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Can vitamins C and E restore the androgen level and hypersensitivity of the vas deferens in hyperglycemic rats?

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Abstract:
Diabetic neuropathy can affect the male reproductive system. The aim of this study was therefore to evaluate whether antioxidant (vitamins C and/or E) treatment could attenuate reproductive dysfunctions in hyperglycemic adult male rats. The animals were randomly assigned to one of four experimental groups: hyperglycemic control (Hy), hyperglycemic + 150 mg/day vitamin C (HyC), hyperglycemic + 100 mg/day vitamin E (HyE) or hyperglycemic + vitamins C and E (HyCE). The normoglycemic group (n = 10) received only the vehicles. The testosterone level and noradrenergic response of the vas deferens were analyzed. Both vitamins significantly decreased the TBARS (thiobarbituric acid reactive species) level in the hyperglycemic groups. There was a significant reduction in the testosterone level in the Hy and HyE groups when compared to the normoglycemic group. However, the testosterone levels were partially recovered in the HyC and HyCE groups. In addition, an increased sensitivity of the α-1 adrenoceptor in the vas deferens of the hyperglycemic control group was observed. Treatment with vitamins partially restored (vitamin E or in combination with vitamin C) or totally (vitamin C alone) this dysfunction. Moreover, the maximum response values to norepinephrine were similar among all groups. Thus, we concluded that vitamin C is more efficient than vitamin E in attenuating the effects of hyperglycemia on the male reproductive system of adult rats.

Key words:
vitamin C, vitamin E, hyperglycemia, testosterone, vas deferens, norepinephrine, male reproductive system, male rat

Introduction

Diabetes, obesity, genetic predisposition and aging are some factors that may lead to a hyperglycemic state. The effects of hyperglycemia may occur through different mechanisms and impact many bodily functions, including reproduction. Sexual dysfunctions, such as decreases in fertility, testosterone levels and sperm count, have been extensively de-

scribed in hyperglycemic males [3, 36, 40, 46]. Autonomic nervous system neuropathy, also known as diabetic neuropathy, has also been described [22, 29, 30, 41, 45] and can damage the ejaculatory process [16, 33, 36, 39]. These neuropathies affect 50–60% of diabetic patients, making them the most common complication in diabetes [9, 19].

Ejaculation is a complex process stimulated by a series of biochemical events and depends on serotonin, dopamine, oxytocin, GABA, adrenaline, acetylcholine [12], testosterone, neuropeptide Y, vasoactive intestinal peptide and nitric oxide, and it is controlled by the sympathetic autonomic nervous system [13, 35]. The central ejaculatory neural circuit comprises the spinal cord and cerebral areas, which form a highly interconnected network. The sympathetic and parasympathetic systems, as well as the somatic spinal centers, under the influence of sensory genital and cerebral stimuli integrated and processed at the spinal cord level, act in synergy to control physiological events occurring during ejaculation [13]. The efferent reflex of the nervous system, responsible for the emission phase of ejaculation, consists of sympathetic efferent fibers of the hypogastric nerve that primarily release noradrenaline, causing propulsive contractions of the epididymis, vas deferens, prostate and seminal vesicle and thus expelling sperm to the prostatic urethra [35]. Many animal studies on the function, biochemistry and sensitivity of α receptors to adrenergic agonists of the vas deferens have shown that the organ machinery is impaired in the hyperglycemic model [22, 28, 29], which can be correlated with ejaculatory dysfunction. Such changes may be related to decreases in testosterone and/or insulin levels [22–24, 28].

Another common consequence of the hyperglycemic state is increased oxidative stress [5, 6, 18, 26, 27], which is extremely toxic to cells and exerts its devastating effects directly, by damaging cellular proteins, lipids, and DNA, or indirectly, by affecting normal cellular signaling and gene regulation [34, 44]. This oxidative stress has a positive relationship with functional, structural and biochemical abnormalities in the autonomic nervous system [9, 15, 19, 34]. To investigate this aspect of hyperglycemia, different types of antioxidants have been utilized to reduce nerve function deficit in experimental conditions and at least partially diminish the complications caused by this disease [34, 44].

