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Enhanced mitochondrial testicular antioxidant capacity in Goto-Kakizaki diabetic rats: role of coenzyme Q

CARLOS M. PALMEIRA,[1] DARIO L. SANTOS,[2] RAQUEL SEIÇA,[3]
ANTÓNIO J. MORENO,[1] AND MARIA S. SANTOS[1]
Center for Neurosciences and Cell Biology of Coimbra, [1]Department of Zoology and [3]Faculty of Medicine, University of Coimbra, 3004-517 Coimbra; and [2]University of Trás-os-Montes e Alto Douro, 5000 Vila Real, Portugal

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Palmeira, Carlos M., Dario L. Santos, Raquel Seiça, António J. Moreno, and Maria S. Santos. Enhanced mitochondrial testicular antioxidant capacity in Goto-Kakizaki diabetic rats: role of coenzyme Q. *Am J Physiol Cell Physiol* 281: C1023–C1028, 2001.—Because diabetes mellitus is associated with impairment of testicular function, ultimately leading to reduced fertility, this study was conducted to evaluate the existence of a cause-effect relationship between increased oxidative stress in diabetes and reduced mitochondrial antioxidant capacity. The susceptibility to oxidative stress and antioxidant capacity (in terms of glutathione, coenzyme Q, and vitamin E content) of testis mitochondrial preparations isolated from Goto-Kakizaki (GK) non-insulin-dependent diabetic rats and from Wistar control rats, 1 yr of age, was evaluated. It was found that GK mitochondrial preparations showed a lower susceptibility to lipid peroxidation induced by ADP/Fe^{2+}, as evaluated by oxygen consumption and reactive oxygen species generation. The decreased susceptibility to oxidative stress in diabetic rats was associated with an increase in mitochondrial glutathione and coenzyme Q9 contents, whereas vitamin E was not changed. These results demonstrate a higher antioxidant capacity in diabetic GK rats. We suggest this is an adaptive response of testis mitochondria to the increased oxidative damage in diabetes mellitus.

diabetes mellitus; sexual dysfunction; oxidative stress; testis mitochondria; glutathione; vitamin E

NON-INSULIN-DEPENDENT DIABETES MELLITUS (NIDDM) is one of the most common metabolic diseases in humans, affecting ~100 million people around the world (2). Furthermore, diabetes mellitus is associated with erectile dysfunction and reduced fertility in men and animal models. The pathophysiological mechanisms of impotence in diabetic patients remain obscure (4, 5, 16, 17, 26).

With the use of animal models, it became possible to predict the development of diabetes and to distinguish the pathogenic mechanisms involved in the onset of the disease. Goto-Kakizaki (GK) rat, a nonobese, spontaneously diabetic animal model produced by repeated selective breeding of Wistar nondiabetic rats and characterized by Goto and coworkers (13, 14, 15), is currently used as an animal model of NIDDM. At the beginning of the diabetes, GK rats do not present severe complications associated with the disease, thus making it an appropriate model to study the events at the onset of diabetes compared with genetically obese diabetic rats, which present severe hyperglycemia and hyperlipidemia (8).

Enhanced oxidative stress and changes in antioxidant capacity are considered to play an important role in the pathogenesis of chronic diabetes mellitus (1, 2, 37). Although the mechanisms underlying the alterations associated with NIDDM are presently not well understood, hyperglycemic levels lead patients to an increased oxidative stress (20) because the production of several reducing sugars (through glycolysis and polyol pathways) is enhanced. These reducing sugars can easily react with lipids and proteins (nonenzymatic glycation reaction) (11), increasing the production of reactive oxygen species (ROS) (19). Mitochondria can contribute to the development of this disease because they generate a great amount of ROS (6), which could stimulate the progression of oxidative stress. Under normal conditions, potentially toxic ROS generated by mitochondria respiratory metabolism are efficiently neutralized by cellular antioxidant defense mechanisms. However, this balance can easily be broken, leading to cellular dysfunction (20, 30).

Currently, there is considerable interest in the roles of vitamin E and coenzyme Q (CoQ) in the protection of membrane lipids against oxidative stress. CoQ has been demonstrated to serve the dual functions of an electron carrier/proton translocator in the respiratory chain (7) and an antioxidant by directly scavenging radicals (35) or indirectly by regenerating vitamin E (32).

