

Effects of hyperglycemia on sperm and testicular cells of Goto-Kakizaki and streptozotocin-treated rat models for diabetes

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Abstract

Diabetes mellitus is a degenerative disease that has deleterious effects on male reproductive function, possibly through an increase in oxidative stress. This study was conducted in order to clarify the mechanisms by which oxidative stress influences animal models for both type 1 (streptozotocin-treated rats, STZ) and type 2 (Goto-Kakizaki (GK) rats) diabetes. We determined the extent of lipid peroxidation, protein oxidation, lactate levels, adenine nucleotides, adenylate energy charge and the activity of glutathione peroxidase, glutathione reductase and lactate dehydrogenase, in isolated testicular cells of control and diabetic rats. We have also correlated these parameters with sperm count and motility. Sperm concentration and motility were decreased in STZ-treated rats. ATP levels were lower in rats treated with STZ for 3 months, in contrast to GK and rats treated with STZ for 1 month, suggesting an adaptative response. STZ-treated rats showed increased lipid peroxidation after 1 week and 3 months of treatment. Glutathione reductase (G-red) activity was found to be higher in GK rats. Glutathione peroxidase activity was lower in GK and rats treated with STZ for 1 month, which is in accordance with the proposal of functional recovery in these animals. We conclude that hyperglycemia has an adverse effect in sperm concentration and motility via changes in energy production and free radical management. Furthermore, both animal models, particularly GK rats and rats treated with STZ for 1 month, present some metabolic adaptations, increasing the efficiency of mitochondrial ATP production, in order to circumvent the deleterious effects promoted by the disease.

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1. Introduction

About 90% of diabetic patients have disturbances in sexual function, including a decrease in libido, impotence and infertility, in the latter case due to testicular

dysfunction associated with sustained hyperglycemia [1,2]. Diabetes mellitus (DM) is a heterogeneous metabolic disorder characterized by hyperglycemia resulting from defective insulin secretion, resistance to insulin action or both [3]. Type 1 diabetes is the consequence of an autoimmune-mediated destruction of pancreatic β -cells, leading to insulin deficiency, while type 2 diabetes is characterized by insulin resistance and relative, rather than absolute, insulin deficiency [4]. Growing evidence indicates that oxidative stress is

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increased in diabetes, due to the overproduction of reactive oxygen species (ROS), and decreased efficiency of antioxidant defences [5,6]. Oxidation of lipids, proteins and other macromolecules (such as DNA) occurs during the development of diabetes [7]. Mitochondrial DNA mutations have also been reported in diabetic tissues, suggesting oxidative stress-related mitochondrial damage [8].

Hyperglycemia leads to the increased production of free radical intermediates via at least four different routes: increased glycolysis; intercellular activation of the sorbitol (polyol) pathway; auto-oxidation of glucose and non-enzymatic protein glycation [9,10]. To control the flux of ROS, aerobic cells have developed an antioxidant defense system, which includes enzymatic and non-enzymatic components [11]. The antioxidant system consists of low molecular weight antioxidant molecules and various antioxidant enzymes [12–15]. Glutathione (GSH), the most prevalent low-molecular weight antioxidant peptide, and GSH-related antioxidant enzymes, such as glutathione peroxidase (G-Px), glutathione reductase (G-Red) and glutathione *S*-transferase (GST), are the most important enzymes of this cellular antioxidant system. G-Px catalyses the reduction of hydrogen peroxide and organic hydroperoxides by transferring electrons from GSH, thus forming GSSG. In turn, GSSG is then reduced back to GSH by G-Red [16]. The stability and capacity of antioxidant status during chronic diabetes seriously influences the outcome of the long-term complications caused by oxidative stress [17].

Mammalian sperm cells present a specific lipidic composition, with a high content of polyunsaturated fatty acids, plasmalogens and sphingomyelins. The lipids in spermatozoa are the main substrates for peroxidation, and Aitken et al. [18] showed that excess amounts of ROS and free radicals have adverse effects on sperm motility and fertility. Furthermore, oxidative damage to lipids and DNA of spermatozoa is associated with declining motility and diminished fertility of human sperm [19–21]. However, the mechanisms of altered spermatogenesis in diabetic men are poorly understood. In this regard, animal models for diabetes are important research tools, since they provide insights which are almost impossible to duplicate in human populations [22,23]. In the present study, we used STZ-induced diabetic rats as models for type 1 diabetes and Goto-Kakizaki (GK) rats as models for type 2 diabetes. STZ-induced diabetic rats are obtained after selective destruction of β cells by streptozotocin (STZ), an antibiotic with diabetogenic effects. STZ-injected rats present many characteristics seen in insulin-dependent diabetic human patients, such

as hypoinsulinemia, hyperglycemia, ketonuria, and hyperlipidaemia [23]. On the other hand, Goto-Kakizaki (GK) rats are currently used as an animal model for type 2 diabetes. This animal is a non-obese, spontaneously diabetic rat [22], produced by selective breeding of Wistar rats, using glucose intolerance as a selection index [24–27]. GK rats exhibit a moderate but stable fasting hyperglycemia, which does not progress to a ketotic state. Furthermore, the GK rat is one of the best characterized animal models of spontaneous non-obese type 2 diabetes mellitus, since it exhibits similar metabolic, hormonal, and vascular disorders as the human disease [22]. Furthermore, in the initial stages of diabetes, GK rats do not exhibit severe complications associated with the disease, constituting an important model to study the initial events of diabetes [28].

In the present work, we have tried to elucidate the mechanisms by which diabetes and associated oxidative stress influence testicular and sperm function. For this purpose we have determined the extent of lipid peroxidation, protein oxidation, activity of glutathione peroxidase and reductase, lactate and pyruvate levels, internal and external energetic charge and lactate dehydrogenase release, in isolated testicular cells of control and diabetic rats. We have also correlated these parameters with epididymal sperm count and sperm motility.

2. Materials and methods

2.1. Materials

All chemicals were obtained from Sigma, St. Louis, MO, USA.

