
<td>3.06B</td>
<td>2.84BC</td>
<td>2.0AC</td>
</tr>
<tr>
<td>Platelets (10³/μL)</td>
<td>808.6A</td>
<td>582.3B</td>
<td>677.9AB</td>
<td>687.7AB</td>
</tr>
</tbody>
</table>

3.3 BIOCHEMICAL ANALYSIS

No significant difference was observed in the levels of total cholesterol (Figure 3), triglycerides (Figure 4) and total proteins (Figure 5) between the groups.

![Figure 3: Total cholesterol dosage graph of the animals studied after euthanasia. The results are expressed as mean and standard deviation (SD).](image-url)
Figure 4: Graph of triglyceride dosage of the animals studied after euthanasia. The results are expressed as mean and standard deviation (SD).

Figure 5: Total protein dosage graph of the animals studied after euthanasia. The results are expressed as mean and standard deviation (SD).

4 DISCUSSION AND CONCLUSION

Streptozotocin (STZ) is widely used to reproduce a type 1 diabetes model (DM1) [11]. Its molecule has similarities to the glucose molecule, causing a cytotoxic effect on pancreatic beta cells [12] [13]. In the present research, through the application of STZ it
was possible to reach a hyperglycemic state and maintain it until the end of animal experimentation.

Silva [14] developed a study in four weeks using 50 mg/kg of STZ, a single dose, which obtained a result of duration of hyperglycemia similar to this work. Several studies also indicate success in mimicking a hyperglycemic model with the use of STZ, single dose and concentrations varying between 50 to 60 mg/kg in animals, contrary to these studies, the present investigation opted for a 2-dose fractional STZ protocol [15] [16] [17] [11] [18].

One of the consequences of the decrease of insulin production by the pancreas is weight loss [19]. The hyperglycemic animals in this study showed a loss of body mass compared to the control group, in addition, polyuria and polydipsia in these groups were notable.

Santos [20] reported similar results using STZ, in which diabetic groups reduced weight in the first four weeks, maintaining weight at the end of the study. According to the review published by King [21], when DM is chemically induced, massive destruction of beta cells occurs, and as a consequence, a decrease in insulin production, resulting in hyperglycemia and weight loss. One hypothesis for this weight loss presented by diabetic groups is hyperglycemia, as a consequence of low internalization of glucose in the cells and with this breakdown of stored proteins and glycogen to obtain energy by the animal organism.

In this experiment, the administration of 15 mg/kg of SIL decreased the inflammation caused by DM, observed through the decrease in leukocytes in the G4 group. The anti-inflammatory effects have already been demonstrated in several studies that relate the production of cytokines and inhibition of oxidative stress through a mechanism dependent on nitric oxide, reducing leukocyte adhesion, and decreasing the severity of inflammation [22] [23] [24].

The administration of 10 mg/kg of SIL did not have the same anti-inflammatory effect, the animals in the G3 group had leukocyte values between the G1 and G2 groups, while the G4 group showed reference values for these cells. Silveiro [25] used SIL 10 mg/kg for the treatment of septic rats. In his research, the drug was not able to change the inflammatory state caused by the infection, corroborating the results found in this study.

On the other hand, Gokakin [26] applied SIL at doses 10 and 20 mg/kg in animal models that had undergone a burn procedure at 98 °C, in his work he proposed that the
drug at dose 10 mg / kg obtained a better anti-inflammatory result. Therefore, the dose of 15 mg/kg used in this research seems to be ideal for mediating anti-inflammatory effects.

Regarding the number of platelets, the diabetic group not treated with SIL (G2) demonstrated thrombocytopenia in relation to the other groups. The G3 and G4 groups showed no statistical difference neither from the G2 group nor from the control group (G1). The literature explains that when the organism has an inflammatory state, there is an exacerbated activation of the coagulation system, which recruits platelets and activates coagulation factors for the inflammation site of the organism [27], with a decrease in the number of platelets in laboratory findings. Thus, confirming the anti-inflammatory action of SIL, due to the G2 group having a greater number of inflammatory cells and thrombocytopenia, showing that the organism of animals in this group was experiencing a more exacerbated inflammatory process than the other hyperglycemic groups.

According to Rocha [28], diabetes increases the risk of hypercholesterolemia, with an increase in triglycerides, total cholesterol, due to the absence of insulin in the liver and adipose tissue, and in his research it was observed after the use of STZ 65 mg / kg, changes in the lipid profile of Wistar rats. Silva et al. [29], also found increased levels of triglycerides and total cholesterol, compared to the control group, in non-obese hamsters diabeticized with a dose of 50 mg / kg of STZ for ten days of experiment. However, in the present study, the hyperglycemic groups did not demonstrate a significant increase in the levels of total cholesterol and triglycerides, without generating a statistical difference with the control group.

The levels of total proteins are linked to the nutritional status of the body, then the greater nutritional deficiency, the lower protein concentration are found. Rocha [28] described in his research that the diabetes model (STZ), causes significant catabolic activity resulting in malnutrition, which can cause a decrease in the concentration of total proteins. However, the results of this study did not showed decrease in total proteins concentration, this fact can be related to the diabetization protocol with lower and fractional concentration. In addition, the difference in results may be related to the time of the experiment, considered a limitation of the study.

Finally, in view of the information presented, it is possible: the confirmation of the success obtained with the use of the streptozotocin protocol to mimic a hyperglycemic model and to maintain this model until the end of the research, the anti-inflammatory effect of SIL that was able to reduce the number of inflammatory cells in the G4 group, which underwent the process of diabetes and received SIL 15 mg/kg as treatment.
Sildenafil, with its use prior to diabetization, did not act to prevent the elevation of blood glucose by the STZ method, as animals in the G3 and G4 groups presented hyperglycemia after the application of this toxin. It is necessary to carry out research with a longer time of animal experimentation and a greater number in groups of animals with variations of three concentrations (10,15,20 mg/kg) of the drug to better elucidate the effects of sildenafil in animal models.
REFERENCES


