**Pharmacological Reports**
2011, 63, 130–138
ISSN 1734-1140



# Ejaculatory dysfunction in streptozotocin-induced diabetic rats: the role of testosterone

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**Abstract:**
Hyperglycemic and hypoinsulinemic states caused by diabetes mellitus are usually related to some type of sexual dysfunction, resulting in infertility in humans and experimental models, mostly due to their effects on ejaculatory function. This study aimed to evaluate the possible role of testosterone in the restoration of normal ejaculatory function in diabetic rats. Male Wistar rats were randomly allocated into 3 experimental groups: control, diabetic (streptozotocin), and diabetic with testosterone supplementation (streptozotocin plus testosterone). The following parameters were assessed at the end of the experiment: body weight, circulating testosterone levels, number of spermatozoa ejaculated in the uterus through natural mating, and weight and *in vitro* isometric contractions of the vas deferens. Diabetic rats showed reduced plasma testosterone levels and ejaculatory dysfunction as observed by a lack in the spermatozoa ejaculated into the uterus of receptive females. In these diabetic rats, no difference was observed in the sensitivity of the vas deferens to norepinephrine, with or without the presence of the cocktail (cocaine plus propranolol). In spite of this, an increased sensitivity to methoxamine through the $\alpha_1$-adrenoceptor was observed. Testosterone supplementation did not restore these parameters to control values. We conclude that, in this experimental model, the lack of testosterone was not directly related to the diabetes-induced ejaculatory dysfunction.

**Key words:**
diabetes, ejaculation, testosterone, norepinephrine, infertility

## Introduction

Diabetes mellitus is one of the most widespread diseases that threatens human health in the modern world, affecting over 171 million people; 366 million people are projected to be diabetic in the year 2030 [47]. Its incidence is increasing rapidly, and its effects on human health are mostly due to the hyperglycemic and hypoinsulinemic states caused by the disease, usually affecting neurological, endocrinological and reproductive functions. Obesity, genetic predisposition and ageing are thought to be the leading factors in this worldwide current scene. The most worrying aspect of diabetes is that it compromises many men at reproductive age, and most of them are not aware of their illness, whether at initial or late stages [38].

Sexual dysfunctions related to the diabetic state have been extensively described [4, 6, 8, 11–13, 23, 25, 27, 48], but their pathophysiological pathways are yet to be clearly elucidated. These studies demonstrated that decreased levels of testosterone in diabetic men and experimental models led to impairment of reproductive parameters, but the direct role of testosterone in the process is unclear.

It is believed that pelvic autonomic neuropathy contributes to impotence and retrograde ejaculation in the male. Gallego et al. [14] stated that the catecholaminergic systems in the diabetic rat are affected in a highly specific manner, with decreased dopamine levels in the dopaminergic nigrostriatal system, increased norepinephrine levels in cardiac ventricles, decreased norepinephrine levels in the stellate ganglia and the blood serum and elevated epinephrine levels in the adrenal gland and diminished epinephrine levels in serum.

Ejaculation is a process stimulated by a series of complex events and depends on coadjuvants, such as testosterone, oxytocin, neuropeptide Y (NPY), vasoactive intestinal peptide (VIP) and nitric oxide (NO) to run effectively. The main role is played by the sympathetic and parasympathetic autonomic neural transmission, leading to a balance of stimulatory (norepinephrine and epinephrine, among others) and relaxant neurotransmitters (acetylcholine locally and serotonin centrally) taking place on the smooth muscle present in the vas deferens and the epididymides, organs through which the sperm passes. At this moment, spermatozoa are still immotile and therefore require emission of seminal fluid and local muscle contractions to continue their passage through the male reproductive system [15, 34].

The effects of diabetes on the rat ejaculatory process, which can lead to a decreased fertility rate *in vivo* [37], can be explained by a secondary complication of diabetes, an autonomic neuropathy syndrome [18, 44]. This pathological situation affects autonomic neurotransmission involved in ejaculation, among other important biological functions, acting both on the vas deferens and the epididymis, 2 organs important to sperm transit through the male reproductive tract, dependent on sympathetic and parasympathetic neurotransmission.

Although the altered ejaculatory parameters in diabetic rats have been analyzed in other studies [31, 35, 41], the role of testosterone in this process is not clearly understood. This study aimed to evaluate whether testosterone supplementation has any effect on restoring the ejaculatory function in diabetic rats. This study is unique in that it evaluates the role of testosterone supplementation in this diabetes-induced setting.

