REPERCUSSÕES DE DIFERENTES INTENSIDADES GLICÊMICAS NO INÍCIO DO DESENVOLVIMENTO EMBRIONÁRIO DE RATAS

ALINE BUENO

Botucatu
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Dissertação apresentada à Faculdade de Medicina de Botucatu – Unesp, Programa de Pós-Graduação em Ginecologia, Obstetrícia e Mastologia. Área de concentração: Tocoginecologia, para obtenção do título de Mestre.

Orientadora: Profa. Dra. Débora Cristina Damasceno

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“Ignorar o inesperado (ainda que fosse possível) seria viver sem oportunidade, sem espontaneidade e sem os ricos momentos dos quais a vida é feita.”
(Stephen Cohen)

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Influence of hyperglycemia at different intensities on the developmental competence of early embryos: In vivo study

Short title: Influence of glycemia on the embryo competence

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Esta dissertação foi redigida de acordo com as normas de publicação da revista **Development,** para a qual será submetida.

Summary

In vitro studies suggest that maternal hyperglycemia insult impairs the early embryogenesis in the preimplantation period. In this paper, we show that streptozotocin given at birth day of life or in adulthood of rats caused hyperglycemic state. Regardless of hyperglycemic intensities (mild or severe diabetes), the embryos of these dams presented development retardation and decreased development competence. Apoptosis was detected using terminal dUTP nick end labeling (TUNEL) assays, and the morulas from mild and severe diabetic rats have a higher incidence of apoptotic cells than control embryos. Tumor necrosis factor-alpha, a cytokine whose synthesis is up-regulated in the diabetic uterus, did not alter the incidence of TUNEL-positive nuclei. The glycemic intensity is related with the increased in the apoptosis indexes in morulas. On the other hand, dams with hyperglycemia, regardless of the glycemic intensity and of the tumor necrosis factor-alpha level presented preimplantation embryos with development retardation and increase of non-viable preimplantation embryos, suggesting that the presence of the hyperglycemia leads to a decreased competence development of preimplantation embryos.

Keywords: diabetes, rat, pregnancy, embryo, tumor necrosis factor – alpha, apoptosis.

Introduction

In the presence of diabetes, metabolic disorders affect essential process in the homeostasis regulation. During pregnancy, alterations in maternal metabolism modify the intrauterine environment (Holemans et al., 2003). With this, newborns of diabetic women have increase of congenital malformations, preterm birth and neonatal or perinatal mortality (Balsells et al., 2009). Experimental studies investigating the effects of maternal hyperglycemia at term pregnancy have shown increased rates of embryonic losses and malformations (Saito et al., 2010; Damasceno et al., 2011). Some investigators suggest that adverse outcomes are due a possible maternal hyperglycemia insult in the zygote during preimplantation period. Wyman et al. (2008) showed that in vivo metabolic changes, before implantation and in early developmental stages, can impair the growth. In vitro studies suggest that abnormal energetic metabolism associated to hyperglycemia impairs the early embryogenesis in the preimplantation period (Moley, 2001; Fraser et al, 2007; Pampfer et al., 1997). Recent data demonstrated that malformation index is higher when one-cell zygote or blastocysts are transferred from severe diabetes dams to non-diabetic mouse, indicating that hyperglycemia presence during first 24 hours after fertilization already alters the embryonic development in the preimplantation period (Wyman et al., 2008). In addition, embryos culture in high glucose concentrations results in morphological, cellular and biochemical alterations. These embryos presented a rise in nuclear fragmentation index and apoptosis incidence (Pampfer et al., 1997; Vercheval et al., 1990; De Hertogh et al., 1991; Moley et al., 1998).

It is known that tumor necrosis factor-alpha TNF-alpha) is a cytokine upregulated in diabetic uterus and this condition inhibits the growth of the blastocysts in vitro to stimulate cellular apoptosis and to reduce cellular proliferation. Thus, the inappropriate liberations of TNF-alpha can induce early embryopathy associated to maternal diabetes (Pampfer 2000). On the other hand, Sinzato et al. (2011) showed that even though plasmatic levels of TNF-alpha remained unchanged during mild diabetic pregnancy, the rate of embryonic loss was higher at term. It suggests that TNF-alpha plasmatic concentrations did not directly reveal the consequences of the hyperglycemia.