2.2. Animals and blood glucose determination

2.2.1. GK rats

Spontaneously diabetic male GK rats, 3 months of age ($n = 23$), were obtained from our local breeding colony (Animal Research Center Laboratory, University Hospitals, Coimbra), established in 1995 with breeding couples from the colony at the Tohoku University School of Medicine (Sendai, Japan; courtesy of Dr. K. Susuki). Control animals were non-diabetic male Wistar rats of similar age ($n = 23$). Animals were kept under controlled light (12-h day/night cycle), temperature (22–24 °C) and humidity (50–60%) conditions and with free access to powdered rodent chow (diet AO4, Panlab, Spain) and water (pH 5.5), except during the fasting periods. In this study, the “Principles of Laboratory Animal Care” (NIH publication 83-25,

revised 1985) were followed. Glycemia was determined from the tail vein using a commercial glucometer (Glucometer-Elite, Bayer).

2.2.2. STZ rats: induction and characterization of STZ-Induced diabetes

Male Wistar rats weighing about 200 g (2 months) were randomly divided into two groups of 10 animals each. In order to induce diabetes, one group was injected intraperitoneally with a single injection of streptozotocin (STZ, 50 mg/kg), after a 16-h fasting period. The volume used was always 0.5 ml/200 g body weight. Streptozotocin [2-deoxy-2-(3-methyl-3-nitrosurea) 1-D-glucopyranose] was prepared prior to use in 100 mM citrate, pH 4.5. In the following 24 h, animals were orally fed with glycosilated serum in order to avoid hypoglycemia resulting from massive destruction of β -cells, and release of intracellular insulin associated with STZ treatment [23]. Animals were kept for 1 week ($n = 13$), 1 month ($n = 21$) and 3 months ($n = 16$) before the experiments. During this period, glycemia was determined from the tail vein. Values were taken in fasting conditions just before STZ administration, and in non-fasting conditions in the following weeks. If feeding blood glucose in the tail vein exceeded 250 mg/dl, animals were used as diabetic. Control animals were non-diabetic male Wistar rats of similar age (3 months, $n = 23$ and 5 months $n = 14$), sham injected with the same volume of citrate solution.

2.3. Rat sperm isolation

After animal sacrifice by cervical dislocation, testes were removed with adherent epididymis and vas deferens. Epididymis and vas deferens were separated from testis and were put into 2 ml prewarmed rat sperm isolation medium containing 17 mM NaHCO_3 , 96.4 mM NaCl; 4.76 mM KCl; 21.58 mM Na-lactate; 5.56 mM glucose; 1.71 mM CaCl_2 ; 1.19 mM KH_2PO_4 ; 1.19 mM MgSO_4 ; 0.5 mM Na-pyruvate; 4.0 mg/ml BSA; 50 μg /ml streptomycin; 75 μg /ml penicillin; 20 mM HEPES; 5 mg/ml phenol red; pH 7.3. Sperm was forced out of the vas deferens with fine forceps by putting pressure on the lower region of the cauda epididymis and “walking” the forceps down the vas deferens, not forcing out excess material, i.e., immature cells. As soon as sperm was released, 10 μl of the concentrated sperm was drawn up and transferred to a coverslip and spermatozoa motility and viability were evaluated. To immobilize spermatozoa, sperm was diluted twice in water and counted using a hemocytometer with Neubauer ruling (400 sq/mm²) using a bright field microscopy. We observed 100 spermatozoa for each sample in all assays.

2.4. Isolation of testicular cells

Testicular cells were isolated from adult rat testes using previously described methodology [30]. The testes were dissected into a Petri dish with EKRB (enriched Krebs–Ringer bicarbonate) medium containing 120.1 mM NaCl, 4.8 mM KCl, 25.2 mM NaHCO_3 , 1.2 mM KH_2PO_4 (pH 7.2), 1.2 mM $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 1.3 mM CaCl_2 , supplemented with 11.1 mM glucose, 1 mM glutamine, 10 ml/l MEM essential amino acid solution (Sigma), 10 ml/l BME nonessential amino acid solution (Sigma), 100 mg/ml streptomycin, 100 U/ml penicillin (K salt) and 20 mM HEPES. Dry collagenase was then added at a final concentration of 0.5 mg/ml, and the testes were incubated for 15–45 min at 32 °C with gentle stirring. Once the seminiferous tubules were dispersed in the medium they were allowed to settle at the bottom of the dish, and the medium was aspirated and discarded. The tubules were then placed in fresh EKRB containing 1 mg/ml DNase I and 0.25 μg /ml trypsin and incubated for 15–45 min with stirring and gentle pipeting. After that, trypsin inhibitor was added to avoid destruction spermatogenic cells. Released spermatogenic cells were pelleted by centrifugation (10 min at 500 rpm in a Sorval RT6000 refrigerated centrifuge) and washed twice in EKRB before being submitted to the next protocol. Round testicular cells were counted by using a hemocytometer with Neubauer ruling (400 sq/mm²) using a bright field microscopy.

2.5. Determination of protein concentration

The concentration of testicular proteins was estimated according the Sedmak method [31] using BSA (bovine serum albumin) as a standard.

2.6. Analysis of adenine nucleotides

Intracellular adenine nucleotides (ATP, ADP and AMP), from testicular cells were determined after cell extraction with 0.6 M perchloric acid supplemented with 25 mM EDTA-Na. Cells were centrifuged at 14,000 rpm in an Eppendorf 5417R centrifuge for 2 min, at 0–4 °C. The supernatants were neutralized with drop wise addition of 3 M KOH in 1.5 M Tris and centrifuged at 14,000 rpm for 2 min, at 4 °C. The resulting supernatants were assayed for internal ATP, ADP and AMP by separation in a reverse-phase high-performance liquid chromatography (HPLC) according to a previously described procedure [32]. The chromatography apparatus was a Beckman-System Gold, consisting of a 126 Binary Pump Model and 166 Variable UV detector controlled by

a computer. The detection wavelength was 254 nm, and the column was a Lichrosphere 100 RP-18 (5 μm) from Merck. An isocratic elution with 100 mM phosphate buffer (KH_2PO_4 ; pH 6.5) and 1.0% methanol was performed with a flow rate of 1 ml/min. Peak identity was determined by following the retention time of standards. Adenylate energy charge (AEC) was determined according the following formula: $\text{ATP} + 0.5 \times \text{ADP} / (\text{ATP} + \text{ADP} + \text{AMP})$.