## Materials and Methods

Adult male and female Wistar rats (90–120 days old) were supplied by the Multidisciplinary Center for Biological Investigation, State University of Campinas (CEMIB-UNICAMP) and were housed in same-sex polypropylene cages (43 × 30 × 15 cm), with laboratory-grade pine shavings as bedding. Rats were maintained under controlled temperatures (23 ± 1°C) and lighting conditions (12L, 12D photoperiod, lights switched off at 7:00 am). Rat chow and filtered tap water were provided *ad libitum*. Experimental protocols followed the Ethical Principles in Animal Research of the Brazilian College of Animal Experimentation and were approved by the Biosciences Institute Ethical Committee for Animal Experimentation/ UNESP Botucatu (Protocol 44/06-CEEA).

Male rats were randomly assigned to one of 3 experimental groups: control (vehicle), diabetic or diabetic + testosterone. Diabetes was chemically induced using a single dose of intravenous 40 mg/kg b.w. streptozotocin (SIGMA Chemical Company, St. Louis, MO) diluted in a citrate buffer 0.01 M, pH 4.6. Five days after the induction, glycemia of all animals was assessed using glucose test strips and a monitoring system (One Touch Ultra, Johnson & Johnson®), and all animals presenting levels higher than 120 mg/dl were considered diabetic. On the same day, one group of diabetic animals were submitted to surgery in which they received a silastic capsule implant filled with testosterone in the subscapular space [10]; animals from the other groups were sham operated.

### EXPERIMENT 1

#### Sexual behavior

Male rats were tested for sexual behavior 15 days before the treatment period and only sexually active ones were selected for the experiment. Rats were randomly assigned to one of 3 groups: control (vehicle, n = 9), diabetic (n = 9), or diabetic + testosterone (n = 8). 3 weeks after diabetes induction animals were tested

for sexual behavior as previously described [37], with the exception that females in natural estrus were utilized and the tests were performed in the morning, from 8:00–12:00 pm.

### Ejaculated sperm counts after natural breeding

The procedure followed in this study was previously described by Kempinas et al. [22], with the following adaptations. At the end of the sexual behavior evaluation, all the male rats, regardless of their performance during the test, were allowed to cohabit with a receptive female for an additional 4 h and then the number of ejaculated sperm was assessed. After the mating period female rats were decapitated and uterine sperm were enumerated. Fine curved forceps were used to elevate the cervix, and the cervix was ligated. Uterine horns were excised, trimmed, washed in Dulbecco's PBS, transferred to a 35-mm Petri dish containing 2 ml of warm Medium 199 (Gibco™, Auckland NZ) and opened using small scissors. The dish was shaken gently for 10 min of dispersion and placed in the incubator at 35°C, thereby allowing the uterine sperm to disperse. The sperm suspension was then transferred to a 15-ml conical tube, and then sonicated for 30 s. A sample of 100 µl was diluted 1:20 with fixative (10% formalin in PBS with 10% sucrose, pH 7.4), and spermatozoa were counted using a hemocytometer.

## EXPERIMENT 2

Twenty-one male rats were randomly assigned to one of 3 experimental groups, with 7 animals in each group: control (vehicle), diabetic or diabetic + testosterone. Due to the death of one animal per diabetic group observed during the treatment period, all experimental groups were standardized to 6 animals per group.

### Body weight and reproductive glands and vas deferens wet weights

Three weeks after diabetes induction, the rats were weighed and then mildly anesthetized with diethyl ether and then killed by decapitation. The vas deferens, ventral prostate and seminal vesicle (without the coagulating glands and both full and without the secretion) were removed and weighed.

### Pharmacological analysis: organ bath studies

The vasa deferentia isolated from rats of the experimental groups were individually set up in 10 ml organ-baths containing continuously nutritive solution, aerated with a mixture of 95% $O_2$ and 5% $CO_2$ and maintained at 30°C to inhibit spontaneous contractions, according to the methods previously described by Pereira [32]. The composition of the nutritive solution consisted of: NaCl 136.0 mM; KCl 5.7 mM; $CaCl_2$ 1.8 mM; $NaH_2PO_4 \cdot H_2O$ 0.36 mM; $NaHCO_3$ 15.0 mM; dextrose 5.5 mM, prepared in glass distilled water [33]. 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 (Sigma Co., USA), methoxamine (Sigma Co., USA), and norepinephrine in the presence of a cocktail containing 6 µM cocaine plus 0.1 µM propranolol (Sigma Co., USA) were obtained by cumulative addition of molar concentrations of the agonists increasing geometrically [42]. The $pD_2$ values, expressed as the negative of the logarithm for the agonist concentration producing 50% ($ED_{50}$) of its maximum effects [26], were determined. In addition, the maximal contractile responses (g of wet tissue) to all agonists were determined.

