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Effect of testosterone on oxidative stress and cell damage induced by 3-nitropropionic acid in striatum of ovariectomized rats

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Abstract

This paper evaluates the effects of testosterone (0.5 mg/kg subcutaneously (s.c.) for 8 days) on oxidative stress and cell damage induced by 3-nitropropionic acid (20 mg/kg intraperitoneally (i.p.) for 4 days) in ovariectomized rats. Gonadectomy triggered oxidative damage and cell loss, evaluated by the detection of caspase-3, whereas 3-nitropropionic acid increased the levels of oxidative stress induced by ovariectomy and prompted cell damage characterized by enhanced levels of lactate dehydrogenase. These changes were blocked by testosterone administration. Our results support the following conclusions: i) ovariectomy triggers oxidative and cell damage via caspase-3 in the striatum; ii) 3-nitropropionic acid exacerbates oxidative stress induced by ovariectomy and leads to cell damage characterized by increased levels of lactate dehydrogenase; iii) testosterone administration decreases oxidative stress and cell damage. Additionally, these data support the hypothesis that testosterone might play an important role in the onset and development of neurodegenerative diseases.

Keywords: Cell damage; Huntington's disease; 3-Nitropropionic acid; Oxidative stress; Testosterone

Cell culture studies by Ramsden et al. (2003), Ahlbom et al. (2001), and Hammond et al. (2001) have demonstrated that testosterone and related androgens can attenuate neuron loss caused by certain insults. Additionally, Lewis et al. (1995) found that orchidectomy reduces the density of CA1 area pyramidal cell spines in male rats, an effect that is partially reversed by estradiol administration. Furthermore, previous studies have demonstrated that patients with neurodegenerative diseases display low levels of androgens (García-Segura et al., 1994; Rubinow and Schmidt, 1996; Hogervost et al., 2001; Militello et al., 2002; Okun et al., 2002, 2004; Markianos et al., 2005), adding to the evidence that androgens play an important role in nueroprotection. Furthermore, certain symptoms of Huntington's disease, such as reduction in muscle and bone mass, depression and cognitive impairment, also are present in elderly subjects with testosterone deficiency (Markianos et al., 2005).

Huntington's disease, an autosomal dominant inherited neurodegenerative disease, is characterized by progressive motor and cognitive deterioration (Martín and Gusella, 1986; Anderson and Marder, 2001; Bonelli and Hoffmann, 2004). Many models of Huntington's disease have been developed. Among these, systemic administration of 3-nitropropionic acid has been found to produce selective lesions that mimic those of Huntington's disease (Brouillet et al., 1995; Borlongan et al., 1995, 1997). This mycotoxin is a suicide inhibitor of succinate dehydrogenase (SDH; EC 1.3.99.1), an enzyme located in the mitochondrial inner membrane, which is responsible for the oxidation of succinate to fumarate, triggering oxidative stress. Oxidative damage is one condition leading to cell death that is thought to be important in several neurodegenerative diseases

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and is relevant to the striatal cell loss observed in Huntington's disease (La Fontaine et al., 2000a,b, 2002).

A recent study by Túnez et al. (2006) has shown a neuroprotective effect of sex steroid hormones (17 β-estradiol) against cell injury and oxidative damage induced by 3-nitropropionic acid on striatum in ovariectomized Wistar rats. This protective action was characterized by a reduction of oxidative stress and cell damage biomarkers.

This paper examines the action of testosterone on damage to striatal nucleus induced by ovariectomy and/or 3-nitropropionic acid. Based on this hypothesis, the main aim of this study is to evaluate the effects of testosterone on oxidative stress and cell damage in an experimental model of Huntington's disease in ovariectomized rats. 3-Nitropropionic acid neurotoxicity, as well as effects of testosterone, was evaluated by measuring biochemical parameters for: i) mitochondrial function: SDH activity; ii) oxidative stress: lipid peroxidation products, protein carbonyls, reduced glutathione (GSH) content and changes in the activities of superoxide dismutase (SOD; EC 1.15.1.1), catalase (EC 1.11.1.6) and glutathione peroxidase (GSH-Px; EC 1.11.1.9); and iii) cell damage: caspase-3-active fragment, caspase-3-associated activity and lactate dehydrogenase (LDH).