Vitamin E (α-tocopherol), a lipid-soluble vitamin, is present in biological membranes and is one of the major biological antioxidants [42]. Vitamin C (ascorbic acid) is a water-soluble vitamin required for multiple biological functions in humans and animals, such as the biosynthesis of collagen, conversion of dopamine to norepinephrine, recycling of α-tocopherol, antioxidant potential [21] and low levels of vitamin C occur in several pathologies [20]. In addition, vitamins C and E have a potential therapeutic role in chronic disease treatment.

In spite of the information above, almost nothing is known about the possible protective effect of vitamins C and E on the isometric contractions of the vas deferens in streptozotocin- induced hyperglycemic rats. This effect may be related to male reproductive system dysfunctions that alter ejaculation and sperm transit through the epididymis. Günes et al. [15] have shown that the use of antioxidants (stobadine and vitamin E) in hyperglycemic rats might be an effective therapy for restoring sympathetic neurotransmission in the vas deferens.

Based on these facts and on the clinical relevance of this subject, as well as on the lack of information in the scientific literature, this study aimed to verify whether treatment with vitamin C and E (alone or in combination) was able to attenuate or eliminate the effects of hyperglycemia on the male reproductive system of adult rats.

Materials and Methods

Animals

Adult male Wistar rats (90 days old; 350–410 g) were supplied by the Multidisciplinary Center for Biological Investigation, State University of Campinas (CEMIB – UNICAMP) and were housed in polypropylene cages with laboratory-grade pine shavings as bedding. Rats were maintained under controlled temperature (23 ± 1°C) and lighting conditions (12 h light/dark photoperiod, lights switched off at 7:00 a.m.). Rat chow and filtered tap water were provided *ad libitum*. The experimental protocol followed the Ethical Principles in Animal Research of the Brazilian College of Animal Experimentation and was approved by the Biosciences Institute Ethics Committee for Animal Experimentation (022/06-CEEA) – UNESP Botucatu.

Hyperglycemic model and experimental protocol

Severe diabetes in rats, which reproduces uncontrolled type 1 diabetes in humans, was chemically induced using a single dose of 40 mg/kg b.w. streptozotocin (Sigma-Aldrich, St. Louis, MO, USA) injected into the tail vein of adult male rats (n = 40). Streptozotocin was diluted in citrate buffer (0.01 M, pH 4.6). Five days after induction, glucose levels of all animals were assessed using glucose test strips and a monitoring system (One Touch Ultra, Johnson & Johnson®). Rats with glucose blood levels higher than 300 mg/dl were considered to be hyperglycemic, as previously described by Guneli et al. [14] These animals were then randomly assigned to one of four experimental groups of 10 animals each: hyperglycemic control (Hy), hyperglycemic + 150 mg/day of vitamin C (HyC), hyperglycemic + 100 mg/day of vitamin E (HyE) or hyperglycemic + 150 mg/day of vitamins C + 100 mg/day of E (HyCE). These doses were adapted from Naziroğlu [26]. Another animal group (n = 10) received no streptozotocin (normoglycemic group, N group) and presented glycemic levels lower than 100 mg/dl. Hyperglycemic animals received the vitamins by gavage (oral route) over 30 consecutive days. Normoglycemic and hyperglycemic groups received only the vehicles (corn oil and water) during the same period.

Preparation of vitamins

Vitamin C (Sigma-Aldrich, St. Louis, MO, USA) was prepared daily by diluting the required quantity in the corresponding volume of warm water. Vitamin E (Sigma-Aldrich, St. Louis, MO, USA) was diluted in corn oil before the experimental period and was used throughout the experiment. The oil and vitamin E were heated to 54°C and combined into a homogeneous mixture. This mixture was stored at 20°C, and its stability was verified by HPLC once a week at the Center for Metabolism in Exercise and Nutrition (CeMENutri) of the Botucatu School of Medicine – UNESP. Both vitamins were stored in dark containers for protection against light.