2.7. Lactate and pyruvate levels

Lactate and pyruvate levels were determined using commercial ELISA-based assay kits for enzymatic quantification of lactate (# LC2389, RANDOX, UK) and UV quantification of Pyruvate (# 8825, BEN srl, Italy), respectively. Both kits were used in accordance with the manufacturers instructions.

2.8. Oxidative stress parameters

2.8.1. Measurement of lipid peroxidation

The extent of lipid peroxidation was determined by measuring the amount of thiobarbituric acid reactive species (TBARS) formed, according to a modified procedure [33]. To measure lipid peroxidation, 500 μl of peroxidation reagent containing 18.8 ml of cold 40% trichloroacetic acid (TCA), 0.375% of thiobarbituric acid (TBA), 0.25 M HCl and 6.8 mM BHT (2,6-diterbutyl-4-metilphenol) were added to 500 μl of the test material. The mixture was heated at 100 $^\circ\text{C}$ during 10 min, and was allowed to cool in ice before a 10 min centrifugation at 3000 rpm in a Sorval RT6000 refrigerated centrifuge. The supernatant was collected and TBARS were spectrophotometrically quantified at 530 nm, against a blank prepared under similar conditions, but in the absence of sample. The amount of TBARS formed was calculated using a molar extinction coefficient of $1.56 \times 10^{-5} \text{ M}^{-1} \text{ cm}^{-1}$ for thiobarbituric acid and expressed as nmol TBARS/mg protein [34].

2.8.2. Analysis of carbonyl groups

The accumulation of oxidized proteins can be evaluated by the content of carbonyl groups that can be spectrophotometrically quantified via reaction with DNPH (2,4-dinitrophenylhydrazine) at 360 nm, [35]. Cells were centrifuged at 14,000 rpm for 6 min, at 0–4 $^\circ\text{C}$. The resulting pellet was solubilized in 1 ml TCA (trichloroacetic acid) and centrifuged (10,000 rpm, 3 min) in an Eppendorf 5417R centrifuge. The sediments were incubated with 500 μl of 10 mM DNPH (freshly prepared in 2 M HCl, and kept in the dark) for 1 h at room

temperature, with vortex agitation every 10 min. Subsequently, 500 μl of 20% TCA was added and samples were centrifuged at 11,000 rpm, for 3 min. The supernatant was decanted and the pellet mixed with 1 ml of a 1:1 ethanol: ethyl acetate solution. The pellet was then incubated with 900 μl of 6 M guanidine (prepared in PBS, pH 6.5), for 15 min at 37 $^\circ\text{C}$ and again centrifuged (10,000 rpm, 3 min). The supernatant was collected and protein oxidation was estimated spectrophotometrically at 360 nm. For all samples a blank was prepared, which was incubated with 2 M HCl instead of DNPH. The carbonyl content was calculated using a molar extinction coefficient of $22 \text{ mM}^{-1} \text{ cm}^{-1}$ for DNPH, and was expressed as nmol DNPH/mg protein.

2.9. Enzymatic activities

Before performing the enzymatic assays cells were lysed with 15 mM Tris (pH 7.4) and submitted to three cycles of freezing/thawing.

2.9.1. Measurement of lactate dehydrogenase release

The viability of testicular cells was assessed by monitoring the leakage of cytosolic lactate dehydrogenase (LDH) to the extracellular medium. LDH activity was measured spectrophotometrically according to a previously described procedure [36], following the rate of conversion of reduced nicotinamide adenine nucleotide (NADH) to oxidized form (NAD^+). The enzymatic assay was performed in the supernatant after cell centrifugation at 14,000 rpm during 6 min in an Eppendorf 5417R centrifuge. The reaction occurred at 30 $^\circ\text{C}$, with constant magnetic stirring, in a cuvette with 200 μl of sample, 1500 μl of 0.244 mM NADH and 150 μl of 9.76 mM pyruvate. Pyruvate and NADH solutions were prepared in Tris/NaCl buffer (Tris 81.3 mM/NaCl 203.3 mM, pH 7.2). The control blank lacked pyruvate. Lactate dehydrogenase activity was calculated by monitoring the absorbance change/min of NADH at 340 nm, using a molar extinction coefficient of $0.63 \text{ l nmol}^{-1} \text{ mm}^{-1}$ for NADH, and its activity was expressed as nmol NADH/min/mg protein.

2.9.2. Glutathione reductase

Glutathione reductase (G-Red) activity was determined according to a modified method [37]. G-Red catalyses the reduction of oxidized glutathione (GSSG) to reduced glutathione (GSH) in the presence of NADPH. Briefly, the enzymatic assay was performed in the supernatant following cell lysis. Each sample (100 μl) was incubated with 500 μl of phosphate buffer

(containing 0.2 M of K_2HPO_4 and 2 mM EDTA, pH 7.0) supplemented with 50 μ l of 2 mM NADPH and 350 μ l H_2O . The measurements were initiated with the addition of 50 μ l GSSG (20 mM), for 400 s, at 30 °C with continuous magnetic stirring, against blanks prepared in absence of GSSG in a Jasco, V-560, UV-vis spectrophotometer. Glutathione reductase activity was calculated by the absorbance change per minute of NADPH at 340 nm, using a molar extinction coefficient of 6220 $cm^2 mol^{-1}$, and its activity was expressed as nmol NADPH/min/mg protein.