### Hormone assays

After decapitation, blood was collected (between 9:00 and 11:30 am) from the ruptured cervical vessels in a heparinized tube 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 frozen at –20°C until the moment of hormonal determination. Plasma testosterone levels were determined by double-antibody radioimmunoassay, using the Testosterone Maia® kit (Biochem Immuno System). All the samples were dosed in the same assay, to avoid inter-assay errors. The lower detection limit for testosterone was 0.064 ng/ml, with a 4% intra-assay error.

### Statistics

To compare results among the 3 experimental groups, statistical tests for analysis of variance were utilized – ANOVA – with the *post-hoc* Tukey-Kramer test or the non-parametric Kruskall-Wallis test, with the *post-*

*hoc* Dunn or Bonferroni test – according to the characteristics of each variable. Differences were considered significant when $p < 0.05$.

## Results

### Experiment 1

The investigation of sexual behavior showed that, while 89% of the control rats ejaculated (8 of the 9 rats), only 33.33% (3 of the 9 rats) of the diabetic animals were sexually active, and only 2 of them ejaculated. Testosterone supplementation in diabetic rats resulted in an increase in the number of animals that presented both sexual and ejaculatory behavior (62.5%, or 5 of the 8 rats). No significant alterations were noted in the other evaluated parameters (data not shown).

The sperm count in the uterus demonstrated that none of diabetic rats ejaculated. In diabetic rats with testosterone supplementation, only 1 in 8 (12.5%) ejaculated, and this group produced around 70.9% less spermatozoa than the control group (diabetic + testosterone: $12.50 \times 10^6$; control group: $42.95 \pm 5.39 \times 10^6$, the mean ± SEM). In the control group, 8 of 9 animals (88.89%) ejaculated.

### Experiment 2

Five days after streptozotocin injection, all animals in the diabetic and diabetic + testosterone groups showed glycemia levels above 120 mg/dl. The same was verified 3 weeks after induction. Rats from the control group showed normal glycemia levels, below 120 mg/dl (Tab. 1).

Three weeks after diabetes induction, diabetic rats without hormone supplementation showed decreased body weight than controls and testosterone supplementation was not capable of restoring these values to normal. The relative vas deferens weight was comparable between groups. However, in the diabetic group the relative weights of the seminal vesicle and the prostate were significantly less than the other groups (Tab. 1).

Testosterone levels were decreased in diabetic animals. In the androgen-supplemented diabetic group the levels of this hormone were statistically equal to those found in control animals and diabetic groups (Tab. 1); although, testosterone supplementation clearly elevated the hormone level compared to the diabetic group.

The *in vitro* biological assay for the vas deferens contractility showed no significant differences to the organ response to norepinephrine (NE) among the 3 experimental groups, with or without the presence of the cocktail containing cocaine plus propranolol. Methoxamine (MET) responses were increased, as the $\alpha_1$ receptor was more sensitive in diabetic animals, in-

**Tab. 1.** Glycemia 5 and 21 days after the beginning of the treatment, testosterone levels, final body weights and relative weights of the vas deferens, ventral prostate and seminal vesicle of rats by experimental group

| | Experimental groups | | |
|---|---|---|---|
| | Control (n = 6) | Diabetic (n = 6) | Diabetic + testosterone (n = 6) |
| Glycemia | | | |
| 5 days after induction (mg/dl) | 88.33 ± 2.96[a] | 526.89 ± 22.92[b] | 520.38 ± 30.65[b] |
| 21 days after induction (mg/dl) | 81.11 ± 1.89[a] | 541.00 ± 10.17[b] | 544.37 ± 15.83[b] |
| Body weight (g) | 392.98 ± 10.96[a] | 296.36 ± 12.64[b] | 309.82 ± 8.30[b] |
| Vas deferens (mg/100 g) | 28.79 ± 1.86[a] | 30.22 ± 1.55[a] | 32.18 ± 1.59[a] |
| Ventral prostate (mg/100 g) | 370.68 ± 15.37[a]<br>94.55 ± 3.69[a] | 189.61 ± 25.69[b]<br>64.87 ± 9.67[b] | 339.56 ± 16.82[a]<br>109.70 ± 4.88[a] |
| Seminal vesicle (full) (g/100 g) | 1.13 ± 0.06[a]<br>0.29 ± 0.01[a] | 0.39 ± 0.04[b]<br>0.13 ± 0.01[b] | 1.18 ± 0.08[a]<br>0.38 ± 0.03[a] |
| Seminal vesicle (empty) (g/100 g) | 0.50 ± 0.05[a]<br>0.13 ± 0.01[a] | 0.27 ± 0.02[b]<br>0.09 ± 0.01[b] | 0.44 ± 0.02[a]<br>0.14 ± 0.01[a] |
| Testosterone levels (ng/ml) | 1.91 ± 0.53 | 0.32 ± 0.09 | 1.42 ± 0.21 |