Since TNF-alpha is a pro-apoptotic factor upregulated in diabetic uterus, we hypothesized that the developmental competence of early embryos can be dependent of the maternal glycemic levels. Therefore, the purpose of this investigation was to evaluate the influence of hyperglycemia at different intensities on the developmental competence of early embryos.

Materials and Methods

Animals and experimental groups

Sprague-Dawley rats (200–220g, n=30) were obtained from State University of Campinas (UNICAMP), São Paulo State, Brazil, randomly divided into 3 experimental groups: mild or severe diabetes and control. Animals were maintained under controlled temperature (22 ± 2°C) and lighting (12h light/12h dark cycle), and were allowed to have free access to food and water. Animals were treated in accordance with the Brazilian College of Animal Experimentation, and authorized by the Ethics Committee for Animal Research of our University (Brazil).

Diabetes induction

Severe diabetes (SD) was induced by a single intraperitoneal injection of streptozotocin (STZ – Sigma Chemical Company, St. Louis, Millstone, USA), at a dose of 50 mg/kg (0.1 M, pH 4.5) in adult life (90 days of life) (Yalcin et al., 2007). To obtain mild diabetes (MD), on day of birth (day 0), female newborn (NB) received a STZ injection subcutaneously administered at a dose of 100 mg/kg. Control animals received an equal volume of citrate buffer. NB remained with their mothers until day 21 of life (weaning period). Glucose tolerance test (GTT) was applied to assess the development of altered glucose metabolism and used as a criterion to maintain the rats in the respective groups (data not shown) (Sinzato et al., 2011).

Mating and pregnancy

In adult life, SD, MD and control female rats were mated overnight with non-diabetic male rats. The morning when spermatozoa were found in the vaginal smear was designated gestational day 0 (D0). After mating the females were kept in individual cages and blood glucose level was measured on D0 and D3 in the afternoon. Blood glucose concentrations were measured by a One-Touch Ultra glucosimeter (Life Scan, Johnson and Johnson®) and the values were expressed in mg/dL. In addition, GTT was performed on D3 to confirm the diabetic status during pregnancy (Sinzato et al., 2011). After an overnight fasting of 12 hours, glucose solution (200g/L) was administered into the stomach of the rats through a gastric catheter, at a final dose of 2g/kg body weight. Blood samples were obtained from a cut tip tail for glycemic determinations (glucose oxidase) at 0, 10, 20, 30, 60 and 120 minutes

(Campos et al., 2007 modified). Glucose responses during the GTT were evaluated by estimation of the total area under the curve (AUC), using the trapezoidal method (Tai, 1994).

Embryos collection and blood samples

At D4 the dams were given an overdose of sodium pentobarbital (Hypnol® 3%) for laparotomy and collection of blood samples. The blood samples were processed to obtain serum for TNF-alpha determination. This measurement was performed using specific kits to Immunoassay System (IMMULITE®). The uterus was removed between the hours 8:00 to 11:00h, and the blastocysts were collected by flushing the uterine horn with 1mL of saline/horn. The recovered embryos were counted and observed under a phase-contrast microscope to reveal any morphological abnormalities. The embryos were classified as anomalous when presented morphological alterations such as: disarrangement or no compactness of the blastomeres, irregular shape of the blastomeres large variation in cell size when compared with the control group (Damasceno et al., 1999).

Terminal transferase-mediated dUTP nick end labeling (TUNEL)

Embryos collected were submitted to the TUNEL reaction (*In Situ* Cell Death Detection Kit with Fluorescein, Roche®, Mannheim, BW, Germany), according to the technique adapted by Paula-Lopes and Hansen (2002). In summary, embryos were fixed and permeabilized. A positive control was performed by treating samples with 1 IU/μL of DNase (Promega Company, Madison, WI, USA). All samples were incubated in micro-drops of the TUNEL Kit containing 10% of the enzymatic solution (deoxinucleotidil terminal transferase enzyme) with 90% of the marking′ solution (2 -deoxyuridine ′5 -triphosphate-dUTP+fluorescein isothiocyanate-conjugated-FITC) for 1h in a humid chamber at 37°C in the dark. The negative control was incubated in micro-drops containing only marking solution. After washing, control and experimental samples stained with Hoechst 33342 were mounted in glycerol on histological slides and observed under a fluorescence microscope. Nuclei with green fluorescence (FITC) were considered TUNEL positive (fragmented DNA). Hoechst 33342 stained all healthy and apoptotic cells, based on the presence of blue fluorescence.