Materials and methods

Materials

The reagents used were of the highest grade available. 3-Nitropropionic acid, testosterone and other reagents were purchased from Sigma (St. Louis, MO, USA).

Animals

Two-month-old female Wistar rats weighing 230–250 g at the beginning of the study were purchased from Charles River, Barcelona, Spain. They were kept under controlled conditions of temperature (20–23 °C), illumination (12-h light/12-h dark cycle, lights on at 08:00 h) and were provided with food (Purina®, Barcelona, Spain) and water ad libitum. All animal care and procedures were in accordance with the European Communities Council Directive (86/609/ECC), R.D. 223/1988, and were approved by the Bioethics Committee of the Institution (University of Cordoba, Spain).

Different experiments were performed to reach the programmed objective. Experiment 1 was designed to study the effect of testosterone on oxidative stress and changes of SDH activity induced by ovariectomy and 3-nitropropionic acid in striatal synaptosomes ($n=6$ animals per group). Experiment 2 was elaborated to quantify the effect of ovariectomy, 3-nitropropionic acid and testosterone on LDH activity in striatal homogenates ($n=6$ animals per group). Experiment 3 was designed to evaluate the effect of ovariectomy, 3-nitropropionic acid and testosterone on caspase-3 activity in striatal homogenates ($n=4$ animals per group). Experiment 4 was performed to determine the histological injury triggered by 3-nitropropionic acid in the striatum ($n=4$ animals per group). At the end of the study the animals were sacrificed under ether anesthesia after 12 h starvation and the brain was removed and frozen immediately at −80 °C.

Surgical intervention and drug administration

Ovariectomy was carried out under anesthesia and asepsis following the bilateral procedure described by Poumeau-Delille (1953). The treatments were administered to animals when the absence of ovary cycle was verified; vaginal smears were daily collected for 7 days. This period usually lasted for twenty days. 3-Nitropropionic acid diluted in 0.1% dimethylsulfoxide (DMSO) was administered intraperitoneally (i.p.) at a dose of 20 mg/kg body weight for 4 consecutive days. Testosterone (0.5 mg/kg body weight) diluted in 0.1% DMSO was administered subcutaneously for 8 days, beginning 4 days before and continuing for 4 days after first injection of 3-nitropropionic acid.

Synaptosome preparation

The brain was removed and the striatum (including caudate and putamen) was isolated and suspended in 2 ml ice-cold isolation buffer (0.32 M sucrose, 20 mM HEPES, 4 μg/ml leupeptin, 4 μg/ml pepstatin, 5 μg/ml aprotinin, 20 μg/ml type II-S soybean trypsin inhibitor, 0.2 mM phenylmethylsulfonyl fluoride, 2 mM EGTA, 2 mM ethylenediaminetetraacetic acid (EDTA), pH 7.2). The homogenate was centrifuged at 450 ×g for 10 min at 4 °C, and the supernatant was transferred to a new tube. The remaining pellet was resuspended in 1.5 ml ice-cold isolation buffer and centrifuged at 450 ×g for 10 min at 4 °C. Both supernatants were mixed and centrifuged at 20,000 ×g for 10 min at 4 °C. The resulting crude synaptosomal pellet was resuspended in 2 ml Locke's buffer (154 mM NaCl, 5.6 mM KCl, 2.3 mm $CaCl_2$, 1.0 mM $MgCl_2$, 3.6 mM $NaHCO_3$, 5 mM glucose, 5 mM HEPES, at pH 7.2) (Springer et al., 1997).

Biochemical parameters

The levels of lipid peroxidation products, measured as malondialdehyde (MDA)+4-hydroxyalkenals (4-HDA) and reduced glutathione (GSH) content were determined in aliquots of striatal homogenates using reagents purchased from Oxis International (Portland, OR, USA), i.e., LPO-586 and GSH-420 kits. Protein carbonyl levels, an index of protein oxidation, were determined according to the method of Levine et al. (1990).

Antioxidative enzyme activity of glutathione peroxidase (GSH-Px; EC 1.11.1.9) was evaluated following the method of Flohé and Gunzler (1984). Catalase (EC 1.11.1.6) activity was assayed by the method of Aebi (1984). Activity of superoxide dismutase (SOD; EC 1.15.1.1) was carried out using reagents purchased from Oxis International (Portland, OR, USA), i.e., SOD-525.