Data are shown as the mean ± SEM. [a,b] – Different letters indicate statistically different results ($p < 0.05$). ANOVA test, with the Tukey-Kramer *post-hoc* test

**Tab. 2.** $pD_2$ of the agonists (NE, MET, NE + cocktail) and vas deferens maximal contractile response to the drugs, obtained from the pharmacological response of the isolated organ of the animals from the 3 experimental groups

| | Experimental groups | | |
|---|---|---|---|
| | Control (n = 6) | Diabetic (n = 6) | Diabetic + testosterone (n = 6) |
| $pD_2$ | | | |
| NE | 5.43 ± 0.09 | 5.55 ± 0.06 | 5.70 ± 0.05 |
| NE + cocktail | 6.08 ± 0.14 | 6.23 ± 0.09 | 6.29 ± 0.07 |
| MET | 4.36 ± 0.11 | 4.91 ± 0.06 | 5.09 ± 0.09 |
| Emax | | | |
| NE | 1.48 ± 0.06 | 1.60 ± 0.12 | 1.37 ± 0.06 |
| MET | 1.45 ± 0.05 | 1.62 ± 0.12 | 1.24 ± 0.09 |
| NE + cocktail | 1.96 ± 0.20 | 1.99 ± 0.12 | 1.78 ± 0.08 |

Data are shown as the mean ± SEM. [1] $pD_2 = -\log [ED_{50}]$; [2] cocktail: cocaine + propranolol; [3] Emax: maximal contractile responses; [a,b] – different letters indicate statistically different results ($p < 0.05$). ANOVA test, with the Bonferroni *post-hoc* test

dicated by the shifting of the curve to the left (Fig. 1) and by the increase of the $pD_2$ value (Tab. 2). Testosterone supplementation was unable to reverse this increased sensitivity observed in diabetic animals. There were no differences in the vas deferens maximal contractile responses to NE independently of the presence of the cocktail, among the experimental groups, although the organ maximal response to MET was decreased in diabetic animals with hormone supplementation when compared to the diabetic group without supplementation (Tab. 2, Fig. 1).

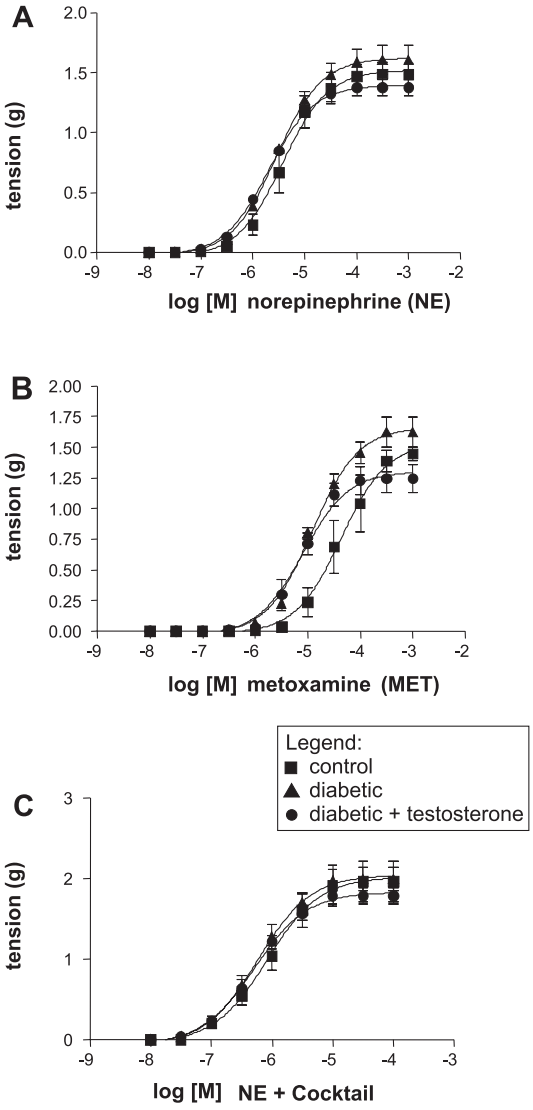


**Fig. 1.** Concentration-response curves for norepinephrine (**A**), methoxamine (**B**) and norepinephrine + cocktail (**C**), obtained from the vas deferens of male rats of the 3 experimental groups (n = 6 per group). Abscissas comprise the molar concentration of the drug in a logarithmic scale; ordinates represent maximum effect percentage. Vertical bars indicate the standard error of the mean

