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Evaluation of quantitative parameters of Leydig cell in diabetic adults rats

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ABSTRACT. The goal of present study was to evaluate the effects of diabetes on quantitative parameters of Leydig cells. Twelve adults Wistar rats were divided in: 1) Diabetic Group (DG), which was induced by a single intraperitoneal injection of streptozotocin (60 mg kg^{-1} of body weight); and 2) Control Group (CG), which received citrate buffer intraperitoneal. After eight weeks of diabetic induction, the animals were weighted, anesthetized and testicles were removed and routinely processed to paraffin embedded. Body weight (40%) and testicular weight (18%) of diabetic rats were significantly lower than control group. Diabetic rats showed an increase in interstitial compartment but the tubular compartment did not differ. The individual volume of Leydig cells and nuclear diameter were lower in DG. However, the population of these cells was increased. In conclusion, diabetes induced by streptozotocin in adult rats promoted alterations in testicular compartments and changes on volume, nuclear diameter and population of Leydig cells, compromising the testicular function.

Keywords: streptozotocin, testes, interstitial cells, morphometry.

Avaliação de parâmetros quantitativos de células de Leydig em ratos diabéticos adultos

RESUMO. O objetivo do presente estudo foi avaliar os efeitos do 'diabetes' nos parâmetros quantitativos de células de Leydig. Doze ratos machos adultos foram divididos em: 1) Grupo Diabético (GD) induzidos por injeção intraperitoneal única de estreptozotocina (60 mg kg^{-1} de peso corporal); e 2) Grupo Controle (GC) receberam tampão citrato, via intraperitoneal. Após oito semanas da indução, os animais foram pesados, anestesiados, os testículos foram removidos e processados rotineiramente em parafina. O peso corporal (40%) e testicular (18%) dos ratos diabéticos reduziu significativamente em relação ao grupo controle. Ratos diabéticos mostraram aumento no compartimento intersticial, mas o compartimento tubular não apresentou diferença significativa. O volume individual e o diâmetro nuclear de células de Leydig reduziram em GD. No entanto, a população dessas células aumentou. Em conclusão, o 'diabetes' induzido por estreptozotocina, em ratos adultos, promoveu alterações nos compartimentos testiculares e mudanças no volume, diâmetro nuclear e população das células de Leydig, comprometendo a função testicular.

Palavras-chave: estreptozotocina, testículo, células intersticiais, morfometria.

Introduction

Diabetes is a metabolic disorder characterized by blood glucose levels above 126 mg dL^{-1}, after fasting for at least eight hours, while normal levels are those below 100 mg dL^{-1}. Actually, the disease is classified in diabetes mellitus type 1, type 2, gestational and others specific types (SEINO et al., 2010). Type 1 diabetes occurs by deficiency in insulin production by damage in pancreatic beta cells and in type 2 diabetes, generally associated with obesity, the insulin production is normal, but there is an insulin resistance on tissues (VAN BELLE et al., 2011).

Male sexual dysfunctions have been studied in diabetic individuals; however, the mechanisms are poorly understood (SHRILATHA; URALIDHARA, 2007; RICCI et al., 2009). Experimental studies in rats reported reductions in the testicular weight (BAL et al., 2011; KIANIFARD et al., 2011; NAVARRO-CASADO et al., 2010), seminiferous epithelium height (KIANIFARD et al., 2011; MALLICK et al., 2007; NAVARRO-CASADO et al., 2010; TRINDADE et al., 2013), impaired seminiferous cell cycle (RICCI et al., 2009) and depletion of germ cells (KAPANOGLU et al., 2013; KHAKI et al., 2010; WANKEU-NYA et al., 2013).

Diabetes may affect the endocrine control of the spermatogenic process (AGBAJE et al., 2007). According to Ballester et al. (2004), lack of insulin promotes alterations in the follicle stimulating hormone levels (FSH) and indirectly reduction in the luteinizing hormone levels (LH) and androgen production.

Changes in testosterone levels in diabetic men may be related to alterations in the Leydig cell function (ARIKAWE et al., 2006; BALLESTER et al., 2004; RICCI et al., 2009; USLU et al., 2009). Ballester et al. (2005) observed that Leydig cell number, testosterone level and reproductive performance were restored in diabetic rats treated with tungstate.

The relationship between quantitative changes in Leydig cells and diabetes are not clear. Some studies have demonstrated a reduced number of these cells (ARIKAWE et al., 2006; BALLESTER et al., 2004; BALLESTER et al., 2005) while others showed a significant increase in the Leydig number (EL-ROUBY et al., 2013; HASSEN et al., 2007; SANGUINETTI et al., 1995). Therefore, the goal of the present study was to evaluate the effects of diabetes on quantitative parameters of Leydig cells.