SDH activity in the striatum was evaluated by the method of Strack et al. (2001) whereas LDH in striatal nucleus homogenates was measured by modification of a colorimetric routine laboratory method (Taffs and Sitkovsky, 1991). Additionally, the caspase-3-associated enzymatic activities in

Table 1
Effects of 3-nitropropionic acid (3-NPA), ovariectomy (OVX) and testosterone (T) on succinate dehydrogenase (SDH) activity in striatal synaptosomes

	SDH (U/mg protein)
Control	0.065±0.006
T	0.059±0.010
OVX	0.049±0.009[c]
3-NPA	0.035±0.005[a]
OVX+T	0.062±0.012[f]
OVX+3-NPA	0.028±0.010[d, e]
OVX+3-NPA+T	0.047±0.009[h]

$n=6$ animals per group. Data are expressed as units per milligram of protein (U/mg protein).
Values are means±S.E.M. [a]$P<0.001$ vs Control; [c]$P<0.05$ vs Control; [d]$P<0.001$ vs OVX; [e]$P<0.01$ vs OVX; [f]$P<0.05$ vs OVX; [h]$P<0.01$ vs OVX+3-NPA.

the striatal homogenates (25 μg) were measured using the corresponding peptide-based substrates Ac-DEVD-AFC (Bachem AG, Bubenforf, Switzerland). Fluorescence was monitored on a GENios Reader (TECAN, Salzburg, Austria) with $\lambda_{Ex/Em}$ at 400/505 nm.

Finally, the protein concentration was determined by the Bradford method, using bovine serum albumin as a standard.

Western blot for caspase-3 processing

Caspase-3 processing was measured in the homogenate of striatum. Proteins (100 μg) were separated by 12% SDS-PAGE and transferred to nitrocellulose. The membranes were incubated with the corresponding commercial primary and secondary antibodies coupled to horseradish peroxidase (Santa Cruz Biotechnology, Inc.).

Tissue processing and histological analysis

At the end of study, animals were sacrificed. The whole brains were rapidly removed and fixed by immersion in 10% buffered formaldehyde. Subsequently they were embedded in paraffin wax, cut into 8 μm thick sections and stained with 0.025% cresyl violet (Nissl-stained). Sections were examined under bright-field illumination on a Leitz Orthoplan microscope.

We analyzed three different rostro-caudal levels (anterior, middle and posterior) spaced 400 μm apart. At each level four sections of 8 μm thickness were obtained. To examine the cell density, the neurons were counted bilaterally on at least two sections at each level at a ×400 magnification by an investigator who was blind for the treatment of the animals.

Statistical analysis

The results of biochemical measurements were accomplished by means of the SPSS® statistical software package. The Shapiro–Wilk test did not show a significant departure from normality in the distribution of variance values. To evaluate variations in data, a one-way analysis of variances (ANOVA) corrected with Bonferroni-test was used. The level was set at $P<0.05$.

Results

Oxidative damage

Ovariectomy significantly reduced SDH activity in striatum, whereas 3-NPA intensified the effect of gonadectomy (Table 1). In addition, 3-nitropropionic acid alone caused a considerable reduction in SDH activity (Table 1). Moreover, testosterone prompted significant increases in SDH activity in the gonadectomized group ($P<0.05$) as well as in the ovariectomy plus 3-nitropropionic acid group ($P<0.01$) (Table 1).

Furthermore, 3-nitropropionic acid significantly increased protein carbonyls and lipid peroxidation products in striatal nucleus synaptosomes ($P<0.001$) (Fig. 1). Ovariectomy, on the other hand, prompted an increase of both lipid peroxidation products ($P<0.001$) and carbonylated protein levels ($P<0.01$) (Fig. 1). The effect of estrogen deficiency caused by