To clarify the role of the antioxidant systems in NIDDM and the role of NIDDM affecting reproductive function (since reproductive function heavily depends on the energy generated by testis mitochondria), we

Address for reprint requests and other correspondence: C. M. Palmeira, Center for Neurosciences and Cell Biology of Coimbra, Dept. of Zoology, Univ. of Coimbra, 3004-517 Coimbra, Portugal (E-mail: palmeira@ci.uc.pt).

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investigated the possible alterations in lipid peroxidation and antioxidant systems of testis mitochondria isolated from control and GK rats of 48 wk of age. We found that GK rats, unlike control animals, presented higher contents of GSH and coenzyme Q9 (CoQ9) and were less susceptible to lipid peroxidation. Together, these results show a higher antioxidant capacity in diabetic GK rats. We suggest that this is an adaptive response of mitochondria to the increased oxidative damage in diabetes mellitus, both in humans and animals.

MATERIALS AND METHODS

Chemicals. Ubiquinone 10 (CoQ10) was obtained from Fluka (Germany). Ubiquinone 9 (CoQ9) and vitamin E were obtained from Sigma Chemical (St. Louis, MO). All other chemicals used were of analytical grade.

Animals. Male spontaneously diabetic GK rats (48 wk of age; nonfasting blood glucose levels 198.6 ± 13.5 mg/dl) were obtained from a local breeding colony of Coimbra (Portugal) that was established in 1995 with breeding couples from the colony at Tohoku University School of Medicine (Sendai, Japan; courtesy of Dr. K. I. Suzuki). Control animals were nondiabetic male Wistar rats of similar age (nonfasting blood glucose levels 93.4 ± 2.9 mg/dl) obtained from our local colony. Animals were killed by decapitation, and the testes were removed and washed in the respective homogenization medium.

Mitochondrial isolation. Testis mitochondria were isolated according to a previously established method (3) with some modifications. Briefly, testes were removed, decapsulated, and homogenized in a medium that contained 0.25 mM sucrose, 5 mM HEPES (pH 7.4), 0.2 mM EGTA, 0.1 mM EDTA, and 0.1% defatted bovine serum albumin. EGTA, EDTA, and bovine serum albumin were omitted from the final washing medium, adjusted to pH 7.2. The mitochondrial pellet was washed twice and suspended in washing medium. Mitochondrial protein was determined by the biuret method, using bovine serum albumin as a standard.

Measurement of lipid peroxidation. Lipid peroxidation was determined as described by Sassa et al. (31). The oxygen consumption was measured in 1 ml of medium (175 mM KCl and 10 mM Tris-Cl, pH 7.4, supplemented with 3 μM rotenone) containing 1 mg of protein, using a Clark-type electrode (YSI 5331, Yellow Springs Instruments) in a closed glass chamber equipped with magnetic stirring and thermostatted at 30°C. Reactions were started by the addition of 1 mM ADP and 0.1 mM $FeSO_4$ after a 2-min incubation period. The saturated concentration of oxygen in the incubation medium was assumed to be 232 μM at 30°C.

ROS generation. ROS were measured according to Royall and Ischiropoulos (29) by following the oxidation of the dye dihydrorhodamine 123 (DHR 123). DHR 123 accumulates in mitochondria (due to its positive charge) and fluoresces when oxidized by ROS to rhodamine 123, detecting the formation of intramitochondrial peroxides. Mitochondria (1 mg/ml) were incubated at 30°C with 5 μM DHR 123 for 15 min in the medium (175 mM KCl and 10 mM Tris-Cl, pH 7.4, supplemented with 3 μM rotenone) to reproduce the same conditions used for the assay with the oxygen electrode. After loading with rhodamine, mitochondria were washed with the same medium and fluorescence was measured in the same medium with excitation at 500 nm and emission at 536 nm. Reactions were started by the addition of 1 mM ADP and 0.1 mM $FeSO_4$ after a 2-min incubation period.

Additionally, we did the same assay in respiring mitochondria, i.e., medium (175 mM KCl and 10 mM Tris-Cl, pH 7.4, supplemented with 3 μM rotenone) supplemented with 5 mM succinate. Addition of antimycin (0.5 μg/mg) results in an increase of fluorescence intensity of ~30%.

GSH and GSSG measurement. GSH and GSSG levels were determined with fluorescence detection after reaction of the supernatant of the H_3PO_4/EDTA-NaH_2PO_4 deproteinized mitochondria solution with *o*-phthalaldehyde, pH 8.0, according to Hissin and Hilf (18).