Material and methods

Experimental design

Twelve adult male Wistar rats (*Rattus norvegicus*, var. *albinus*), 70 days of age (200-300 g), were assigned to the following groups: Diabetic Group (DG; n = 6) and Control Group (CG; n = 6). Each group had free access to pelleted food and water until the end of the experiment. They were kept in a 12 hours reverse light dark^{-1} cycle, with controlled humidity (50%) and temperature (22°C) in the vivarium of the Anatomy Department of Federal University of Pernambuco (UFPE). This study was approved by the ethics committee on animal experimentation of the UFPE (58/08 from 9/17/2008).

After fasting for 14 hours, diabetes was induced by a single intraperitoneal injection of streptozotocin (60 mg kg^{-1}) according to Silva et al. (2011). The Control Group received citrate buffer (vehicle) by the same way. Animals were considered diabetic when presented glucose levels above 200 mg dL^{-1}. The glycemic index was checked weekly and before euthanasia by blood collection from the caudal vein (Glucometer Kit- Accu Chek Active).

After eight weeks, rats were weighed, anesthetized with xylazine (5 mg kg^{-1}) and ketamine (80 mg kg^{-1}) and subjected to euthanasia (FANTONI; CORTOPASSI, 1996). All testicles were collected, weighed and fixed by immersion in 4% paraformaldehyde in phosphate buffer solution. Samples were routinely embedded in paraffin and fragments with 5 μm thickness were stained with hematoxylin-eosin. Quantitative testicular analysis was performed according to Valença et al. (2013) and the gonadosomatic index (GSI = [testicular weight/body weight] x 100]) was calculated as proposed by Caldeira et al.(2010).

Morphometric analysis

Volumetric density (%)

Volume densities of the testicular tissue components were obtained by 441-intersection grid placed in the ocular of a light microscope. Ten fields, randomly chosen (6615 points), were scored for each animal at 400X magnification. The intersection points over testicular parenchyma tissue were considered as following: tubular compartment (tunica propria, seminiferous epithelium and lumen) and intertubular space (Leydig cells, connective tissue, blood and lymphatic vessels). According to Valença et al. (2013), the volume of each testicular component (mL) was established by the product of percentage points of testicular component (volumetric density %) and testis volume (testis net weight) (Figure 1).

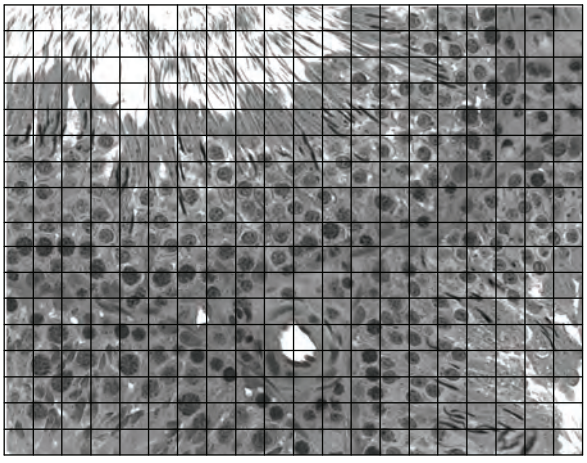

Figure 1. Schematic drawing of the method used to measure the volumetric density of testicular tissue components. Percentage points of the testicular component (volumetric density %) x testis net weight/ 100 = volume of the testicular component (ml).

Nuclear diameter of leydig cells

Thirty nuclear profiles were captured at 1000x magnification and analyzed with the software LAZ EZ 1.6.0. The nuclear diameter was obtained by means of two diametrically opposite measurements, according to Saraiva et al. (2006).

Volumetric density of leydig

The Leydig cells volumetric density (%) was measured using a reticule with 441 points (coupled in microscope) at 1000x magnification. A thousand points were counted, considering nucleus and cell cytoplasm. After this, the individual volume of the Leydig cell was determined with mathematical models, using the values of nuclear diameter and proportion in the testis.

Nuclear volume = 4/3 πR^3 R = nuclear radius;

Cytoplasmic Volume = % cytoplasm x nuclear volume/ % nucleus;

Cell Volume = nuclear volume + cytoplasmic volume;

The Leydig cell population was obtained using the values of individual volume of Leydig cell, volumetric density (%) and total volume (mL) occupied by Leydig cells in the testes.

Statistical analysis

Data were analyzed using t-student or Mann-Whitney tests in the program Sigmastat 3.5 after testing for normality. Data were expressed as mean (±) standard deviation and median (minimum and maximum values). All statistical analysis was outlined for $p < 0.05$.