Statistical analysis

Gamma and Poisson distributions were performed when the data presented no homogeneous distribution (Gauss curve) such as blood glucose level, GTT, TNF-alpha

determination and mean number of cell per embryo. For comparison of the embryo development stage and TUNEL labeling cell among the groups, Chi-square test was applied.

Results

On days 0 and 3 of pregnancy, there was a significant increase in the mean glycemia values of both mild and severe diabetic rats compared with those of control rats (Figure 1). The mild diabetic group presented increased glycemic values at all timepoints measured in the GTT compared with those of control group (Figure 2A). The AUC of mild diabetic rats was higher in relation to that found in the control group (Figure 2B). In our lab, previous studies with severe diabetes showed that this group presented elevated glycemic levels and alteration at all timepoints (glycemia > 400 mg/dL) in the GTT and the AUC was increased compared with those of control and mild diabetic groups (Iessi, 2012). Subsequently, GTT and AUC were not applied in SD group in this work.

Regarding embryo developmental stages on day 5 of pregnancy (D4), we observed an increased number of morulas and embryos classified as non-viable in both diabetic groups (MD and SD) compared with those of control group. Furthermore, there was reduction in percentage of blastocysts in MD (17.1%) and SD (14.1%) groups compared with those of control group (59.0%) (Table 1).

The comparison among mean of cell total number in morulas of control, mild and severe diabetic rats D4 showed that both diabetic groups (MD and SD) presented decrease in this variable compared with that of control group. In addition, morulas recovered of SD group presented statistically reduced mean of cell total number compared with those of morulas recovered from MD group (Figure 3A).

The occurrence of cell death was analyzed using a technique that allowed for the detection of fragmented DNA (TUNEL-positive nuclei). In morulas from MD group we observed increase in TUNEL-positive nuclei index (34.6%) compared with that of control group (19.6%). Morulas from SD group showed TUNEL-positive nuclei index approximately fourfold higher in relation to that of control group. This index was also statistically increased in the SD group (77.6%) compared with that of MD group (34.6%) (Figure 3B and Figure 4)

The TNF-alpha level was increased in the SD group (4.7 pg/mL) compared with that of control group (1.06 pg/mL). No change in TNF-alpha level was observed between MD and control groups (Figure 5).

Discussion

Our results show that the glycemic levels observed in the study rats during early pregnancy were compatible with the severe and mild diabetes. The mild diabetes group presented glycemic values ranging from 120 to 300 mg/dL, while the severe diabetic group showed higher levels of hyperglycemia, in agreement with our previous studies (Sinzato et al., 2011; Damasceno et al., 2011) and others (Wentzel et al., 2008; Jawerbaum et al., 2005).

Glucose intolerance during early pregnancy was confirmed by GTT and AUC values. The rats with mild diabetes presented glycemic levels > 140 mg/dL at least at two timepoints. Previous studies carried out at our laboratory showed that GTT glycemic values were higher(> 400mg) in the severe diabetic group at all timepoints indicating not only glucose intolerance but also diabetes.

In both diabetic groups, the number of preimplantation embryos at morula stage was increased while the mean number of blastocysts was decreased, characterizing development delay. Moreover, as the number of embryos classified as non-viable increased, embryonic viability dropped.

Fraser et al. (2007) demonstrated that there is an inverse association between in vitro glucose levels and the number of expanded and hatching blastocysts, supporting the hypothesis that hyperglycemia impairs early embryogenesis in the preimplantation period resulting in embryonic loss (Moley, 1999; Wyman et al., 2008; Fraser et al., 2007).