## Discussion

In humans it has been suggested that androgen supplementation for 3–6 months is needed to enable recovery of impaired sexual function [3, 16], whereas rats require much shorter periods to recover, at least in part, male reproductive parameters [5, 20]. The present work supports this idea because 3 weeks of testosterone supplementation was able to restore the weights of the ventral prostate and seminal vesicle and the increased maximal contractile responses to MET in vas deferens from diabetic rats, which is also observed in castration studies [2]. Another reason for using a short course of testosterone supplementation is that we did not want to prolong the duration of the experiment because in this experimental model (streptozotocin-induced diabetes), the glycemic levels are very high, and as time passes the animals become weaker [45].

However, 3 weeks of testosterone supplementation was not able to restore the ejaculatory dysfunction observed in diabetic rats, as shown by the lack of ejaculated sperm in the uterus of receptive females and hy-

persensitivity of the post-junction $\alpha_1$-adrenoceptor to its synthetic agonist MET in the vas deferens. While Steger [40] reported that testosterone was not able to restore ejaculatory function in diabetic rats, a study performed by Longhurst [24] demonstrated that a longer period of testosterone supplementation (8 weeks) was able to restore contractile parameters in the vas deferens but only partially prevented the decreased response to nerve stimulation observed in these animals.

Experimental studies also demonstrated reduction in plasma testosterone levels in diabetic animals [18, 37, 39], as seen in the present work, whereas others have reported normal levels of this hormone in induced diabetes [19]. These disparities are probably due to different experimental models and diabetic induction procedures.

In contrast to the erectile process, whose neurological and vascular mechanisms have been well elucidated, the pathophysiology of the ejaculatory process remains yet to be completely investigated. In humans, the emission and the ejection phases are regulated by an integrated and time-coordinated activity of the parasympathetic and sympathetic systems, which finally leads to sperm propulsion from the urethra [43]. In the present study, the increased sensitivity of the vas deferens to MET that was not abolished by testosterone supplementation showed that this reproductive alteration observed in diabetic animals was not directly related to the reduced testosterone levels. This result agrees with those that showed an increased sensitivity of adrenergic receptors in diabetic rats [31, 35, 41] and indicate that probably the diabetic organism is trying to compensate for the illness-induced damage by preserving or trying to preserve the capacity to produce descendents *via* improving the contractility of the vas deferens to facilitate sperm release. This hypothesis is also corroborated by the findings of Kamata et al. [21] who observed in heart ventricular muscle preparations from 4-week diabetic rats that the MET-induced smooth muscle contractions were significantly enhanced over a 10-week period, thus demonstrating a physiological change in the heart expressed by elevated mRNA levels for both the $\alpha_{1a}$-adrenoceptor and the $\alpha_{1a}$-adrenoceptor protein. It has long been known that $\alpha_1$-adrenoceptors play an important role in the control of vas deferens motility. Antagonist data in rats [9] and humans [29] demonstrate that this motility enhancement is largely mediated by $\alpha_{1a}$-adrenoceptors. Accordingly, $\alpha_{1a}$ knockout mice exhibited markedly reduced contractile responses to NE or field stimulation *in vitro*, and in $\alpha_{1A/B/D}$ triple knockout mice this response was abolished [36]. Based upon these findings, it appears that functional $\alpha_1$-adrenoceptors, particularly $\alpha_{1a}$-adrenoceptors, are essential for the physiological contraction of the vas deferens and hence for sperm delivery from the testes to the urethra. Sanbe et al. [36] also demonstrated an important role of $\alpha_1$-adrenoceptors, particularly $\alpha_{1a}$-adrenoceptors, in vas deferens function, and that inhibition of these effects can lead to male infertility. Despite the similarity, in the present study, between NE and MET contractile responses in the presence of propranolol (plus cocaine), the contractile response to MET through $\alpha_1$-adrenoceptors was augmented in diabetic rat vas deferens. These results suggest the absence of a β-receptor contribution to the contractile response of NE. Thus, we expect that NE-induced contractile responses in the presence of propranolol should be similar to the augmented α-adrenergic response sensitivity in vas deferens provoked by MET in diabetic rats. Other receptors that are not $\alpha_1$-adrenergic may also be involved in this alteration. Another possible explanation for differences in responses between NE in the presence of the cocktail (propranolol plus cocaine) *versus* MET may be the presence of postjunctional $\alpha_2$-adrenoceptors that are not excited by MET. This postjunctional $\alpha_2$-adrenoreceptor type that is undetectable in normal vas deferens may be found in reserpinized as well as denervated rat vas deferens using [$^3$H]clonidine [46]. It was seen that postjunctional $\alpha_2$-adrenoceptors, which are undetectable in normal vas deferens, were found in reserpinized as well as denervated rat vas using [$^3$H]clonidine, but it was not subsequently confirmed by means of [$^3$H]rauwolscine binding [1, 28].