2.9.3. Glutathione peroxidase

Glutathione peroxidase (G-Px) activity was determined spectrophotometrically [38]. In this method, G-Px catalyses the oxidation of glutathione in the presence of *tert*-butyl hydroperoxide (*t*-BHP). Oxidized glutathione is converted to the reduced form in the presence of glutathione reductase and NADPH, while NADPH is oxidized to $NADP^+$. Briefly, the enzymatic assay was performed in the supernatant after cell lysis and centrifugation (as above). Each sample (30 μ l) was incubated for 5 min, in the dark, with 100 μ l of phosphate buffer (containing 0.25 mM KH_2PO_4 , 0.25 mM K_2HPO_4 and 500 μ M EDTA, pH 7), 100 μ l GSH (freshly made and protected from light), 100 μ l glutathione reductase (1 unit), and 460 μ l H_2O . The quantification occurred after the addition of 100 μ l of NADPH (2.5 mM) and 100 μ l of *t*-BHP, at 30 °C with continuous magnetic stirring. The measurements were made against blanks prepared in the absence of NADPH in a Jasco, V-560, UV-vis spectrophotometer. Glutathione peroxidase activity was calculated by the absorbance change per minute of NADPH at 340 nm, using a molar extinction coefficient of, 6220 $cm^2 mol^{-1}$, and its activity was expressed as nmol NADPH/min/mg protein.

2.10. Statistical analysis

All statistical analyses were done using the SPSS (Statistical Package for the Social Sciences Program), version 12.00, software for windows (SPSS Inc., Chicago, IL, USA). All variables were checked for normal distribution. Results are presented as mean \pm S.E.M. of the number of experiences indicated and statistical significance between diabetic and control rat groups was accessed using the Mann-Whitney *U*-test. Multiple comparisons were performed using one-way ANOVA, followed by the post hoc Tamhanes and $p \leq 0.05$ was considered significant. Bivariate correlation (*r*) in different groups was

evaluated by calculating the Pearson correlation coefficient with a two-tailed significance (*p*).

3. Results

3.1. Glycemia

Blood glucose (non-fasting) levels, were significantly higher in diabetic rats when compared to respective controls ($p < 0.001$). However, GK rats presented mild hyperglycemias (110–330 mg/dl), while STZ-treated rats presented severe hyperglycemias (>293 mg/dl), $p < 0.001$ (Fig. 1).

3.2. Epididymal sperm count and motility

An effect of age was noted in control Wistar rats, with 5-month-old rats (controls for rats treated with STZ for 3 months) showing higher sperm counts and motility than 3-month-old rats (controls for all other experimental groups) ($p < 0.01$). Rats treated with STZ for 1 and 3 months showed significantly lower sperm count and motility than controls. Interestingly, sperm of rats treated with STZ for 1 week showed similar motility to that observed in Wistar controls (data not shown). Since sperm mature for several days to weeks in the epididymis [39], most spermatozoa analysed in this case are probably already epididymal at the time of

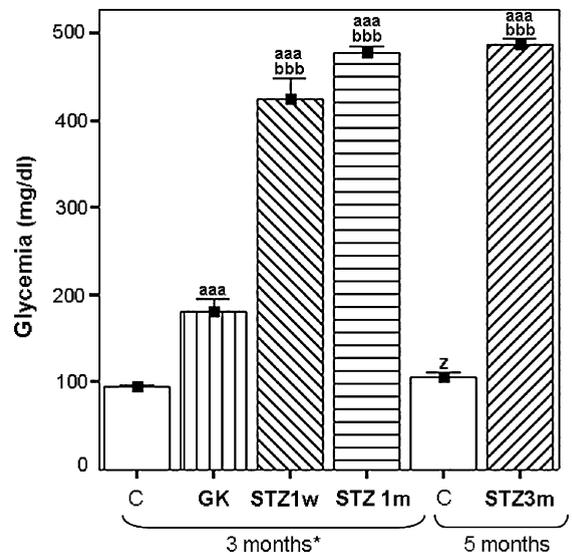


Fig. 1. Glycemia in diabetic and Wistar control rats. Glycemia was determined as described in Section 2. Data is expressed as means \pm S.E.M. Statistical significance: (z) $p < 0.05$ compared with 3-month-old Wistar controls, (aaa) $p < 0.001$ compared with Wistar control rats, (bbb) $p < 0.001$ compared with GK rats. Three months*: rats treated with STZ for 1 week are 2 months and 1 week old.

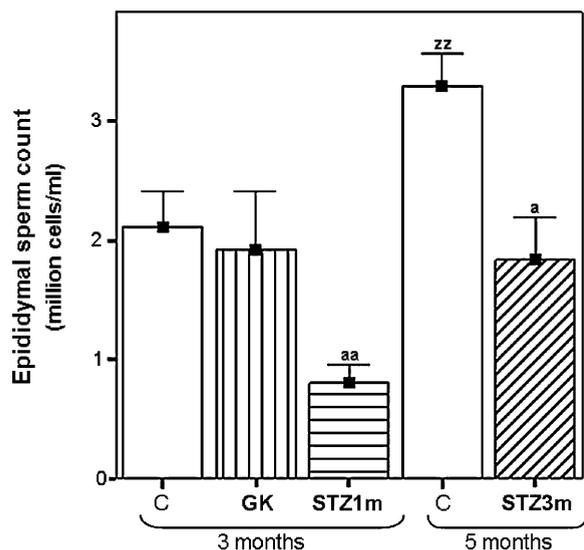


Fig. 2. Epididymal Sperm Count in Diabetic and Wistar control rats. Epididymal sperm count was determined as described in Section 2. Data is expressed as means \pm S.E.M. Statistical significance: (zz and aa) $p < 0.01$ compared with 3-month-old Wistar controls, (a) $p < 0.05$, compared to 5-month-old Wistar controls.

streptozotocin injection. Thus we did not take into consideration rats treated with STZ for 1 week in analyses that involve sperm. On the other hand, sperm from GK rats showed a decrease in sperm count and motility compared with Wistar controls, however these differences were not statistically significant (Figs. 2 and 3). No differences were found in testicular round cell count (data not shown), suggesting that hyperglycemia affects the later stages of spermatogenesis.