Body and some reproductive organs weights

At the end of the treatment, 9 or 10 rats from each experimental group were weighed, slightly anesthetized with 3% sodium pentobarbital (Hyptonol® – 0.1 ml/kg b.w.) and sacrificed by decapitation. Both the left vas deferens and seminal vesicle (without the coagulating gland) were removed and freed of secretion. Their relative weights were determined on an analytical balance. The right vas deferens was immediately used for pharmacological analysis.

Plasma testosterone level

After decapitation, blood was collected (between 9:00 and 11:30 a.m.) from the ruptured cervical vessels in a heparinized tube (Liquemine, Hoffmann-La Roche, Switzerland) for the determination of plasma testosterone levels. The plasma was obtained after centrifugation (2,400 rpm, 20 min, 3.5°C) in a refrigerated device and was frozen at –20°C until measurement. Plasma testosterone levels were ascertained by double-antibody radioimmunoassay using a Testosterone Maia® kit (Biochem Immuno Systems, Allentown, PA, USA) at the Neuroendocrinology Laboratory, Dental School of Ribeirão Preto, University of São Paulo – USP. All of the samples were dosed in the same assay to avoid inter-assay errors. The lowest detection limit for testosterone was 0.064 ng/ml, with a 4% intra-assay error.

Thiobarbituric acid reactive species (TBARS) analysis

The remainder of the heparinized blood (3 ml) was properly prepared and stored at –80°C to measure the concentration of TBARS, which indicates the level of lipid peroxidation by oxidative stress. Lipid peroxides were estimated in washed erythrocytes using thiobarbituric acid (TBA). One milliliter of washed erythrocytes was added to a test tube containing 1.0 ml of 3.0% sulfosalicylic acid and was then shaken for 10 s, centrifuged at 15,557 × g for 3 min and kept at rest for 15 min. The sample was then diluted to 500 µl with 0.67% TBA solution. The mixture was heated to 80°C for 30 min, and absorbance was measured at 535 nm. Results were expressed as nM of TBARS per gram of hemoglobin (nM/g Hb), indirectly representing a lipid peroxidation index according to Ferreira et al. [8].

Pharmacological analysis of the isolated vas deferens

The right vas deferens isolated from 5 or 6 rats of each experimental group was individually set in 10 ml

organ baths containing a continuous nutritive solution, aerated with a mixture of 95% O_2 and 5% CO_2 and kept at 30°C according to the methods previously described by Pereira [31]. The composition of the nutritive solution was (in mM): 136.0 NaCl, 5.7 KCl, 1.8 $CaCl_2$, 0.36 $NaH_2PO_4.H_2O$, 15.0 $NaHCO_3$, and 5.5 dextrose. This solution was prepared in glass-distilled water [32]. A resting tension of 1.0 g was applied to the tissue, with changes in isometric tension measured *via* force-displacement transducers. After an initial resting period of 45 min, complete concentration-response curves for norepinephrine (NE) (arterenol bitartrate, Sigma) and tyramine (Ty) (Sigma, USA) were obtained by the cumulative addition of molar concentrations of the agonists that were geometrically increased [43]. The pD_2 values, expressed as the negative of the logarithm for the agonist concentration that produced 50% (ED_{50}) of its maximum effect [25], were ascertained. In addition, the maximal contractile response (g of wet tissue) to NE was determined.

Statistical analysis

To compare the results among the five experimental groups, statistical tests for analysis of variance were utilized – ANOVA, with the *post-hoc* Tukey test or the non-parametric Kruskal-Wallis test with the Dunn *post-hoc* test – according to the characteristics of each variable. Differences were considered significant when $p < 0.05$.

Results

Five days after the streptozotocin injection, animals began to show characteristic signs of hyperglycemia, such as polyphagia, polydipsia and polyuria, which persisted throughout the experimental period. In addition, these animals also exhibited glucose levels above 300 mg/dl, characteristic of a hyperglycemic state (Tab. 1). The normoglycemic group showed normal glucose levels below 100 mg/dl (Tab. 1).