Despite the effort presented by the organism, in the current study, fewer diabetic rats reached ejaculation than control rats during the sexual behavior test, corroborating Scarano et al. [37]. Steger [40] demonstrated that streptozotocin-treated rats had reduced plasma testosterone levels, but this factor alone does not appear responsible for changes in copulatory behavior, given that testosterone supplementation did not reverse the adverse effects of diabetes on sexual behavior. Moreover, after natural mating, no spermatozoa were observed in the uterus of receptive females that had been placed with diabetic rats. Testosterone supplementation was not efficient in reversing this situation, even though the testosterone level was normal.

Regarding the contractile machinery of the vas deferens, Oztürk et al. [30] demonstrated that diabetes compromises the calcium-dependent contractility. Contractile responses to KCl and calmidazolium were decreased, demonstrating that the organ's machinery is impaired in the diabetic model, which can be correlated with altered ejaculatory function. The authors also found that this situation could be only partially reverted by insulin supplementation, which suggests that other factors beside lower insulin levels are affecting the organ's function. The present study demonstrated that another possible factor involved in this process may be the decrease in testosterone plasma levels, but this possibility does not explain by itself the alterations observed in the diabetic rat, as the androgen-supplementation was unable to recover normal $\alpha_1$-receptor responses, suggesting that testosterone did not play a main role in the ejaculatory process but is most likely being an adjuvant in the process.

According to Güneş et al. [17], diabetic rats present alterations (autonomic nerval termination) in adrenergic nerves, including degenerative damage that could be responsible for an increase in the vas deferens contractile responses to electric stimulation. This damage could compromise NE neuronal uptake and therefore explain the increase in sensitivity of the $\alpha_1$-receptor in diabetes. However, there were no differences among the experimental groups as to the vas deferens responses to exogenous NE in the presence of the cocktail containing cocaine plus propranolol, thus demonstrating the absence of participation of post-junctional β-adrenoceptor and of the neuronal uptake process in the organ response of the diabetic and diabetes-plus-testosterone animals.

Given the degenerative problems observed in diabetic rat nerve endings [14, 17, 41] and decreased NE levels found in blood serum [14] together with the results herein presented (an increase in sensitivity of the $\alpha_1$-adrenoceptor in diabetes), we can suggest that this damage to the ejaculatory process has a possible neurogenic cause, but the myogenic hypothesis cannot be excluded. Thus, the diabetic state damages the ejaculatory process dependent also on the contractility of the vas deferens resulting in no sperm ejaculation, due to a number of different mechanisms, probably including problems related to the functioning of NE release at the synaptic cleft, caused primarily by autonomic neuropathy, because androgen supplementation could not recover these parameters as observed in controls.

**Acknowledgments:**
We are grateful to Dr. Débora Cristina Damasceno and Dr. Gustavo Tadeu Volpato of the Laboratory of Experimental Research of Gynecology and Obstetrics, Department of Gynecology and Obstetrics, Botucatu Medical School, São Paulo State University – UNESP, for the diabetes induction procedures. We are also grateful to Dr. Janete Aparecida Anselmo Franci of the Neuroendocrinology Laboratory, Dental School of Ribeirão Preto, University of São Paulo – USP, for the hormone assays. This work would not have been possible without the financial support of the following sponsors: CAPES-PROEX; FAPESP – The State of São Paulo Research Foundation and CNPq – National Council for Scientific and Technological Development.

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**Received:** January 2, 2010; **in the revised form:** May 10, 2010; **accepted:** June 9, 2010.