3.3. Analysis of adenine nucleotides and adenylate energy charge

3.3.1. Adenine nucleotides

ATP levels were higher in 5-month-old Wistar control rats than in 3-month-old Wistar control rats

Table 1
ATP, ADP and AMP levels in rat testicular cells

	ATP (pmol/mg protein)	ADP (pmol/mg protein)	AMP (pmol/mg protein)
Wistar controls (3 months)	1386.9 \pm 288.4	1976.6 \pm 374.1	2276.6 \pm 449.1
GK rats (3 months)	2013.4 \pm 286.4	1597.05 \pm 162.1	2681.1 \pm 680.3
STZ (1 week after treatment)	1253.7 \pm 291.2	2647.04 \pm 806.3	4194.9 \pm 1279.4
STZ (1 month after treatment)	2298.2 \pm 353.6	2781.5 \pm 429.6	4680.9 \pm 819.4 a
Wistar controls (5 months)	3521.3 \pm 550.6 zz	3882.8 \pm 581.9 z	2253.05 \pm 558.4
STZ (3 months after treatment)	276.7 \pm 18.6 a,bbb,ddd	1035.8 \pm 22.54 abd	4508.5 \pm 1648.6

Data is presented as means \pm S.E.M. for each condition studied. (zz) $p < 0.01$ when compared with 3-month-old Wistar controls; (a) $p < 0.05$ when compared with 5-month-old controls. (bbb and ddd) $p < 0.001$ when compared with GK rats and with rats treated with STZ for 1 month, respectively. (z) $p < 0.05$ when compared with 3-month-old controls (abd) $p < 0.05$ when compared with 5-month-old Wistar controls, GK rats and rats treated with STZ for 1 month, respectively. (a) $p < 0.05$ when compared with 3-month-old controls.

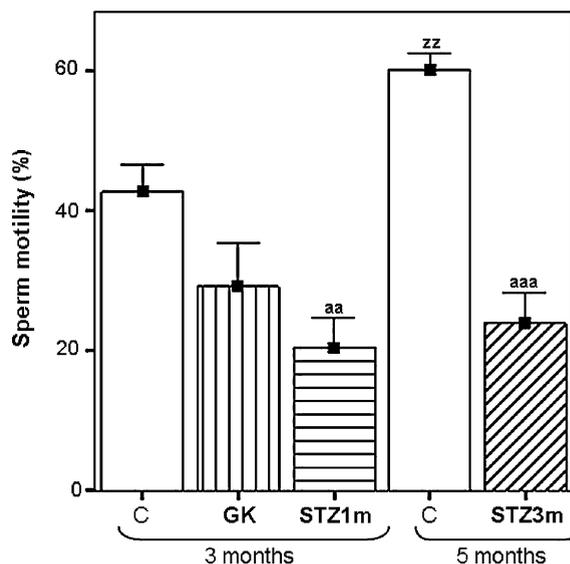


Fig. 3. Motility of epididymal sperm from diabetic and control rats. Sperm motility was determined as described in Section 2. Data is expressed as means \pm S.E.M. Statistical significance: (zz and aa) $p < 0.01$ compared with 3-month-old Wistar controls and (aaa) $p < 0.001$ compared with 5-month-old Wistar controls.

($p < 0.01$). Rats treated with STZ for 3 months showed lower levels of ATP when compared with controls ($p < 0.05$), GK rats ($p < 0.001$) and rats treated with STZ for 1 month ($p < 0.001$). The same patterns were observed with ADP levels. AMP levels showed no age-dependent changes in control rats, but showed a tendency to increase in rats treated with STZ for 1 month ($p < 0.05$). Rats treated with STZ for 3 months showed similar levels to the last group (Table 1).

3.3.2. Adenylate energy charge (AEC)

AEC in testicular cells from GK rats were very similar to Wistar controls. Conversely, AEC was lower in testicular cells of rats treated with STZ for 1 week ($p = 0.058$) and in rats treated with STZ for 3 months ($p < 0.05$, Fig. 4).

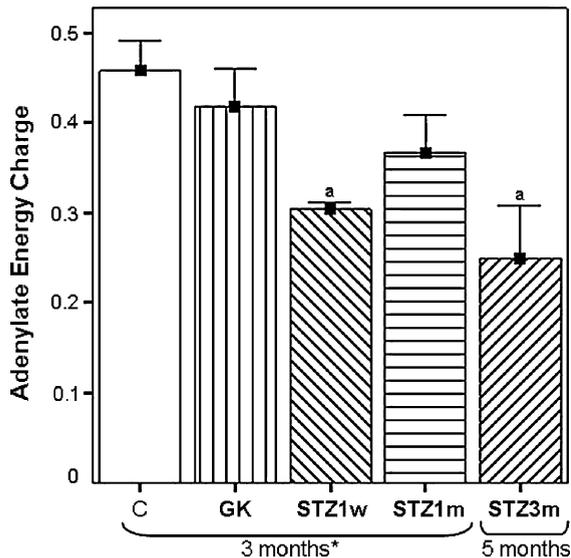


Fig. 4. Adenylate energy charge (AEC) in testicular cells of diabetic and control rats. AEC was determined as described in Section 2. Data is expressed as means \pm S.E.M. Statistical significance: (a) $p < 0.05$ compared with Wistar control rats. This graph presents only one control bar because there were no differences between 3- and 5-month-old Wistar control rats.

3.4. Lactate and pyruvate levels

Lactate levels were significantly lower in GK rats when compared with Wistar controls ($p < 0.01$). Also, rats treated with STZ for 3 months showed lower lactate levels when compared to their respective controls ($p < 0.05$), and higher levels when compared to GK and rats treated with STZ for 1 month ($p < 0.01$) (Fig. 5). No differences were found in pyruvate levels (data not shown).