Extraction and quantification of CoQ9, CoQ10, and vitamin E. Aliquots of mitochondria containing 1 mg of protein/ml were extracted according to the method described by Takada et al. (34). The extract was evaporated to dryness under a stream of N_2 and resuspended in absolute ethanol. CoQ content was determined by reverse-phase HPLC (Spherisorb RP18, S5ODS2 column). Samples were eluted with methanol:heptane (10:2 vol/vol) at a flow rate of 2 ml/min. Detection was performed by an ultraviolet (UV) detector at 269 nm. Vitamin E was extracted and quantified by following the method described by Vatassery et al. (36). The extract was evaporated to dryness under a stream of N_2 and resuspended in *n*-hexane. Vitamin E content was determined by reverse-phase HPLC (4.6 × 200 mm; Spherisorb S10w column). Samples were eluted with *n*-hexane modified with 0.9% methanol at a flow rate of 1.5 ml/min. Detection was performed by a UV detector at 287 nm.

Data analysis and statistics. Data are expressed as means ± SE of the indicated number of experiments, each obtained with a different animal. Statistical significance was determined using the paired Student's *t*-test and by using the one-way ANOVA Student-Newman-Keuls posttest for multiple comparisons. $P < 0.05$ was considered significant.

RESULTS

Mitochondrial oxidative stress induced by ADP/Fe^{2+}. GK testis mitochondria show a lower susceptibility to oxidative stress induced by the oxidizing agents

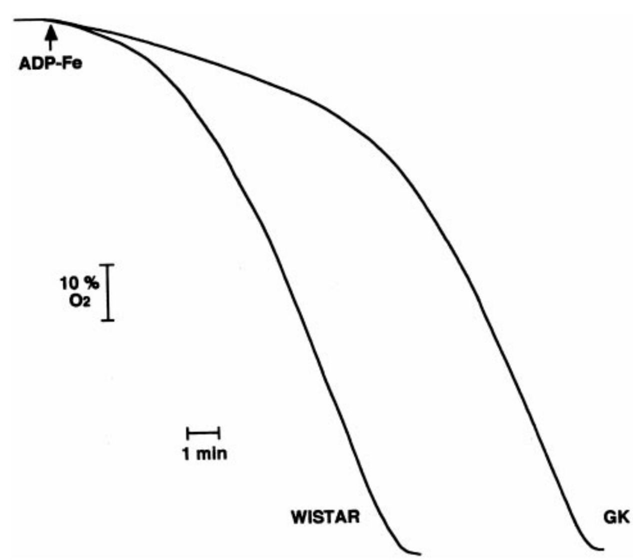

Fig. 1. Rate and extent of mitochondrial oxidation induced by ADP/Fe^{2+} in Goto-Kakizaki (GK) and control testis preparations evaluated as oxygen consumption. Details are described in MATERIALS AND METHODS. In the absence of peroxidation inducer (ADP/Fe^{2+}), oxygen consumption was similar in both preparations. Traces represent typical direct recordings from 5 different preparations (control Wistar and GK rats).