Results

Glycemic index, body weight, testicular weight and gonadosomatic index

The diabetes induced by streptozotocin was efficient since the average glycemic level in the DG [526.5 (439-579) mg dL^{-1}] increased 84% ($p = 0.002$) when compared with CG [85.5 (83-90) mg dL^{-1}].

According to Figure 2, adult male rats with high glucose levels presented reduction in body (263 g ± 20.8; $p \leq 0.001$) and testicular weight [1.422 (0.992-1.660) g; $p = 0.026$] when compared with the control group [BW = 427 g ± 22.01; TW = 1.708 (1.693-1.786) g]. There was no significant difference in the gonadosomatic index between experimental groups, even though this index was higher in diabetic rats (1.05 % ± 0.146) than in non-diabetic rats (0.81% ± 0.313).

The volumetric density of the seminiferous tubule in DG (92.06% ± 0.699) did not differ from CG (93.06% ± 0.298). However, intertubular space increased in the diabetic group (8.34% ± 0.499; p= 0.007) when compared with non-diabetic animals (6.46% ± 0.256) (Figure 3).

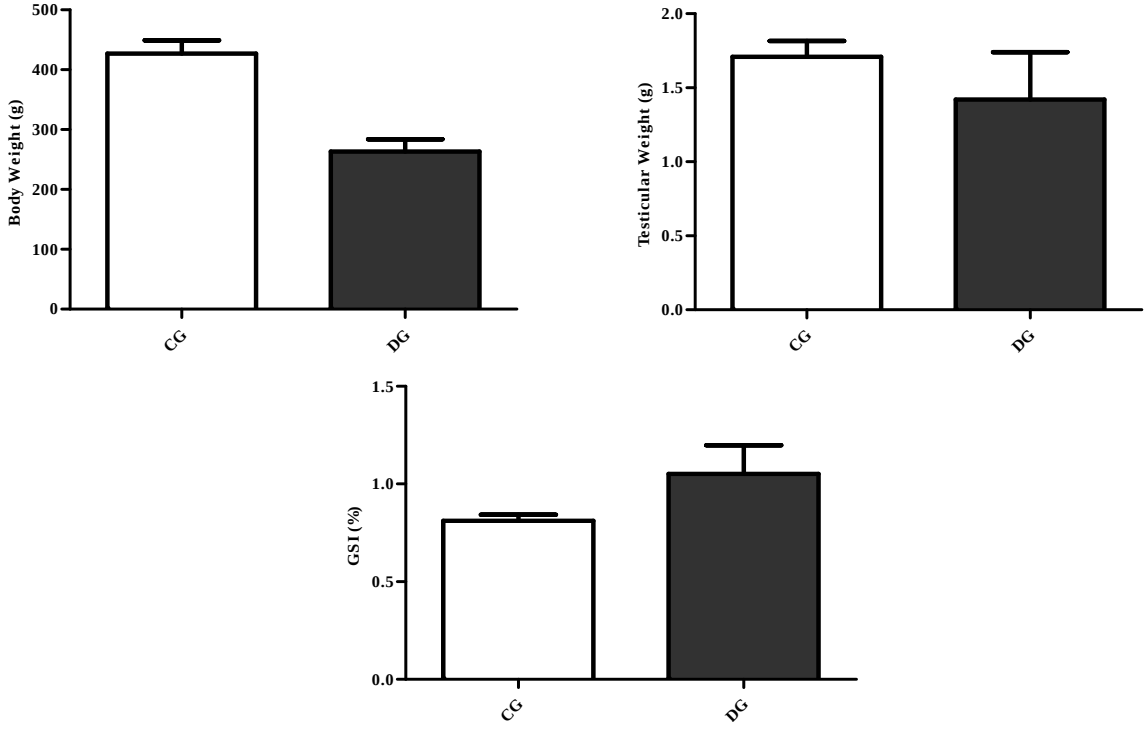

Figure 2. Body weight (g), testicular weight (g) and gonadosomatic index (GSI) in adult rats of control and diabetic groups. Average and standard deviation ($p < 0.05$).