Tab. 1. Body weight, wet vas deferens, empty seminal vesicle and vesicle secretion weights and glycemia levels

	Normoglycemic (n = 10)	Hyperglycemic (n = 10)	Hyperglycemic + vitamin C (n = 09)	Hyperglycemic + vitamin E (n = 10)	Hyperglycemic + vitamins C and E (n = 10)
Body weight (g)	417.40 ± 9.02[a]	303.40 ± 9.80[b]	303.44 ± 7.27[b]	298.40 ± 10.06[b]	309.30± 9.00[b]
Vas deferens (mg)	102.48 ± 4.03[a]	93.43 ± 4.70[a,b]	80.39 ± 3.11[b]	82.71 ± 3.19[b]	83.44 ± 4.50[b]
Empty seminal vesicle (g)	0.57 ± 0.05[a]	0.26 ± 0.02[b]	0.30 ± 0.03[b]	0.27 ± 0.02[b]	0.30 ± 0.03[b]
Seminal vesicle secretion (g)	0.81 ± 0.06[a]	0.28 ± 0.11[b]	0.30 ± 0.11[b]	0.14 ± 0.04[b]	0.18 ± 0.04[b]
Glycemia 5 days after induction (mg/dl)	94.00 ± 3.33[a]	401.60 ± 20.03[b]	386.00 ± 18.30[b]	378.70 ± 21.66[b]	365.3 ± 10.35[b]
Final glycemia (mg/dl)	93.00 ± 2.12[a]	555.40 ± 11.20[b]	543.11 ± 17.56[b]	562.40 ± 15.00[b]	573.60 ± 10.25[b]

Values expressed as the means ± SEM. [a, b] Indicate statistically different results ($p < 0.05$); ANOVA with the Tukey *post-hoc* test

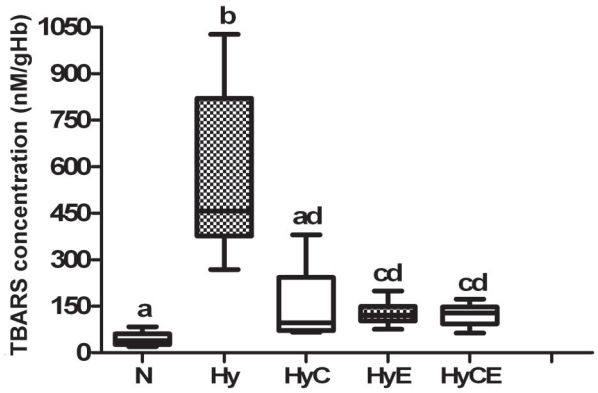

Fig. 1. Blood oxidative stress level. Values expressed as median, quartiles, minimum and maximum values (vertical bars). [a, b, c, d] Indicate statistically different results ($p < 0.05$); Kruskal-Wallis test with the Dunn *post-hoc* test. N = normoglycemic group; Hy = hyperglycemic group; HyC = hyperglycemic + vitamin C; HyE = hyperglycemic + vitamin E; HyCE = hyperglycemic + vitamin C + vitamin E

Tab. 2. Responses to norepinephrine (NE) and tyramine (Ty) (pD_2), and maximal response to norepinephrine (E_{max} to NE) of isolated vas deferens

Groups	pD_2[1]		E_{max} to NE (n = 06)
	NE (n = 06)	Ty (n = 05)	
Normoglycemic	5.31 ± 0.05[a]	4.10 ± 0.12[a]	1.52 ± 0.05
Hyperglycemic	5.63 ± 0.04[b]	4.49 ± 0.04[b]	1.54 ± 0.11
Hyperglycemic + Vitamin C	5.35 ± 0.08[a]	4.43 ± 0.05[a,b]	1.44 ± 0.19
Hyperglycemic + Vitamin E	5.40 ± 0.07[a,b]	4.50 ± 0.08[b]	1.50 ± 0.18
Hyperglycemic + Vitamins C and E	5.46 ± 0.08[a,b]	4.45 ± 0.08[b]	1.67 ± 0.25