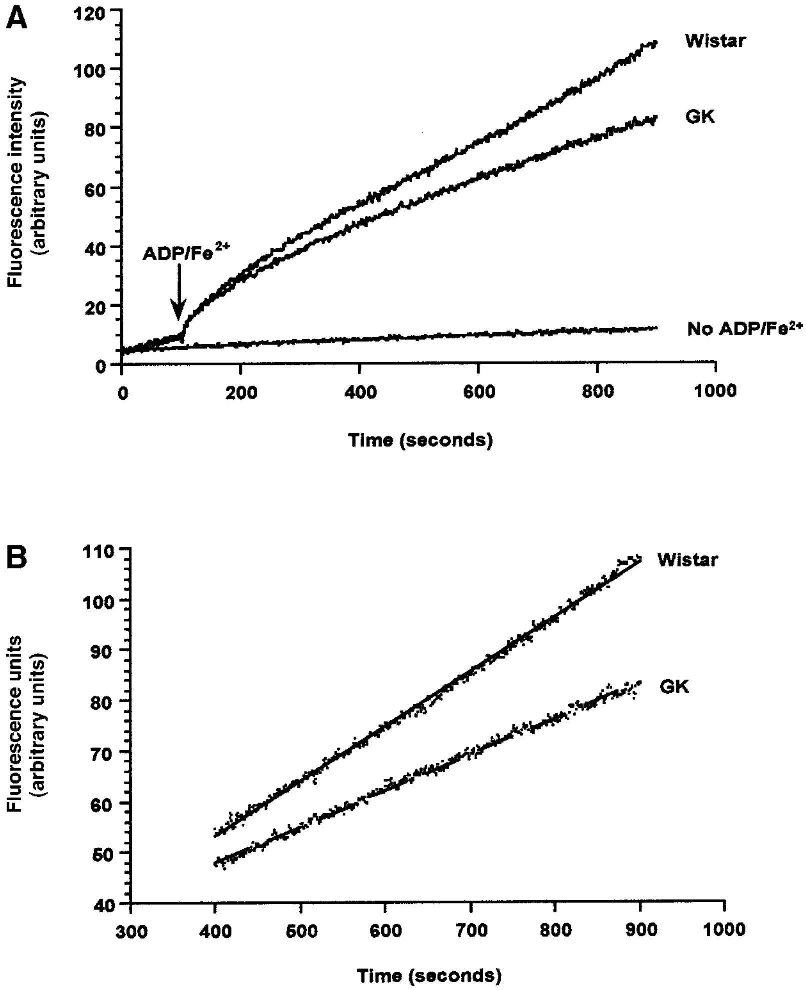

Fig. 2. *A*: effect of oxidative stress induced by ADP/Fe^{2+} on the levels of reactive oxygen species (ROS) formation in testis mitochondria from Wistar and GK rats. *B*: extent of ROS formation (arbitrary units per minute). Details are described in MATERIALS AND METHODS. Traces represent typical direct recordings from 3 different preparations (Wistar and GK rats).

ADP/Fe^{2+}, as assessed by oxygen consumption (Fig. 1) and ROS formation (Fig. 2). As shown in Fig. 1, diabetic mitochondria show a slow oxygen consumption in the first 8 minutes after the addition of ADP/Fe^{2+}, and after this period of time, the oxygen consumption increases drastically. Conversely, control mitochondria have a higher oxygen consumption immediately after ADP/Fe^{2+} addition. The initial slow oxygen consump-

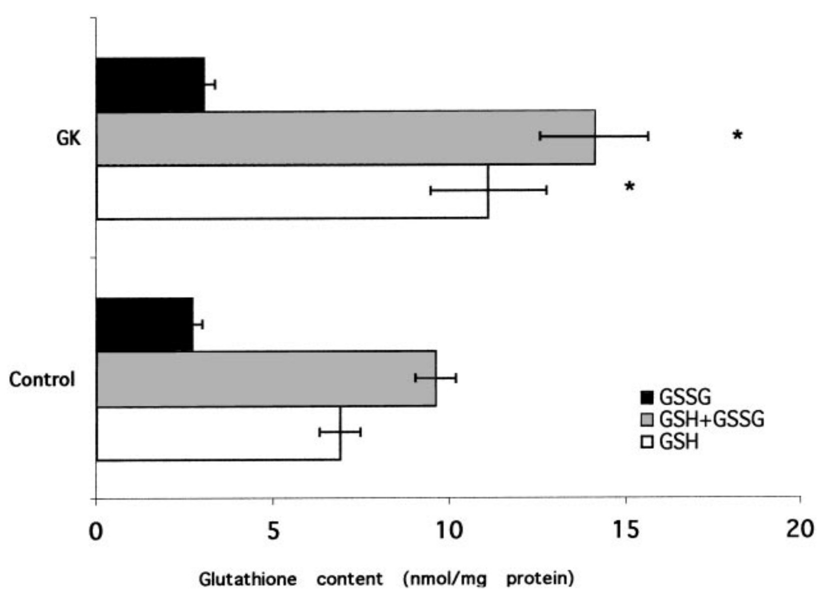

Fig. 3. Glutathione levels of GK and control testis mitochondria preparations. GSH and GSSG levels were determined with fluorescence detection as described in MATERIALS AND METHODS. Data are means ± SE of 5 different preparations (control Wistar and GK rats). *Values statistically different from control nondiabetic Wistar rats ($P < 0.05$).