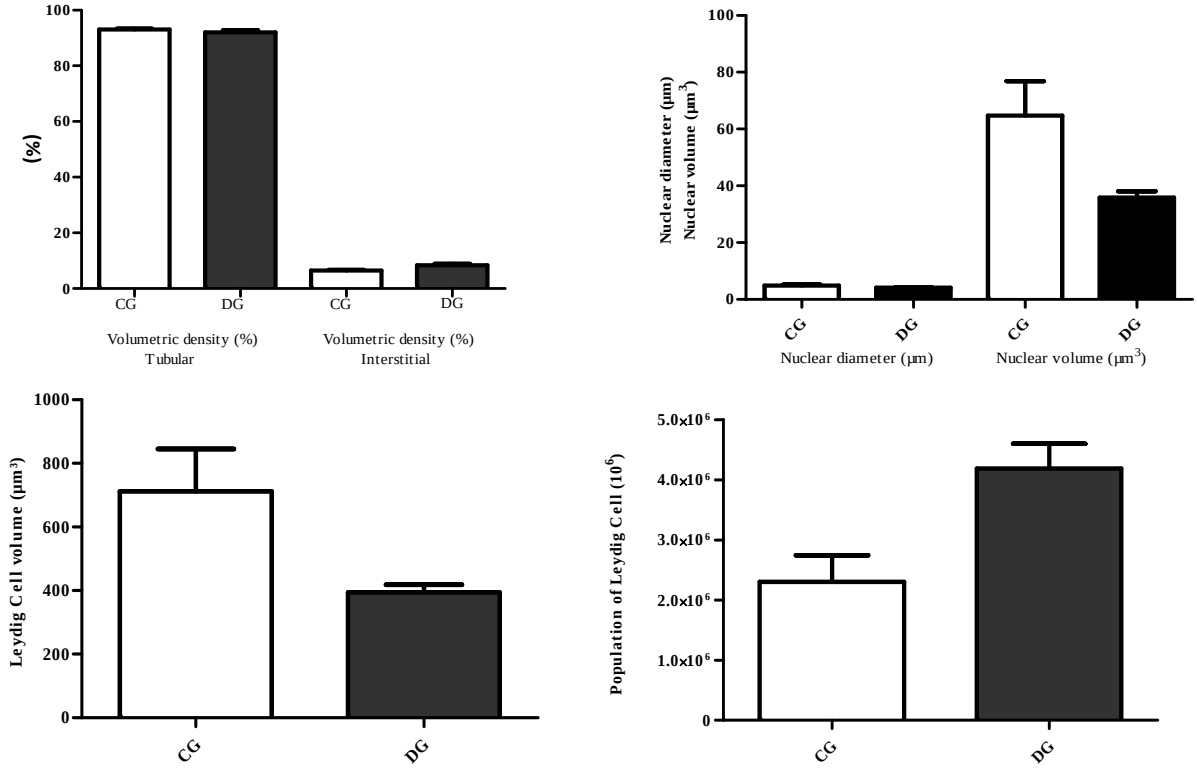

Figure 3. Volumetric density of testicular compartments (%), nuclear diameter (μm), nuclear volume (μm^3), volume (μm^3) and population of Leydig cells in adult rats of Control and Diabetic groups. Mean and standard deviation ($p < 0.05$).

Nuclear diameter, individual volume and population of leydig cells

According to Figure 2, Leydig cells nuclear diameter (μm), nuclear volume (μm^3) and cell volume were decreased in DG (ND = 4.08 μm ± 0.08; p = 0.033; NV = 35.88 μm^3 ± 2.18; p = 0.041; CV = 394.7 μm^3 ± 24.03; p = 0.041) when compared to CG (ND = 4.88 μm ± 0.312; NV = 64.74 μm^3 ± 12.1; CV = 712.1 μm^3 ± 133.1). The population of Leydig cell per gram of testis was 82 % higher in DG (418.6 ± 41.2 x 10^6; p = 0.01) than CG (230.3 ± 44.1 x 10^6).

The interstitial compartment is illustrated in Figure 4, which presents the arrangement of Leydig cells in animals of the control and diabetic groups.

Discussion

Diabetes is a metabolic disorder associated with some characteristic symptoms as glycosuria, weight loss and polydipsia. Over time, high glucose levels promotes various complications including retinopathy, neuropathy, nephropathy, cardiovascular symptoms and sexual dysfunction (SEINO et al., 2010; VAN BELLE et al., 2011).