A large number of in vitro studies have shown a relationship between high glycemia and impaired embryonic development. However, in this in vivo study, glycemia presence rather than glycemia intensity (mild or severe diabetes) had influence on the preimplantation embryo development delay and the incidence of embryos classified as non-viable.

Pampfer et al. (1997) showed a decreased total number of cells in blastocysts exposed to high glucose concentrations and/or TNF- alpha in vitro due to higher apoptosis indexes. In this study, the mean total number of cells per morula was reduced in both diabetic groups, but more markedly in the severe diabetic group. Furthermore, there was a decrease in cell number in preimplantation embryos exposed to intermediate levels of maternal hyperglycemia regardless of maternal TNF- alpha levels. The apoptosis index was much higher in the severe diabetic group than in the mild diabetic group. These findings are in line with those obtained in vitro where blastocysts were subjected to high glucose concentrations (Shen et al., 2009;

Leunda-Casi et al., 2002). Contrarily to Fraser et al. (2007), our study demonstrated that apoptosis rates in morulas increased with maternal glycemic levels, suggesting that embryonic cell viability is related to hyperglycemia intensity in vivo. On the other hand, serum TNF-alpha levels in dams with mild diabetes did not differ from those in the control group. No association was observed between the levels of this cytokine and apoptosis rates, suggesting that TNF-alpha levels in the maternal serum do not reflect the changes that occur at this stage of embryonic development. These results are in agreement with Sinzato et al. (2011), who found that although mild diabetic dams showed no alterations in TNF-alpha maternal plasma concentrations, there was increase in embryonic loss and decrease in the rate of live fetuses at the end of the pregnancy.

TNF- alpha has been demonstrated to be upregulated in the uterus of rats with higher hyperglycemia levels (> 300 mg/dL – severe diabetes) (Pampfer et al., 1997). However, the increased levels observed in this study did not correlate also with the high number of embryos that were non-viable or showed development delay. Kawamura et al. (2007) demonstrated that preimplantation embryos present stage-selective susceptibility to the detrimental effect of TNF-alpha, and that the high expression of the receptor TNFr1 occurs at the expanded blastocyst stage. Thus, they are less sensitive to high concentrations of TNF-alpha during earlier developmental stages such as two-cell, morula and young blastocyst. This may explain the lack of correlation between TNF-alpha levels and the impaired embryonic development observed in this investigation.

No correlations were found among glycemia, TNF-alpha levels and developmental stage in all study groups (data not shown), suggesting that other hyperglycemia-derived factors, such as the exacerbation of oxidative stress, may be involved. Increase in apoptotic events and impaired embryonic development are known to be associated with violations of the oxidant/antioxidant balances by high glucose concentrations (Eriksson et al., 2003, 1993; Zangen et al., 2002). Within this framework, it is noteworthy that Sivan et al. (1997) found that superoxide dismutase activity in 11.5-day-old embryos from STZ induced diabetic rats was reduced in those with neural tube defects, and normal in the embryos without neural tube defects.

Conclusion

In contrast to in vitro models, in vivo experimentation allowed us to investigate the influence of maternal hyperglycemia and several hyperglycemia-derived factors on early embryo development levels integrally. Our results show that rates of apoptosis in morulas associated with hyperglycemic intensity, whereas preimplantation embryo development delay and increase in the number of non-viable preimplantation embryos associated with diabetes; regardless of hyperglycemia intensity and TNF-alpha levels