[1] $pD_2 = -\log[ED_{50}]$. E_{max} = maximal contractile response (g/100 g of tissue). Values expressed as the mean ± SEM. [a, b] Indicate statistically different results ($p < 0.05$); ANOVA with the Tukey *post-hoc* test

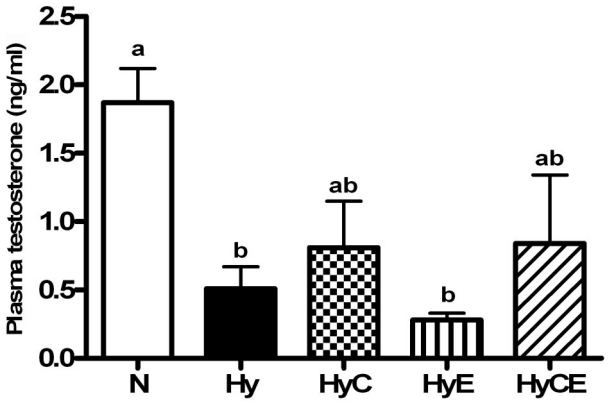

Fig. 2. Plasma testosterone level. Values expressed as the means and SEM. (vertical bars). [a, b] Indicate statistically different results ($p < 0.05$); Kruskal-Wallis test with the Dunn *post-hoc* test. N = normoglycemic group; Hy = hyperglycemic group; HyC = hyperglycemic + vitamin C; HyE = hyperglycemic + vitamin E; HyCE = hyperglycemic + vitamin C + vitamin E

The lipid peroxidation level (TBARS) (Fig. 1) was significantly increased in the hyperglycemic control group relative to the other groups. The treatments with vitamin C and E (isolated or in combination) were able to significantly reduce this parameter in relation to the hyperglycemic group. However, TBARS levels in the normoglycemic group were similar only in the vitamin C-treated group.

Body and reproductive organ weights are shown in Table 1. There was a significant reduction in body weight, empty seminal vesicle weight, and seminal vesicle secretion weights in the hyperglycemic groups compared to the normoglycemic group. Vitamin C treatment was able to partially recover the empty seminal vesicle weight. However, the vas deferens relative weight increased significantly in the hyperglycemic control group relative to the normoglycemic group. This alteration was completely reverted by treatment with vitamin C alone or in combination with vitamin E. This reversion was partial with vitamin E alone. Plasma testosterone levels (Fig. 2) were reduced in all hyperglycemic groups compared to the normoglycemic group and partially recovered by treatment with vitamin C, alone or in combination with vitamin E.

Although the maximum response values to NE were statistically similar among all groups (Tab. 2), the *in vitro* isometric contractions of the vas deferens revealed a significant increase in the pD_2 value of NE in the hyperglycemic control group compared to the normoglycemic group. This result revealed the hypersensitivity of the post-junction α-1 adrenoceptor. However, vitamin C alone completely recovered this hypersensitivity because the pD_2 value of NE was similar to the normoglycemic group (Tab. 2 and Fig. 3A). This parameter was only partially recovered in the hyperglycemic groups treated with vitamin E (alone or in combination) (Tab. 2 and Fig. 3A). The Ty curve dislocation to the left (Fig. 3B) and the increase of the pD_2 value (Tab. 2) in the hyperglycemic control and hyperglycemic plus vitamin E (alone or in combination with vitamin C) groups (Tab. 2 and Fig. 3B) confirmed this hypersensitivity. This sensitivity was partially recovered in the vitamin C treated group because Ty pD_2 values in this group were similar to all other groups (Tab. 2 and Fig. 3B).