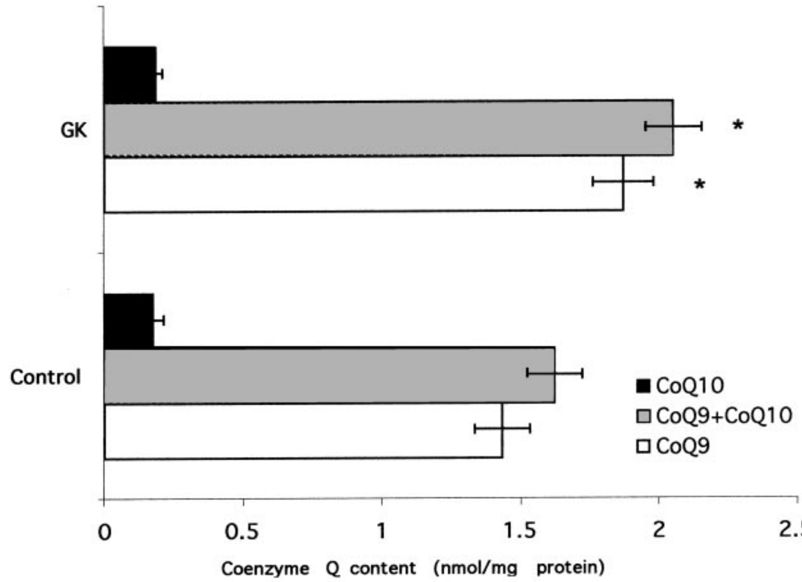

Fig. 4. Coenzyme Q9 (CoQ9) and coenzyme Q10 (CoQ10) levels of GK and control testis mitochondria preparations. Coenzyme Q levels were measured by HPLC as described in MATERIALS AND METHODS. Data are means ± SE of 5 different preparations (control Wistar and GK rats). *Values statistically different from control nondiabetic Wistar rats ($P < 0.05$).

tion following ADP/Fe^{2+} addition until rapid oxygen consumption is considered to be the time required for the generation of a sufficient amount of ROS derived from ADP/Fe^{2+}, such as the perferryl complex ADP/Fe^{3+}/O_2^-, which is responsible for the induction of lipid peroxidation. The contribution of oxidative stress to the formation of ROS in our mitochondrial preparation, namely peroxides, is shown in Fig. 2. When testis mitochondria were submitted to the oxidizing agents ADP/Fe^{2+}, the increased rate of free radical formation was higher in Wistar (6.5 arbitrary units per minute) compared with GK rats (4.3 arbitrary units per minute). Similar results were obtained with respiring mitochondria, which means that mitochondrial function is responsible for the observed difference in free radical generation.

Mitochondrial GSH content. Because glutathione in the reduced form (GSH) is known to play an important role in protecting cells and organelles from oxidative stress, GSH and GSSG contents were evaluated both in GK and control mitochondrial preparations (Fig. 3). We found a higher GSH content in GK testis mitochondria compared with control mitochondria (11.1 ± 1.6 and 6.9 ± 0.5 nmol/mg protein, respectively), whereas no changes in GSSG content in both preparations were observed. Consequently, the total mitochondrial content in glutathione (GSH + GSSG) was increased in GK diabetic rats (14.2 ± 1.5 and 9.6 ± 0.6 nmol/mg protein in GK and control, respectively).

Mitochondrial CoQ and vitamin E contents. CoQ in rat mitochondria consisted of two main homologues, CoQ9 and CoQ10. CoQ10 content is similar in both preparations (Fig. 4). Interestingly, CoQ9 content in GK mitochondria is significantly higher than in control (1.9 ± 0.1 and 1.45 ± 0.1 nmol/mg protein, respectively). CoQ9 increased content paralleled that of the total CoQ content in GK diabetic mitochondria (Fig. 4). Additionally, vitamin E levels were determined, and no difference was found between the diabetic GK rat mitochondria and the control nondiabetic animals (Fig. 5).