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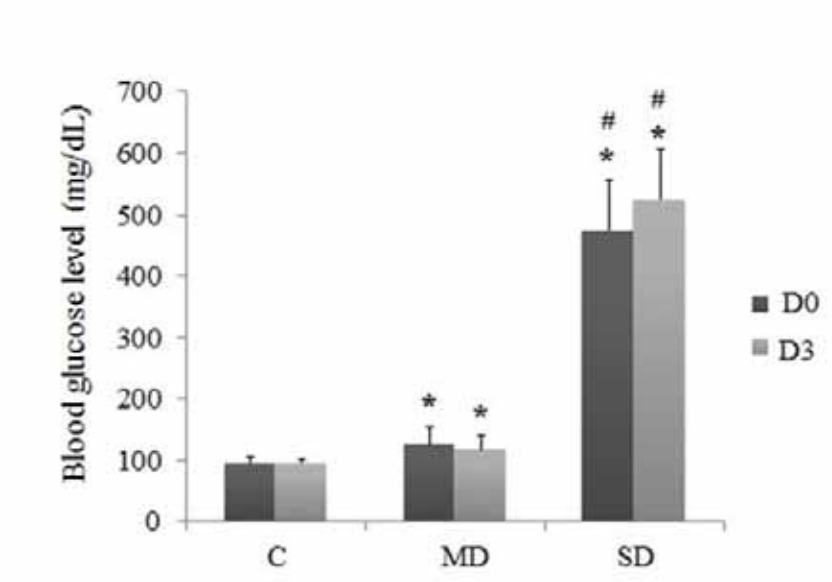


Figure 1. Blood glucose level during early pregnancy (day 0 and day 3) of mild (MD) and severe (SD) diabetic and control (C) groups. Data were reported as mean ± standard deviation.*****$p<0.05$ – compared with C group; $^{\#}p<0.05$ – compared with MD group (Gamma distribution).

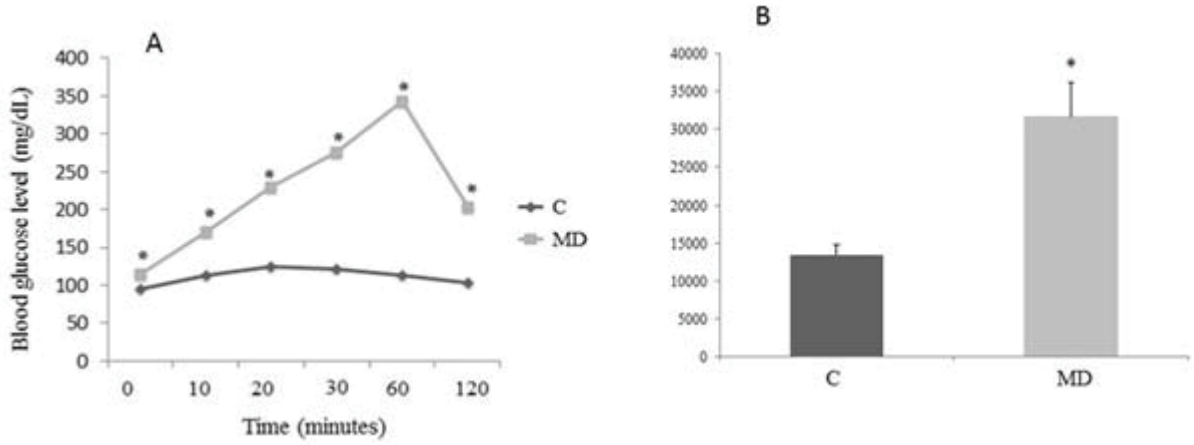


Figure 2. Oral glucose tolerance test (GTT – Figure A) and the under the curve area (AUC – Figure B) of mild diabetic (MD) and control (C) groups on day 3 of pregnancy. Data were reported as mean ± standard deviation. *****$p<0.05$ – compared with C group (Gamma distribution).

Table 1. Developmental stages of rat early embryos recovered on D4 of mild (MD) and severe (SD) diabetic and control (C) groups

	C (n=13)	**MD** (n=13)	**SD** (n=13)
Embryos recovered	100	88	99
Morulas	30/100 (30.0)	46/88* (52.2)	48/99* (48.4)
Blastocysts	59/100 (59.0)	15/88* (17.1)	14/99* (14.1)
Non-viables embryos	11/100 (11.0)	27/88* (30.7)	36/99* (36.3)

Data were reported as mean ± standard deviation.
*****$p<0.05$ – compared with C group (Chi-Square test)
(Percentage the early embryos analyzed)