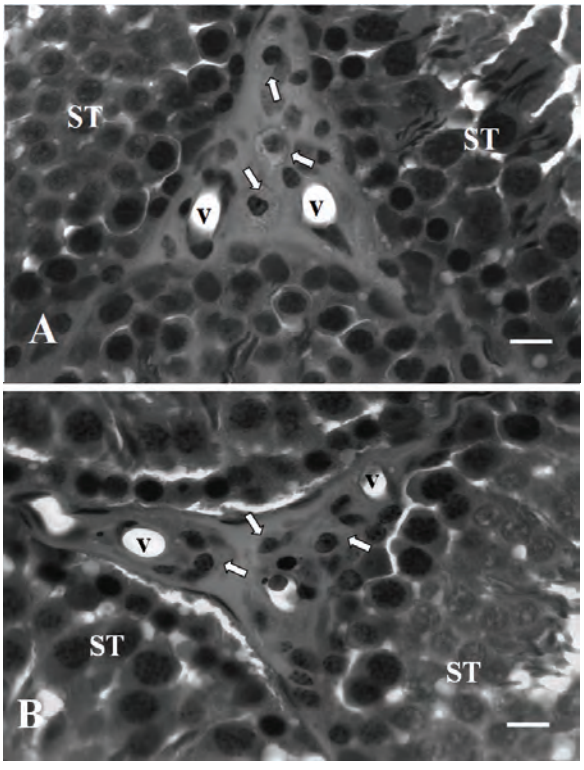

Figure 4. Photomicrographs for section of testis of the control group (A) and diabetic group (B) presenting the interstitial compartment and presence of Leydig cells (white arrows). Seminiferous tubule (ST); Blood vessel (V). Bar: 8.67 μm.

In the present study, the body weight reduction observed in diabetic animals corroborated Ricci et al. (2009). This reduction can be related to excessive urinary water and electrolytes loss (CHIASSON et al., 2003). Furthermore, low levels of insulin increases fat mobilization by activation of lipases, promoting triglycerides breakdown and release of free fatty acids (CHIASSON et al., 2003).

Testicular weight is a parameter related to seminiferous tubules length, Sertoli cells population and sperm production (FRANÇA; RUSSELL, 1998). The testicular weight reduction (22.6%) observed in the current study corroborate previous studies in diabetic animals (BALLESTER et al., 2004; KHAKI et al., 2010; KIANIFARD et al., 2012; YULUG et al., 2013).

The gonadosomatic index (GSI = [testicular weight/ body weight] x 100) indicates the percentage of body weight allocated to the testis (CALDEIRA et al., 2010). In the current experiment, the GSI did not differ between groups, but in diabetic animals, the reduction in body and testicular weight was proportional, justifying the absence of difference. However, the GSI of the diabetic rats tended to increase, as previously described by Kianifard et al. (2011).

In this study, no change was observed in the tubular compartment between groups. Nevertheless, Ricci et al. (2009) observed reduced size and atrophy of seminiferous tubules in diabetic animals. These changes are probably related to reduction in FSH levels since low insulin levels affect directly testosterone and FSH levels (BALLESTER et al., 2004; KIANIFARD et al., 2012).

In the current study, the interstitial compartment was increased (29 %) in diabetic rats. This improvement may be related with increased thickness in the walls of blood vessels and higher deposition of extracellular matrix, observed in diabetics (BALLESTER et al., 2004; HASSEN et al., 2007).

Population of Leydig cells per gram of testis and individual volume vary among species. In mammalians, the volume of each Leydig cell ranges from 1000 μm^3 to 2000 μm^3 as well as the number per testis (FRANÇA; RUSSEL, 1998).

Smooth endoplasmic reticulum (SER) is the most extensive organelle of Leydig cells (SHIRAI et al., 2013). The individual volume reduction of Leydig cells observed in the current study could be related to reduction of SER in the Leydig cells of the diabetic rats (KIANIFARD et al., 2012). For instance, Leydig cells function can be impaired since endoplasmic reticulum is directly related to androgen production (HASSEN et al., 2007). Furthermore, the low levels of LH and testosterone observed in diabetics by Ballester et al. (2004) can be related to the reduction of Leydig cells volume in this study. Therefore, these hormonal alterations influence the mechanisms related to proliferation, differentiation and function of Leydig cells, promoting changes in the number of these cells (KHAKI et al., 2010; NASROLAHI et al., 2013; SCHOELLER et al., 2012).

The Leydig cells number was increased in diabetic rats in the present study and corroborates Sanguinetti et al. (1995), Hassen et al. (2007) and El-Rouby et al. (2013). However, other studies pointed out a reduction in the number of these cells (ARIKAWE et al., 2006; BALLESTER et al., 2004; BALLESTER et al., 2005). On the other hand, Cameron et al. (1985) found no structural differences, in the number or function of Leydig cells. El-Rouby et al. (2013) mentioned that this variation between studies might be related to various factors as induction protocol, duration of the experiment and differences in species.

Conclusion

In conclusion, streptozotocin-induced diabetic rats show changes in the volumetric density between testicular compartments. In addition, absence of normal insulin levels affects the individual volume and population of Leydig cells.

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