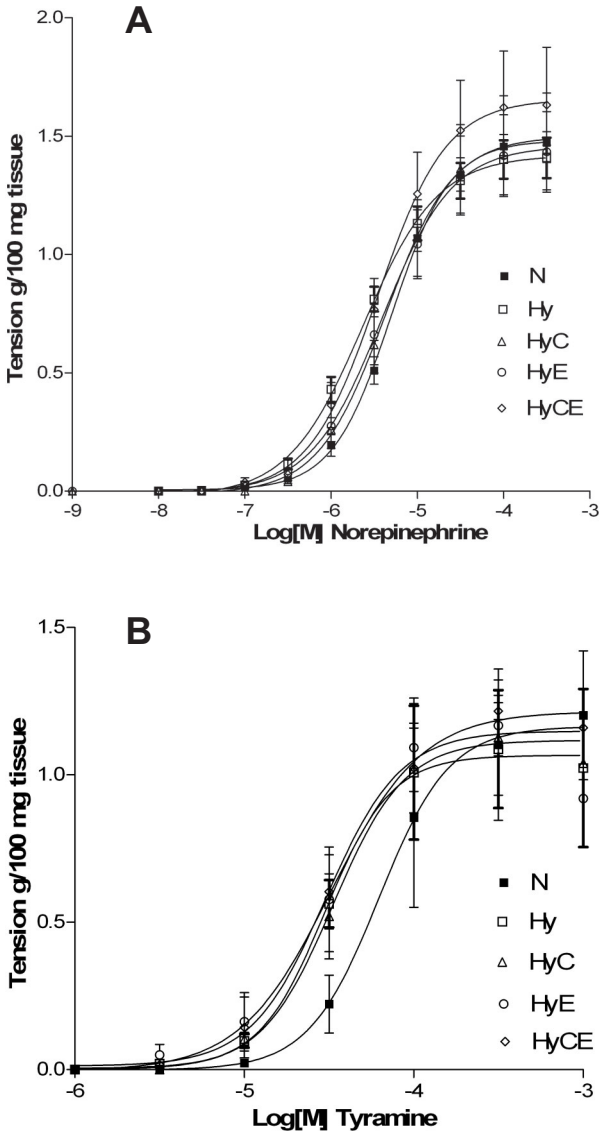

Fig. 3. Cumulative concentration response for norepinephrine (**A**) and tyramine (**B**) in vas deferens isolated from rats of different experimental groups. Abscissas show the molar concentration of the drugs on a logarithmic scale. Ordinates show effects produced by the drug, expressed as grams per 100 mg tissue. Vertical bars indicate SEM. N = normoglycemic group; Hy = hyperglycemic group; HyC = hyperglycemic + vitamin C; HyE = hyperglycemic + vitamin E; HyCE = hyperglycemic + vitamin C + vitamin E

Discussion

In this study, the antioxidant potential of vitamin C and E in hyperglycemic animals was demonstrated, as lipid peroxidation in erythrocytes was significantly reduced in the vitamin-treated hyperglycemic groups. These results corroborated previous studies that showed both vitamin C [5] and E [11] expressed antioxidant potential by reducing the oxidative stress level in erythrocytes of streptozotocin-induced hyperglycemic rats. Our results clearly demonstrated that vitamin C and E supplementation eliminated lipid peroxidation in hyperglycemia and provided evidence that vitamins C and E may have a therapeutic role in diseases that are mediated by reactive oxygen species. Furthermore, the reduction of lipid peroxidation in erythrocytes may have occurred in the organs of the male reproductive system and in the nervous system because previous studies reported that vitamins C and E concomitantly attenuated lipid peroxidation in different tissues of hyperglycemic rats [5, 10].

The decreased body weight in hyperglycemic animals could be attributed to metabolic changes that arise from the absence or decrease of insulin in the blood [37, 39], which is characteristic of a hyperglycemic condition. In addition, the hypersensitivity of the post-junction α-1 adrenoceptor to NE in the hyperglycemic control group shown in this study was corroborated by previous studies [30, 41]. Our results suggest that a hyperglycemic organism is trying to compensate for the damage caused by the illness by preserving the contractile capacity of the vas deferens in order to facilitate sperm release. Therefore, this α-1 adrenoceptor hypersensitivity to exogenous NE in the hyperglycemic control group might have been due to diminished release of endogenous NE in the nerve endings of these animals, as evidenced by the Ty data. Oztürk et al. [29] also showed that intrinsic activities for both NE- and Ty-induced contractions of the vas deferens in rats were increased in short-term alloxan diabetes (72 h) and decreased in long-term alloxan diabetes (8 weeks). These authors also reported that α-adrenergic responsiveness in diabetic rat vas deferens depended on the time elapsed. Moreover, Günes et al. [15] demonstrated an increased α-adrenergic responsiveness of diabetic vas deferens to exogenous NE. This response might be attributable to new receptor synthesis or activation of post-receptor events due to diabetes-induced neuropathy rather than an enhancement in α-adrenoceptor affinity. In contrast, Kamata and Kirisawa [17] showed that the dose-response curve for the contractile response of the vas deferens to NE was significantly shifted to the right and that the apparent affinity (pD_2 values) was significantly decreased in streptozotocin-induced hyperglycemic rats.