3.5. Oxidative stress parameters

3.5.1. Lipid peroxidation and protein oxidation

Testicular cells of rats treated with STZ for 3 months showed a tendency towards higher susceptibility to lipid peroxidation, assessed by higher TBARS production, although this was not significant. Rats treated with STZ for 1 month showed lower TBARS levels when compared with controls and rats treated with STZ for 1 week ($p < 0.05$) (Fig. 6). No differences in carbonyl groups were found (data not shown).

3.6. Enzymatic activities

3.6.1. Lactate dehydrogenase release

We found no significant differences among different rat groups in the residual LDH activity

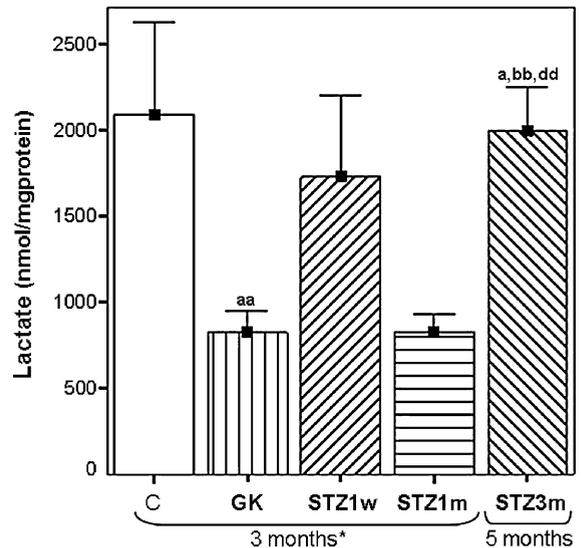


Fig. 5. Lactate levels in testicular cells of diabetic and control rats. Lactate levels were determined as described in Section 2. Data is expressed as means \pm S.E.M. Statistical significance: (a) $p < 0.05$ and (aa) $p < 0.01$ compared with Wistar control rats; (bb) $p < 0.01$ compared with GK rats; (dd) $p < 0.01$ compared with rats treated with STZ for 1 month. There were no differences between 3- and 5-month-old Wistar control rats.

monitored in the supernatant of our cultures (data not shown), suggesting that testicular cell viability is not compromised during the experiment.

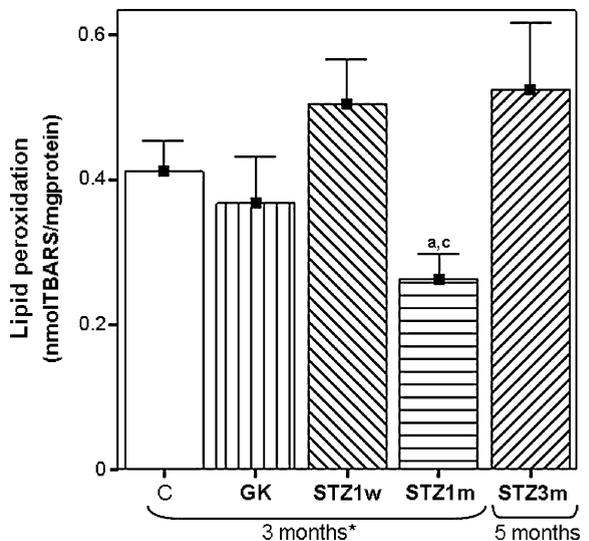


Fig. 6. Lipid peroxidation in diabetic and control rats. TBARS levels were determined as described in Section 2. Data is expressed as means \pm S.E.M. Statistical significance: (a) $p < 0.05$ compared with Wistar control rats; (c) $p < 0.05$ compared with rats treated with STZ for 1 week. There were no differences between 3- and 5-month-old Wistar control rats.

3.6.2. Glutathione reductase activity

Wistar control rats showed an increase in G-Red activity with age ($p < 0.05$). G-Red activity in the testicular cells of GK rats was higher when compared with Wistar controls ($p < 0.05$). In STZ-treated rats we observed that treatment for 3 months resulted in increased G-Red activity, compared with treatment for 1 week ($p < 0.05$) and 1 month ($p < 0.01$). Since we found no differences between STZ-treated rats and their controls these differences are likely due to age (Fig. 7).

3.6.3. Glutathione peroxidase activity

Our results show that G-Px activity decreases in testicular cells of GK rats ($p < 0.05$) and rats treated with STZ for 1 month ($p < 0.05$). However, G-Px activity was higher in rats treated with STZ for 3 months, compared to GK rats ($p < 0.05$) and STZ-treated rats for 1 month ($p < 0.01$). Therefore an enhancement of enzyme activity is evident in the later stage of the disease (Fig. 8).

3.7. Correlations

Both in controls and hyperglycemic rats, LDH activity was negatively correlated with ATP and positively correlated with TBARS, stressing that energy supply and oxidative stress are important factors for the maintenance of cellular viability, and suggesting the validity of our experimental approach. Also, G-Px activity correlated

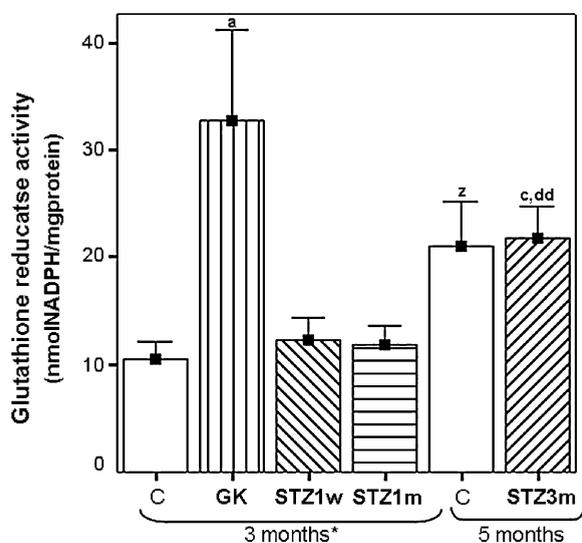


Fig. 7. Glutathione reductase activity in testicular cells of diabetic and control rats. Activity was determined as described in Section 2. Data is expressed as means \pm S.E.M. Statistical significance: (z and a) $p < 0.05$ compared with 3-month-old Wistar controls; (c) $p < 0.05$ compared with rats treated with STZ for 1 week, (dd) $p < 0.01$ compared with rats treated with STZ for 1 month.

