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Atrazine affects the morphophysiology, tissue homeostasis and aromatase expression in the efferent ductules of adult rats with mild alterations in the ventral prostate

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HIGHLIGHTS

- Atrazine alters efferent ductules morphology with mild effects on rat ventral prostate.
- Atrazine induces aromatase expression in the efferent ductules but not in the ventral prostate.
- Atrazine effects in testis may be secondary to alterations in the efferent ductules.

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ABSTRACT

The widely used herbicide atrazine is a potent endocrine disruptor known to cause increased aromatase expression and transient increase in testicular weight followed by remarkable testis atrophy. However, whether the effects of atrazine on the testes are primary or secondary to dysfunctions in other components of male reproductive tract remains unknown. Given the high sensitivity of the efferent ductules to estrogen imbalance and the similarity to alterations previously described for other disruptors of these ductules function, and the testicular alterations observed after atrazine exposure, we hypothesized that the efferent ductules could be a target for atrazine. Herein we characterized the efferent ductules and the ventral prostate of adult Wistar rats treated with 200 mg/kg/day of atrazine for 7, 15, and 40 days. Additionally, we evaluated if the effects of atrazine in these organs could be reduced after discontinuation of the treatment. Atrazine exposure resulted in mild effects on the ventral prostate, but remarkable alterations on the efferent ductules, including luminal dilation, reduced epithelial height, and disruption of the epithelial homeostasis, which coincides with increased aromatase expression. Together with our previous data, these results suggest that at least part of the testicular effects of atrazine may be secondary to the alterations in the efferent ductules.

1. Introduction

Exposure to estrogenic or antiestrogenic compounds and impairment of estrogen receptors by genetic or chemical inactivation are among the main causes of male infertility and have been shown to cause major dysfunction of the efferent ductules (Hess et al., 1997a, 2000; Lee et al., 2001; Mckinnell et al., 2001; Oliveira et al., 2001, 2002; Zhou et al., 2001; Lee et al., 2009; Hess et al., 2011; Nanjappa et al., 2016). The efferent ductules are the segment of the male reproductive tract presenting the highest levels of estrogen in the luminal fluid and the highest expression of estrogen receptor in the epithelium (Hess et al., 1997b). Their main function is to reabsorb fluid coming from the testis, which occurs under the control of estrogens (Hess et al., 1997a, 2000; Lee et al., 2001; Oliveira et al., 2001, 2002, 2005; eira et al., 2002; Oliveira et al., 2005). Disturbance in fluid reabsorption leads to the accumulation of fluid in the ductule lumen and consequent reflux to the testis, thus resulting in luminal dilation of the seminiferous tubules followed by testicular atrophy and, consequently, infertility (Hess et al., 1997a; Oliveira et al., 2001; Oliveira et al., 2002).

Atrazine (2-chloro-4-ethylamino-6-isopropylamino-*s*-triazine)

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is a widely used herbicide shown to be a potent endocrine disruptor and cause adverse effects on the male genital system (Kniewald et al., 2000; Betancourt et al., 2006; Hayes et al., 2006, 2010; Swan, 2006; Suzawa and Ingraham, 2008; Rey et al., 2009; Belloni et al., 2011; Hayes et al., 2011). Potential risks for animal health include the increase in estrogen and reduction in the testosterone levels (Stoker et al., 2000; Friedmann, 2002; Victor-Costa et al., 2010). Among the effects of atrazine on male rats are testicular alterations, such as a transient increase in weight followed by a remarkable testicular atrophy (Victor-Costa et al., 2010), with a concurrent increase in aromatase immunoexpression in Leydig cells (Martins-Santos et al., 2017). Although evidence suggests that this key enzyme for estrogen production could be a target for the herbicide (Crain et al., 1997; Sanderson et al., 2000, 2001, 2002; Heneweer et al., 2004; Laville et al., 2006; Sanderson, 2006; Fan et al., 2007a, 2007b; Holloway et al., 2008; Tinfo et al., 2011), atrazine's mechanism of action remains to be elucidated. Moreover, most of the atrazine's effects on aromatase were demonstrated *in vitro* (Sanderson et al., 2000, 2001, 2002; Heneweer et al., 2004; Betancourt et al., 2006; Laville et al., 2006; Fan et al., 2007a, 2007b; Holloway et al., 2008; Suzawa and Ingraham, 2008; Higley et al., 2010; Tinfo et al., 2011; Quignot et al., 2012; Fa et al., 2013; Caron-Beaudoin et al., 2016).

One important question that remains unanswered is whether the atrazine effects in the testis are primary or secondary to changes in other testicular segments of the male reproductive tract. Given the high sensitivity of the efferent ductules to estrogen imbalance, the similarity to the alterations previously described for other disruptors of these ductules function (Nakai et al., 1992, 1993; Hess, 1998; Gotoh et al., 1999), and the testicular alterations observed after atrazine exposure, we hypothesized that the efferent ductules could be a target for atrazine. We investigated this hypothesis by evaluating the effects of atrazine on the morphology, aromatase expression, cell proliferation, and apoptosis of the efferent ductules. For comparison purposes, we also evaluated the effects of atrazine on the ventral prostate, which is an important target for androgens and estrogens (Ellem and Risbridger, 2010).