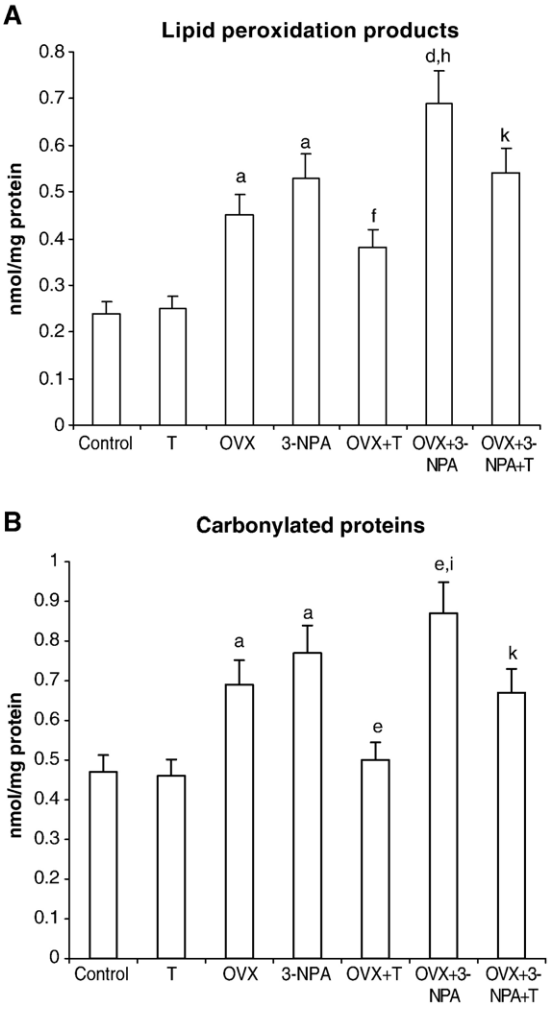

Fig. 1. Changes in the levels of lipid peroxidation (Panel A) and protein carbonylation (Panel B) in striatal synaptosomes of rats treated with 3-nitropropionic acid (3-NPA) alone or in combination with ovariectomy (OVX) and/or testosterone (T). Values are means±S.E.M., $n=6$ animals per group. Results are presented in nanomole per milligram of protein (nmol/mg protein). [a]$P<0.001$ vs Control, [d]$P<0.001$ vs OVX, [e]$P<0.01$ vs OVX, [f]$P<0.05$ vs OVX, [h]$P<0.01$ vs 3-NPA, [i]$P<0.05$ vs 3-NPA, [k]$P<0.01$ vs OVX+3-NPA.

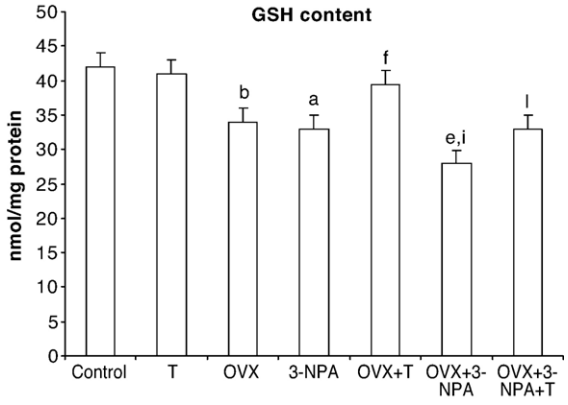

Fig. 2. Effects of 3-nitropropionic acid (3-NPA), ovariectomy (OVX) and testosterone (T) on GSH content in striatal homogenates. Values are means ± S.E.M., $n=6$ animals per group. Results are represented in nanomole per milligram of protein (nmol/mg protein). [a]$P<0.001$ vs Control, [b]$P<0.01$ vs Control, [e]$P<0.01$ vs OVX, [i]$P<0.05$ vs 3-NPA, [l]$P<0.05$ vs OVX+3NPA.

ovariectomy was intensified by 3-nitropropionic acid (Fig. 1). Additionally, both intervention (gonadectomy) and 3-nitropropionic acid administration reduced GSH content and catalase and GSH-Px activity, and enhanced SOD activity (Figs. 2 and 3). The administration of testosterone prevented the increase in lipid peroxidation products, carbonylated proteins and SOD activity in the ovariectomy and ovariectomy plus 3-nitropropionic acid groups (Figs. 1 and 3), whereas it reversed the effect on GSH content and GSH-Px and catalase activity (Figs. 2 and 3).

Cell damage

Ovariectomy did not induce changes in LDH activity, whereas 3-nitropropionic acid administration significantly enhanced LDH activity in ovariectomized rats (Table 2). Testosterone prevented the effects of 3-nitropropionic acid administration in ovariectomized rats (Table 2).