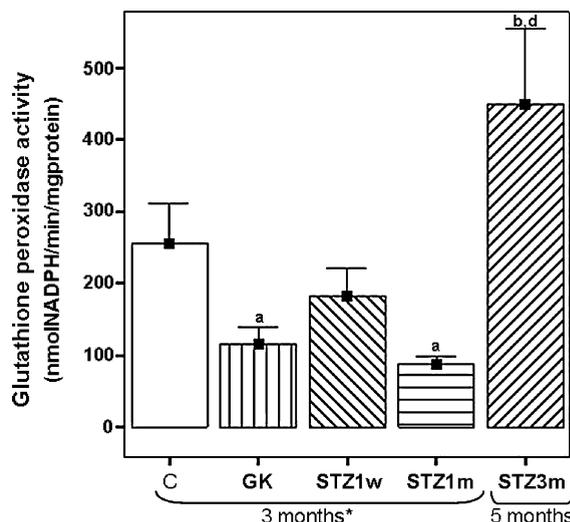


Fig. 8. Glutathione peroxidase activity in testicular cells of diabetic and control rats. Activity was determined as described in Section 2. Data is expressed as means \pm S.E.M. Statistical significance: (a) $p < 0.05$ compared with Wistar controls; (b) $p < 0.05$ compared with GK rats; (d) $p < 0.05$ compared to rats treated with STZ for 1 month. There were no differences between 3- and 5-month-old Wistar control rats.

positively with LDH activity, TBARS and lactate levels, meaning that antioxidant defenses are triggered when the cell condition worsens, and that probably G-Px represents an active mechanism in protection against radicals originated by lipid peroxidation.

On the other hand, some important correlations were found that are likely due to the diabetic condition. A stronger negative correlation between ATP and TBARS was found in diabetic animals, mainly due to GK rats (data not shown), and the

Table 2

Correlations in parameters monitored for Wistar control rats (C) and hyperglycemic rats (H)

		LDH	TBARS	G-Px	G-Red	Lactate
ATP	C	-0.519*	-0.197	0.121	0.550*	0.061
	H	-0.522**	-0.498**	-0.457**	-0.43	-0.331
LDH	C		0.454**	0.608**	-0.109	0.529**
	H		0.506**	0.524**	0.168	-0.13
TBARS	C			0.631**	0.002	0.521**
	H			0.478**	-0.009	0.358**
G-Px	C				0.075	0.646**
	H				0.282*	0.304*
G-Red	C					0.270
	H					0.181

Pearson correlation coefficients (r) obtained in Wistar control rats (C) and hyperglycemic rats (H) rats are presented. * $p \leq 0.05$ and ** $p \leq 0.01$.

correlation between ATP with G-Px became negative, mainly due to rats treated with STZ (data not shown). Although not significant, the correlation between ATP and lactate also became negative in hyperglycemic rats. G-Px correlation with G-red also becomes evident, mainly due to rats treated with STZ (data not shown), suggesting the up-regulation of the glutathione pathway in the hyperglycemic testis. All bivariate correlations, calculated by the Pearson correlation coefficient, are presented in Table 2.

4. Discussion

Increasing evidence suggests that diabetes has an adverse effect on male reproductive function [2], and that oxidative stress may be involved [29]. According to Mahboob et al. [40], antioxidant enzyme-dependent defences may play an important role by scavenging free radicals produced under oxidative stress. The current study was conducted in order to clarify the relationship between oxidative stress originated by a diabetic condition, and parameters related to spermatogenesis and sperm function. For this purpose we used animal models for Type 1 (STZ-treated rats) and Type 2 (GK rats) diabetes, as well as control animals (Wistar rats). Overall, more severe effects were seen in rats treated with STZ for 3 months, while GK rats showed milder effects, possibly due to moderate hyperglycemia, or adaptations through selective breeding.

An age-dependent sperm concentration and motility increase was observed in control rats, but given that sexual maturity is reached by 8 weeks of age, 3-month-old rats may have not yet achieved the maximum potential for sperm production [41,42]. GK rats showed no differences in sperm parameters, while sperm concentration and motility decreased in rats treated with STZ, in accordance with some previous results [72,73], which have also described STZ-induced impairment in mating behaviour and sperm ejaculation. Furthermore, Soudamani et al. also found that STZ-induced diabetes has detrimental effects on the maintenance and establishment of fully differentiated epididymal epithelium during sexual maturation [74].

Since we found no differences in testicular cell concentration between different groups, the decrease in sperm concentration is likely due to the influence of severe hyperglycemia in late stages of spermatogenesis, possibly through an increase in ROS. The consequences of such oxidative damage could include loss of motility due lipid peroxidation [43–45], induction of DNA damage in the sperm nucleus, and errors in spermiogenesis affecting fertilizing potential [45].