2. Materials and methods

2.1. Animals

Sexually mature male Wistar rats (100 days old), acquired from the Animal Facility at the Instituto de Ciências Biológicas, Universidade Federal de Minas Gerais (UFMG), Brazil, were used in this study. The rats were maintained under constant conditions of light (12/12 h light/dark cycles) and temperature (22 °C), receiving pelletized chow (Nuvital Nutrientes S.A., Colombo, PR, Brazil) and water *ad libitum*. All experimental procedures followed the guidelines of UFMG's animal care and research of the Institutional Ethical Committee on Animal Use (https://www.ufmg.br/bioetica/ceua/). The study was approved by the Institutional Ethical Committee for Animal Experimentation (Protocol number 287/2008).

2.2. Treatment

For 7, 15, and 40 days, sexually mature male Wistar rats received a daily dose of 200 mg/kg of atrazine (Gesaprim 500 Ciba Geigy, Syngenta, São Paulo, Brazil) diluted in corn oil. The control group received only corn oil. The dosages and treatment periods were based in previous studies showing alterations in testicular morphology and steroidogenesis (Victor-Costa et al., 2010; Martins-Santos et al., 2017).

In order to determine if the possible alterations caused by atrazine are permanent or transitory, after the 40-days treatment, a group of rats was maintained in the animal facility under the same environmental conditions for further 75 days (ATZ 40d Rec), without any treatment. Control of the recovery group (Cont 40d Rec) consisted of rats receiving only corn oil for 40 days and maintained for further 75 days (Martins-Santos et al., 2017).

2.3. Tissue preparation

Subsequently to each treatment and recovery periods, the rats were weighed, anesthetized with intraperitoneal injection of sodium pentobarbital (80 mg/kg) and ketamine chlorydrate (10 mg/kg) and then perfused intracardially with Ringer solution, followed by 10% (v/v) of neutral buffered formalin (NBF). After fixation, the efferent ductules and ventral prostate were dissected, immersed in NBF, and stored at 4 °C. Fragments of both segments were routinely processed for histological and immunohistochemical analyses.

2.4. Histology and morphometry

The tissue fragments were dehydrated with ascending concentrations of ethanol solutions, embedded in paraffin (Histosec pastilhas, Merck KGaA, Darmstadt, Germany), cut into 5-μm-thick sections and placed on glass slides. The sections were stained with hematoxylin and eosin (HE), and 1% toluidine blue-sodium borate solution for histological analysis.

The luminal diameters of the proximal and distal efferent ductules were measured at the widest region of five randomly chosen transversal sections per animal, by using a scale of 1000 μm coupled to the eyepiece of the microscope (Nikon Eclipse E200, Melville, USA). Measurements were made at 400X magnification.

The height of the proximal efferent ductules epithelium was measured from the basement membrane to the microvillus tip, in areas of straight sections from 25 cells with evident nuclei (in five tubule sections). Pictures were taken by using Pannoramic Viewer software (3DHISTECH Ltd., Budapest, Hungary). The proximal area was selected for this measurement based on previous studies showing that this is the region more sensitive to disruption on the estrogen responsive system (Oliveira et al., 2002).

2.5. Immunohistochemistry

Immunohistochemistry was performed to detect aromatase in the efferent ductules and the ventral prostate and to determine possible alterations in cell proliferation and apoptosis by using MCM7 and caspase-3 as markers, respectively.

To this end, fragments of NBF-fixed tissues were embedded in paraffin, cut into 5-μm-thick sections, mounted on glass slides, dewaxed, re-hydrated in graded ethanol, and incubated in methanol containing 0.6% H_2O_2 to inactivate the endogenous peroxidase. After microwaving in 0.1 M citrate buffer, pH 6.0, for antigen retrieval, the endogenous biotin activity was blocked by avidin and biotin-blocking solution (avidin/biotin blocking kit; Vector Laboratories, Burlingame, USA). To block non-specific antibody binding, the sections were incubated with 10% normal goat serum, followed by an overnight incubation at 4 °C with the primary antibodies: polyclonal rabbit anti-human aromatase (Sigma-Aldrich, Saint Louis, USA), diluted 1:500; monoclonal mouse *anti*-CDC47/MCM7 (Ab-2, Thermo Fisher Scientific, Rockford, USA), diluted 1:500; or polyclonal rabbit anti-cleaved caspase-3 (Millipore Corporation, California, USA), diluted 1:200. Negative controls were incubated with phosphate buffer saline (PBS) instead of the primary antibodies. Tissues were then incubated for 1 h with a biotinylated secondary antibody: goat anti-rabbit (for aromatase and caspase-3) or goat anti-mouse (for MCM7) (Dako, Carpinteria, USA), both diluted 1:100. This step was followed by incubation with avidin-