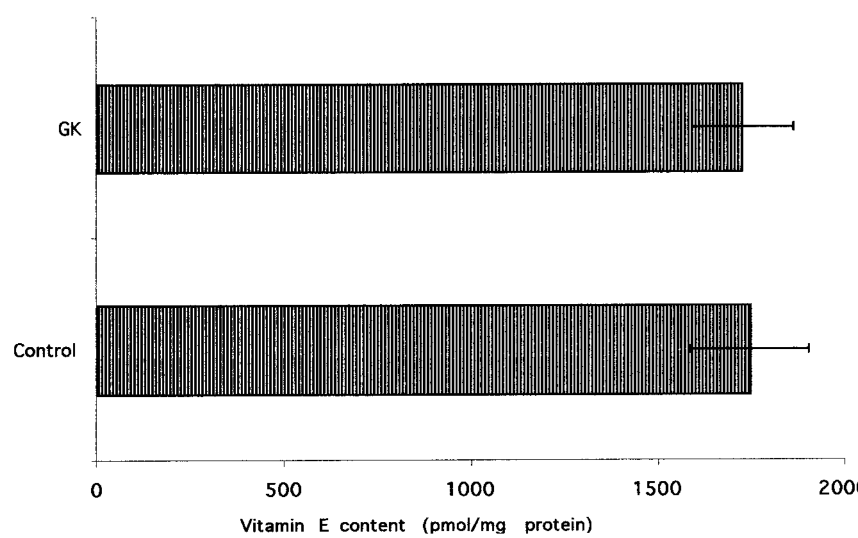

Fig. 5. Vitamin E levels of GK and control testis mitochondria preparations. Vitamin E levels were measured by HPLC as described in MATERIALS AND METHODS. Data are means ± SE of 5 different preparations (control Wistar and GK rats).

DISCUSSION

Several reports show unequivocally that sexual behavior and reproductive tract function are markedly affected by diabetes mellitus (12, 17) and that increased oxidative stress leads to the impairment of spermatogenesis in rat testis (25). Because of constant hyperglycemic levels, NIDDM patients are exposed to an increased oxidative stress (20) because the production of several reducing sugars (through glycolysis and polyol pathways) is enhanced. These reducing sugars can easily react with lipids and proteins (nonenzymatic glycation reaction) (11), increasing the production of ROS (19).

A previous report from our group (10) on the susceptibility of liver mitochondrial preparations to lipid peroxidation in vitro showed that GK mitochondrial preparations were less susceptible to oxidative stress than control preparations, in agreement with the observations of others (21, 33) using mitochondria from streptozotocin-induced diabetic rats with brief periods of induced diabetes.

In this study we found that GK testis mitochondrial preparations were less susceptible to in vitro oxidation, evaluated by oxygen consumption and ROS generation. This lower susceptibility is correlated with an increase in mitochondrial GSH and CoQ9 contents. It is well known that GSH plays an important role in the metabolism of hydroperoxides and free radicals (28). Moreover, GSH plays a crucial role in mitochondrial function because mitochondria lack catalase, and, therefore, peroxides are reduced only by glutathione peroxidase. The higher GSH content in GK testis preparations suggests that diabetic mitochondria could be protected from damage mediated by free radicals. Previous studies have shown that variations in the total CoQ content or in the type of CoQ homologue are associated with alterations in mitochondrial function (23, 24). One of the functions of CoQ is to act as an antioxidant, either by directly scavenging radicals (35) or indirectly by regenerating vitamin E (32). Interestingly, CoQ9 content in diabetic GK testis mitochondria is also increased. This fact reinforces the observed lower susceptibility of GK diabetic testis mitochondria to oxidative stress.

It should be pointed out that GK rats exhibit a moderate but stable fasting hyperglycemia, which does not progress to a ketotic state. Therefore, at this age, GK rats do not present severe complications associated with the disease, thus making it an appropriate model to study the events at the onset of diabetes, compared with genetically obese diabetic rats, which present with severe hyperglycemia and hyperlipidemia (8).

Because the energy requirements of developing sperm proximal to the blood-testis barrier are met primarily through the consumption of lactate, whereas mature sperm rely on carbohydrates for their energy needs (27), possible damage to the testis mitochondria induced by oxidative stress could cause a decrease in the energy level available for developing sperm and, in this way, may be responsible for the reported impairment of testicular function in diabetes (9, 17, 22).

In conclusion, the results from the present study show that GK diabetic testis mitochondria are less susceptible to the induction of oxidative stress. The higher content in GSH and CoQ9 contributes to the observed antioxidant protection in this animal model of diabetes. Despite upregulated defense mechanisms, oxidative damage could be sufficient to impair testicular function in diabetes given that, as suggested by McVary et al. (26), diabetic sexual dysfunction could reflect central and peripheral neuropathic disease processes.

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