3-Nitropropionic acid-induced degeneration and cell loss was observed in comparison with the control groups (Fig. 4A–B). In the 3-nitropropionic group, pyknotic neurons stained darkly; no nucleus or nucleolus was visible, and the cells were shrunken and sickle/raisin-shaped (Fig. 4B). Additionally, some neurons displayed swollen cytoplasm and a smaller nucleus with condensed chromatin. Moreover, neuropil vacuolization was observed. Ovariectomy enhanced the processing of procaspase-3 to active caspase-3 fragment (Fig. 4D) and caspase-3-associated activity (Fig. 4C) compared with the control group. However, 3-nitropropionic acid administration did not enhance caspase-3 activation in the ovariectomized rats. The administration of testosterone reduced caspase-3 activation, although the differences in values were not statistically significant with respect to the ovariectomy group (Fig. 4C, D).

Discussion

Steroids are biomolecules with a structure made up of cyclopentanoperhydrophenanthrene. Two steroids derived from cholesterol are testosterone (4-pregnen-17-hydroxy-3-one) and progesterone (Calderón Guzmán et al., 2005). Different effects have been described for testosterone; this agent can both enhance or protect against toxicity, depending on the experimental model used (Nishino et al., 1998; Ahlbom et al., 2001). This study demonstrates the protective property of testosterone against oxidative stress induced by gonadectomy and 3-nitropropionic acid, as well as on cell damage biomarkers such as LDH and caspase-3. However, the interpretation of the role played by this hormone is complex.

The results of this study show that ovariectomy induces high levels of oxidative stress, characterized by increases in lipid peroxidation products, protein carbonylation content and SOD

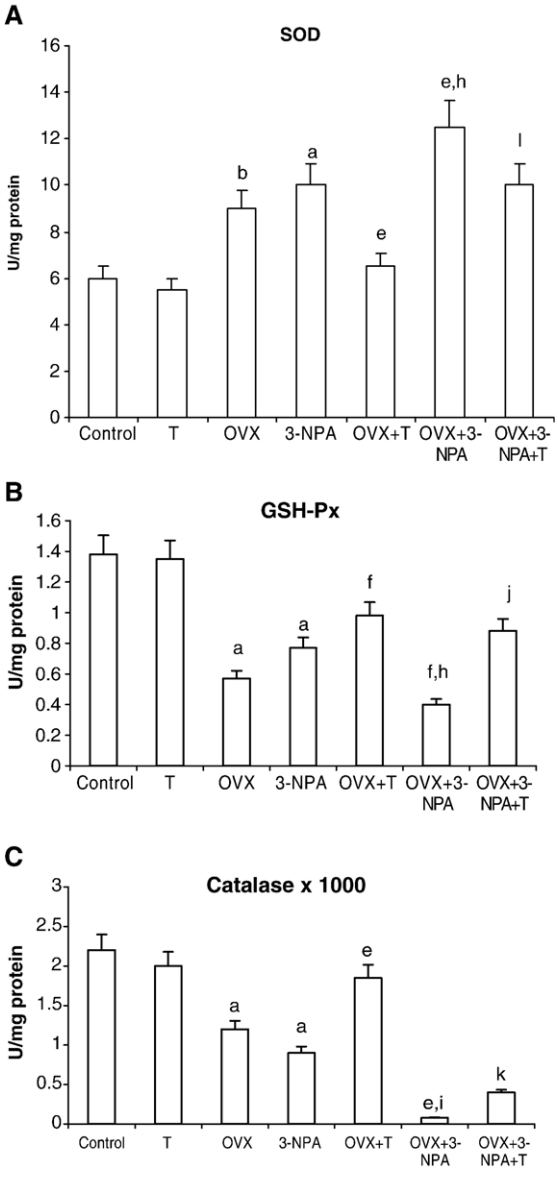

Fig. 3. Changes in the levels of SOD (Panel A), GSH-Px (Panel B) and catalase (Panel C) activities in striatal synaptosomes of rats treated with 3-nitropropionic acid (3-NPA) alone or in combination with ovariectomy (OVX) and/or testosterone (T). Values are means ± S.E.M., $n=6$ animals per group. Results are represented in units per milligram of protein (U/mg protein). [a]$P<0.001$ vs Control, [b]$P<0.01$ vs Control, [e]$P<0.01$ vs OVX, [f]$P<0.05$ vs OVX, [h]$P<0.01$ vs 3-NPA, [i]$P<0.05$ vs 3-NPA, [j]$P<0.001$ vs OVX+3-NPA, [k]$P<0.01$ vs OVX+3-NPA.