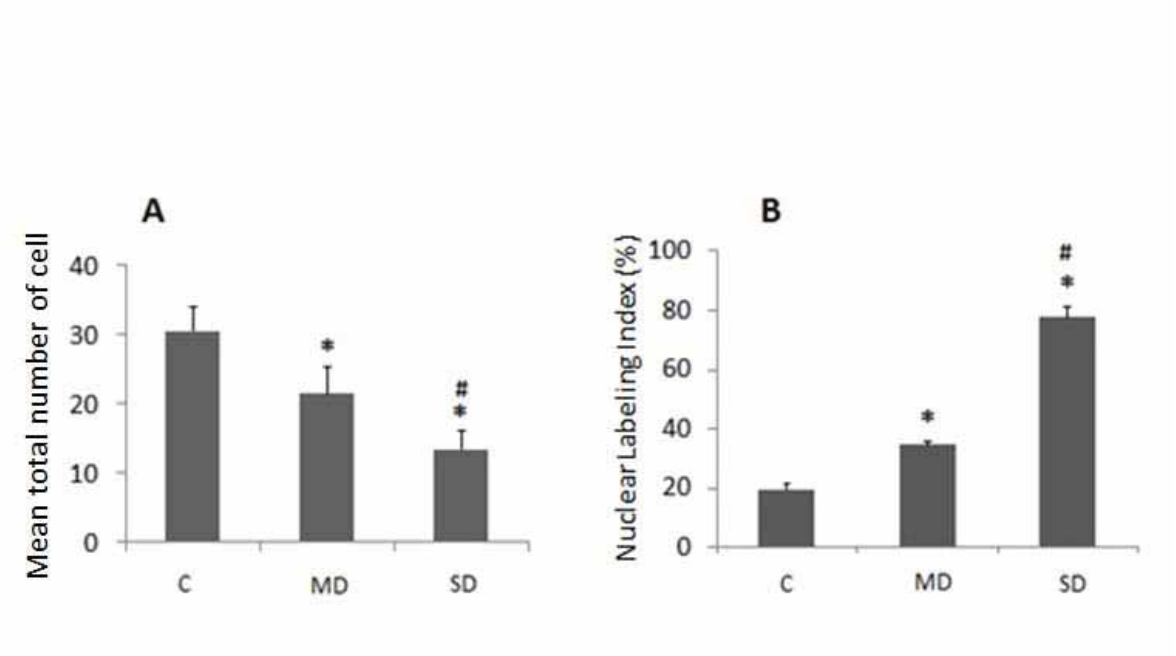


Figure 3. Mean of total number of cell (A) and TUNEL labeling cell (B) of morulas recovered on D4 of mild (MD) and severe diabetic (SD) and control (C) groups. Data were reported as mean ± standard deviation *****$p<0.05$ – compared with C group; $^{\#}p<0.05$ – compared with MD group (Figure 4A – Poisson distribution; Figure 4B - Chi-Square test).