A major issue in hyperglycemic STZ-treated rats is a decrease in testicular ATP levels, leading to a compromised adenylate energy charge during spermatogenesis. Furthermore, the negative correlation observed between LDH and ATP levels in Wistar controls becomes stronger in hyperglycemic rats, mostly due to rats treated with STZ, stressing that cell energy supply is paramount for viability. Interestingly, GK rats and rats treated with STZ for 1 month showed a slight (non-significant) increase in ATP levels, when compared to other diabetic groups, suggesting that some kind of adaptation is underway in these rats, in order to increase ATP production. Metabolic adaptation may occur by an enhancement in oxidative phosphorylation in order to increase ATP production [28]. This adaptation was no longer seen in rats treated with STZ for 3 months, probably due to continuous severe hyperglycemia. Indeed, Ferreira et al. [28] demonstrated that liver mitochondria from GK rats presented improved respiratory activity and ATP synthase activity, as opposed to STZ-treated rats 9 weeks after diabetogenic treatment (corresponding to our rats treated with STZ for 3 months), which had decreased ATP synthase activity. If the respiratory enzymes of the electron transport chain are damaged by free radicals, the production of ATP is concurrently decreased [46]. Furthermore, a decline in ATP generation negatively affects glycolysis (since ATP is needed in the first stages), decreasing the use of glucose, fructose, and several amino acids, and leading to severe energetic impairment [47,48]. GK rats may also have the same metabolic adaptation due to genetic factors. Moreover, 3-month-old Wistar controls had inferior ATP levels when compared to 5-month-old controls, suggesting that similar changes occur with age [49–51], keeping in mind that age is also associated with increased oxidative stress [52–58].

A decrease in energy charge (decrease in ATP and increase in AMP) would switch off anabolic pathways, and switch on catabolic pathways, such as glycolysis and fatty acid oxidation [59]. We found that GK rats had similar AEC values to Wistar control rats, consistent with the proposed metabolic adaptations mentioned earlier. Among STZ-treated rats, despite a lower AEC in rats with 1 week and 3 months of STZ treatment, rats treated with STZ for 1 month presented a slight increase in AEC, in agreement with what was observed in terms of ATP levels. Furthermore, we suggest that rats treated with STZ for 1 week represent the acute phase of the disease, and have not yet adjusted their metabolism.

In Sertoli cells, glucose is metabolised via cytosolic glycolysis to lactate, which is then used primarily by the

germ cells as a substrate for ATP production in mitochondrial oxidative phosphorylation [60]. The observed lowered lactate levels in GK rats and rats treated with STZ for 1 month may be explained by the variations observed in ATP, since if lactate levels drop if it is being used to produce ATP. Although not significant, it is interesting to note that the correlation between ATP and lactate became negative in hyperglycemic rats. Rats treated with STZ for 3 months showed higher lactate levels when compared to GK rats and rats treated with STZ for 1 month, but not when compared to control rats, although other observations relate the severity of diabetes with higher lactate levels [61]. In fact, hyperglycemia seems to enhance non-oxidative metabolism (glucose conversion to lactate), i.e. the Crabtree effect, by increasing glucose-6-phosphate levels (G6P). Increased glucose metabolism to lactate is associated with an increase in the NADH/NAD⁺ ratio. Under this condition of markedly accelerated glycolysis, oxidation of glyceraldehyde 3-phosphate (GAP) to 1,3-biphosphoglycerate (1,3-DPG), coupled to the reduction of NAD⁺ to NADH, appears to become the rate-limiting step in glycolysis. In the cytosol, NADH is oxidized to NAD⁺ by lactate dehydrogenase (LDH), coupled by reduction of pyruvate to lactate. Thus, the increase in the ratio of NADH/NAD⁺ will reflect an increased lactate/pyruvate ratio. The mechanism by which an increased rate of glycolysis increases free cytosolic NADH/NAD⁺ ratio (redox imbalance) appears to result from an imbalance between the rate of oxidation of GAP to 1,3-DPG and the rate of reduction of pyruvate. This indicates that increased glycolysis, as a consequence of hyperglycemia, is closely related to an increase in the NADH/NAD⁺ ratio due to impaired oxidation of NADH to NAD⁺ [10].

If metabolic adjustments seem to be occurring what is the possible role of hyperglycemia in oxidative stress? Diabetic rats, with the exception of GK rats and rats treated with STZ for 1 month, had increased lipid peroxidation, in concordance with previous reports [10,40,62–65]. In rats treated with STZ for 1 month we observed a decrease in TBARS, when compared to controls and rats treated with STZ for 1 week, which may again represent an adaptive response to a chronic condition, in that the cells adapt to lower ROS production and/or increase inactivation of free radicals. In diabetic rats the negative correlation between TBARS and ATP levels became marked, demonstrating that the energetic state of the cell is important for the maintenance of proper levels of ROS. Additionally, a stronger positive correlation of TBARS levels with LDH activity was evident in diabetic rats, drawing a

parallel between cellular viability and lipid peroxidation, and stressing that oxidative stress is related to cell loss.

In terms of possible enzymatic defences against the generation of ROS, conflicting reports, note both increased and decreased G-Px and G-Red activity in diabetic individuals [40,66–68]. In type 1 diabetes (STZ-treated rats), and discarding possible age-induced effects, we observed a decrease in G-Px activity in the early stages of the disease, followed by a significant increase. Moreover, for both enzyme activities correlations with ATP levels seen in Wistar controls are inverted in diabetic rats, mostly due to rats treated with STZ, and G-Px continues to be highly correlated with TBARS levels and LDH, implying that activity is higher when the cell condition is worse, and that G-Px represents an active mechanism in cell protection. Conversely, in type 2 diabetic rats (GK rats) these two enzymes had dissimilar patterns of variation, with a significant increase in G-Red activity, concomitant with a decrease in the activity of G-Px. Interestingly, this decrease mirrored what is seen in the early stages following STZ injection, thus stressing that there is some adaptation to hyperglycemia in STZ-treated rats. It is also possible that GK rats may need to regenerate GSH that might be oxidized by a G-Px independent pathway [69–71], thus justifying the higher activity of this enzyme.

Taken together these results allow us to conclude that hyperglycemia has adverse effects in energy levels, sperm concentration and sperm motility in model animals for type 1 diabetes, and that diabetic rats present some metabolic adaptations, augmenting the efficiency of ATP production and reducing ROS, in order to compensate the impairments promoted by high glucose levels typical of the disease. These adjustments are evident in GK rats and rats treated with STZ for 1 month and apparently disappear in the later stages of the disease.

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