Table 2
Effects of 3-nitropropionic acid (3-NPA), ovariectomy (OVX) and testosterone (T) on lactate dehydrogenase (LDH) activity in striatal homogenates

	LDH (IU/mL/μg protein)
Control	690±103
OVX	746±85
OVX+T	620±98
OVX+3-NPA	1772±248[e]
OVX+3-NPA+T	560±157[h]

n=6 animals per group. Results are represented in international units per millilitre per microgram protein (IU/ml/μg protein).
Values are means±S.E.M. [e]P<0.01 vs OVX; [h]P<0.01 vs OVX+3-NPA.

activity; it also triggers decreases in GSH content and catalase and GSH-Px activity. Furthermore, these experimental conditions, especially 3-nitropropionic acid, induced a reduction in SDH activity. These results support the evidence found previously by our group (Túnez et al., 2006), where similar effects were observed for ovariectomy. Additionally, 3-nitropropionic acid exacerbated oxidative damage induced by ovariectomy. The oxidative stress induced by 3-nitropropionic acid has been previously observed in rats both by our group (Montilla et al., 2004; Túnez et al., 2004a,b, 2005, 2006) and by others (La Fontaine et al., 2000a,b, 2002).

Testosterone was found to block oxidative injury triggered by ovariectomy and ovariectomy plus 3-nitropropionic acid. This backs up findings from other authors who have demonstrated the possible protective effect of androgens, especially testosterone, on the recovery of specific populations of motoneurons (Nordeen et al., 1985; Kujawa et al., 1989), as well as the results of Ahlbom et al. (2001), who reported that testosterone protects cerebellar granule cells from oxidative stress through a receptor-mediated mechanism. Additionally, this effect is indirectly supported by reports which claim that other androgens such as dehydroepiandrosterone (DHEA) might act as neuroprotective steroids (Veiga et al., 2003; Túnez et al., 2005).

Depletion of endogenous female sex steroid hormones by ovariectomy was found to increase neuronal vulnerability characterized by caspase-3 activation. Caspase-3 is considered to be a key protease, responsible for many of the biological and morphological features of apoptosis (Nishino et al., 2000). These results are consistent with other studies, which found that ovariectomy enhances neuronal death (Kume-Kick et al., 1996; Nishino et al., 1998, 2000; Mogami et al., 2002; Túnez et al., 2006). Furthermore, 3-nitropropionic acid led to alterations in LDH levels in the striatum of gonadectomized rats, whereas it did not affect the caspase-3 pathway. These findings concur