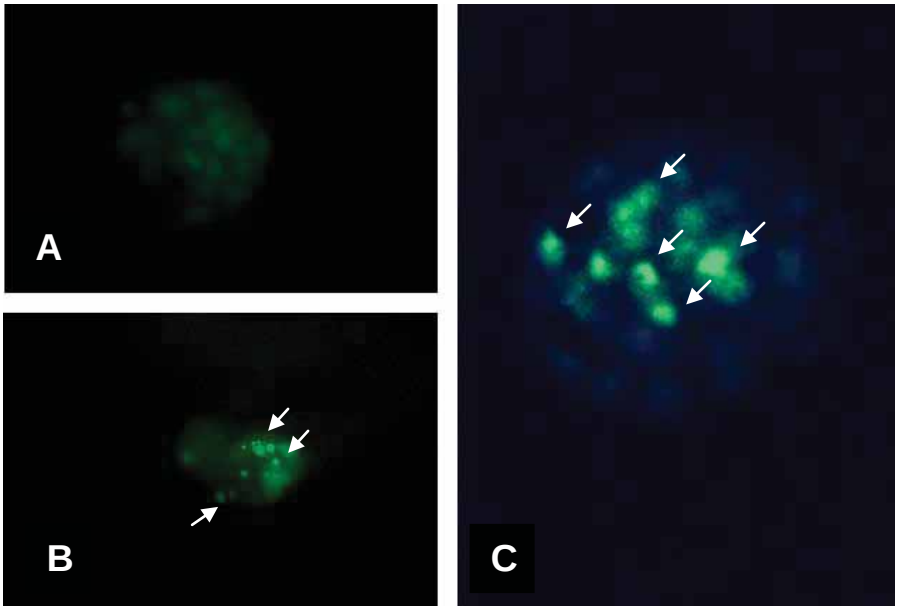


Figure 4. TUNEL staining in of morulas recovered on D4 of control, severe and mild diabetic groups. Arrows indicate TUNEL-positive nuclei in different groups.

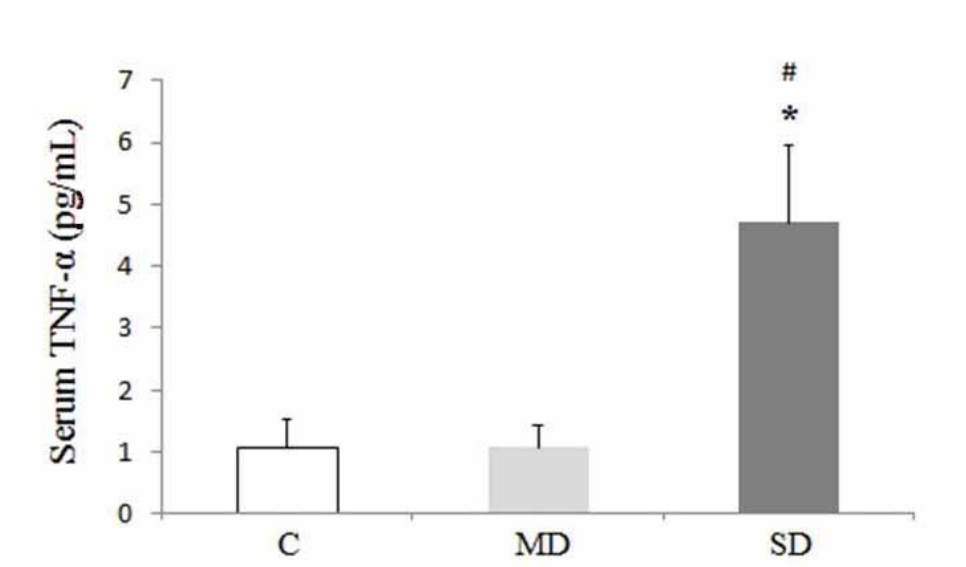


Figure 5. Serum TNF-alpha levels of mild (MD) and severe diabetic (SD) and control (C) groups. Data were reported as mean ± standard deviation. *****$p<0.05$ – compared with C group; $^{\#}p<0.05$ – compared with MD group (Gamma distribution).

Anexos

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Comissão de Ética em Experimentação Animal





Criada através da Portaria DFM nº 30 de 26/04/99

Certificado

Certificamos que o Protocolo n.º 790 sobre o projeto de pesquisa "Repercussões de diferentes intensidades glicêmicas no início do desenvolvimento embrionário de ratas", de autoria de Aline Bueno, orientada pela Profª. Drª. Débora Cristina Damasceno, com a participação de Aline de Oliveira Netto, Bruna Dallaqua, Felipe Hiroshi Saito, Iracema de Mattos Paranhos Calderon, Isabela Lovizutto Iessi e Marilza Vieira Cunha Rudge, está de acordo com os Princípios Éticos na Experimentação Animal adotado pelo Colégio Brasileiro de Experimentação Animal (COBEA), com a ressalva de que os "ratos", são provenientes de Biotério convencional, sem condições de atestar a Sanidade dos mesmos.

Projeto de Pesquisa aprovado em reunião da CEEA em 25/03/2010.

Profª Drª Regina H. Garcia Martins
Presidente da CEEA

Alberto Santos Capelluppi
Secretário da CEEA

Distrito Rubião Junior, s/nº - Botucatu – S.P. CEP: 18.618-970 Fone/Fax: (0xx14) 3811-6143 e-mail secretaria: capellup@fmb.unesp.br

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