In the present study, vitamins C and E were observed to partially (vitamin E alone or in combination) or totally (vitamin C) recover α-1 adrenoceptor hypersensitivity to NE in hyperglycemic rats. In a similar way, Günes et al. [15] demonstrated that antioxidant treatment (stobadine and vitamin E) significantly decreased hemoglobin glycosylation and that stobadine completely returned NE-induced contractions to basal levels, whereas vitamin E alone had no effect.

We suggest that vitamin C and E supplementation may have an important therapeutic role in diabetic neuropathies, especially those related to the male reproductive system. Several animal studies have demonstrated that antioxidants can prevent or reverse nerve dysfunctions induced by hyperglycemia. Additionally, several antioxidants (e.g., vitamin C, vitamin E, resveratrol, α-lipolic acid, taurine) have demonstrated amelioration of the nerve function in experimental diabetes [19, 34, 44].

In relation to Ty response, vitamin C alone was more efficient in inducing NE release in hyperglycemic animals. The NE stock in the sympathetic nerve endings was likely partially increased, resulting in a greater release of NE by Ty. This effect was not evident in the vitamin E-treated hyperglycemic groups despite the decreased α-adrenoceptor hypersensitivity to NE in both groups. However, despite the change in the sensitivity of the organ to NE, the hyperglycemic rats did not show any damage in maximal contractile capacity induced by NE. Previous studies using streptozotocin-induced hyperglycemia showed that at least some of the rats could ejaculate during sexual behavior tests [33, 36, 39]. Further studies using the same experimental design of our study could be conducted testing the sexual behavior of the hyperglycemic rats and compare the *in vivo* results with those *in vitro*.

The significant reduction of plasma testosterone in the hyperglycemic group was corroborated by previous studies [4, 16, 36]. These studies reported that testosterone diminished with insulin shortage. Nevertheless, the HyC and HyCE groups presented here partially increased plasma testosterone levels. This was consistent with the previous finding that vitamin C [2, 38] and vitamin E [7] played key roles in the steroidogenic process that stimulates testosterone synthesis. However, in the vitamin E-treated hyperglycemic group, neither this stimulation nor testosterone release occurred. The dose of vitamin E used in this group was therefore likely inadequate to stimulate these processes. Nevertheless, this decrease in plasma testosterone (total or partial) observed in hyperglycemic groups probably caused reduction in the empty seminal vesicle and vesicle secretion weights. This change could indicate hyperglycemia-induced function impairment of these androgen-dependent organs [1, 36]. In our study, vitamin C attenuated the seminal vesicle relative weight loss compared to other hyperglycemic groups, probably due to body weight reduction.

The observed decrease in plasma testosterone may cause hypersensitivity of the post-junction α-1 adrenoceptor of the vas deferens because contractile responses of androgen-sensitive smooth muscles are highly dependent upon the endocrine status of the animal. The decreased testosterone levels were at least partially responsible for the changes in the weight and contractility of the vas deferens of streptozotocin-hyperglycemic [22] and castrated [24] rats. In addition, the impairment of the vas deferens contractile response in hyperglycemic animals could be only partially reverted by insulin supplementation [28].

In this experimental protocol, vitamins C and E partially to fully ameliorated the negative effects of hyperglycemia on vas deferens sympathetic neurotransmission and plasma testosterone levels in rats. In addition, vitamin C was more efficient than vitamin E in attenuating the effects of hyperglycemia on the male reproductive system of adult rats.

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