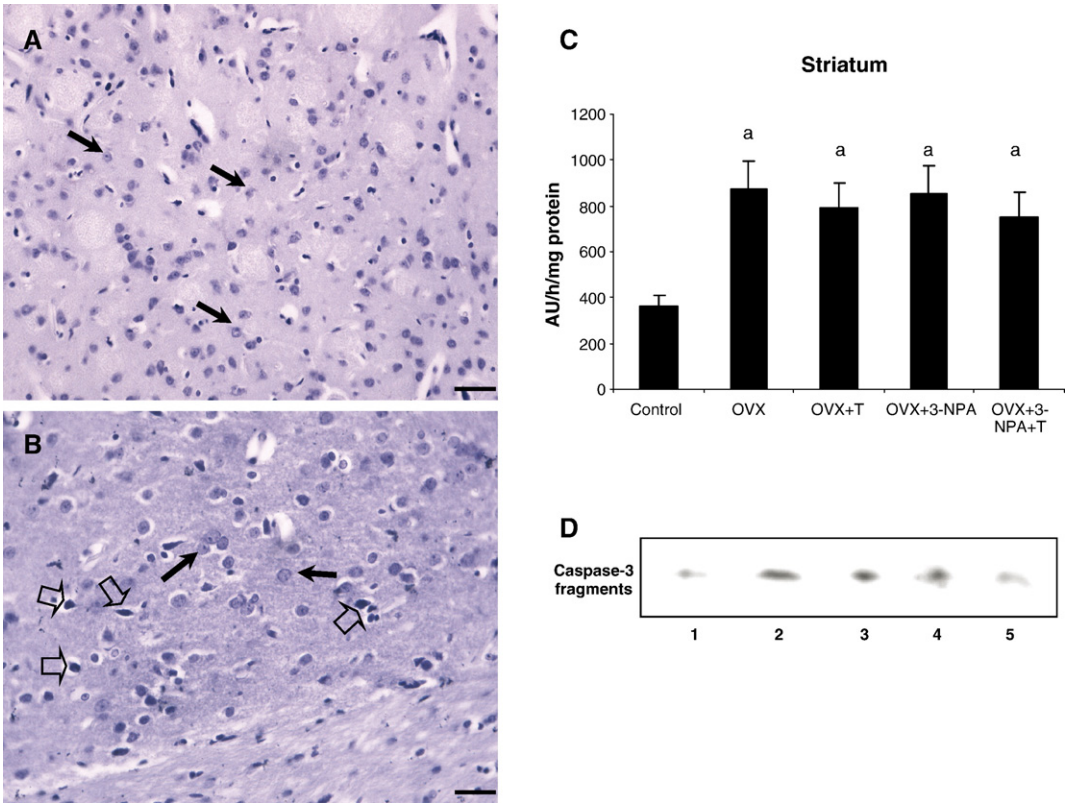

Fig. 4. Light microscopic views of cresyl violet-stained paraffin sections. Control group (Panel A) and 3-nitropropionic acid group (Panel B). The arrows indicate: normal neurons (arrows) and pyknotic neurons (open arrows). Original magnification ×400. Furthermore, the effects of 3-nitropropionic acid (3-NPA), ovariectomy (OVX) and testosterone administration (T) on caspase-3-associated activity (Panel C) are shown. Values are means±S.E.M., n=4 animals per group. Results are represented in arbitrary units per hour per milligram protein (AU/h/mg protein). [a]P<0.001 vs Control. Finally, caspase-3-active fragment (Panel D) in the striatum was evaluated by Western blotting as described in the Materials and methods section. Lane 1: control group, lane 2: OVX group, lane 3: OVX+T group, lane 4: OVX+3-NPA group, and lane 5: OVX+3-NPA+T group. Ovariectomy enhanced caspase-3-active fragment, whereas the administration of testosterone seems to reduce its expression. The image is representative of four rats.

with previous studies, which demonstrate that 3-nitropropionic acid induces mitochondrial dysfunction; as well as cell degeneration, loss and death (Vis et al., 2002; Bizat et al., 2003; Pang et al., 2003; Strauss and Morton, 2003; Túnez et al., 2006). Moreover, testosterone administration prevented cell damage triggered by ovariectomy and 3-nitropropionic acid. These results indirectly are in agreement with reports from Ramsden et al. (2003), who found that depletion of endogenous sex steroid hormones by orchidectomy increases neuronal vulnerability. These authors showed that androgen replacement in orchidectomized animals protected neurons. Similar data were reported by Azcoitia et al. (2001) following testosterone administration.

Taken together, these data seem to indicate that the effects observed on the quantified biomarkers of oxidative stress and cell damage, as well as the effect of SDH activity, would partly be the result of a cytoprotective effect induced by testosterone. However, our data present an important limitation in describing the possible pathways or mechanisms involved in these neuroprotective effects. Nevertheless, the studies reported indicate the routes involved: i) aromatization to estradiol (Azcoitia et al., 2001; Ramsden et al., 2003; Huppenbauer et al., 2005; Hiltunen et al., 2006); ii) transformation to dihydrotestosterone (DHT) (Raber et al., 2002; Huppenbauer et al., 2005); iii) action via androgen receptor-specific system (Raber et al., 2002; Leranth et al., 2003; Huppenbauer et al., 2005); and iv) antioxidative action (Ahlbom et al., 2001; Chisu et al., 2006).

In short, this study demonstrates the neuroprotective and antioxidative effect of testosterone against tissue injury induced by 3-nitropropionic acid, characterized by oxidative stress and cell damage, preventing at least some of the alterations observed in rats exposed to this acid. This situation seems to suggest a possible therapeutic value for testosterone replacement therapy in neurodegenerative diseases. However, further research is required to assess the mechanisms involved.

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