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The systemic and gonadal toxicity of 3-methylcholanthrene is prevented by daily administration of α-naphthoflavone

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ABSTRACT

In the present study, we investigated the effect of 3-methylcholanthrene (3MC) on sexual maturity and the ability of α-naphthoflavone (αNF) to prevent this action. To this end, immature rats were daily injected intraperitoneally with 3MC (0.1 or 1 mg/kg) and/or αNF (80 mg/kg). Body weight, vaginal opening and estrous cycle were recorded and ovaries were obtained on the day of estrus. Ovarian weight, ovulation rate (measured by the number of oocytes within oviducts), and follicular development (determined by histology) were studied. No differences were found in body weight, ovarian weight, day of vaginal opening, or the establishment of the estrous cycle among the different groups of rats. However, animals treated with 3MC, at both doses, exhibited a lower number of primordial, primary, preantral and antral follicles than controls. Also, 3MC inhibited the ovulation rate and induced an overexpression of both the *Cyp1a1* and *Cyp1b1* genes, measured by chromatin immunoprecipitation assay. The daily treatment with αNF alone increased the number of follicles in most of the stages analyzed when compared with controls. Moreover, the αNF treatment prevented completely not only the 3MC-induced decrease in all types of follicles but also the 3MC-induced overexpression of *Cyp* enzymes and the genetic damage in bone marrow cells and oocytes. These results suggest that (i) daily exposure to 3MC during the pubertal period destroys the follicle reserve and alters the ovulation rate; (ii) the 3MC action seems to be mediated by an aryl hydrocarbon receptor-dependent mechanism; (iii) daily administration of αNF has a clear stimulatory action on the ovarian function; and (iv) αNF may prevent both the systemic and gonadal 3MC-induced toxicity.

1. Introduction

Polycyclic aromatic hydrocarbons (PAHs) are a heterogeneous group of chemicals released into the environment from different anthropogenic activities such as synthesis of pesticides, bleaching paper using chlorine products, or incomplete combustion in the incineration of organic compounds like wood, oil, tobacco, oils or simply part of the gases emitted by cars, especially those with diesel engine. The most widely studied PAHs include: pyrene derivatives, benzo[a]pyrene (BaP), 9,10-dimethylanthracene (DMBA) and other pyrene or anthracene derivatives. Early research argued that the highly potent tumorigen 3-methylcholanthrene (3MC) is generated from cholesterol by pyrolysis of phytosterols during different cooking processes and other incomplete combustion processes (Fieser and Fieser, 1949; Kröller, 1964; Rodgman and Perfetti, 2013). Although 3MC may be prepared by a series of chemical reactions from a sterol-derived compound structurally related to cholesterol, it is considered that tobacco smoke, as other combustion processes, contains numerous PAHs, including 3MC, because of pyrolysis of the organic material (Rodgman and Cook, 2009; Pleil et al., 2010). Most of these compounds can cause different toxic responses in different systems by activating the aryl hydrocarbon receptor (AhR), although some seem to act by different pathways (Fujii-Kuriyama and Mimura, 2005). AhR is a

Abbreviations: PAH(s), polycyclic aromatic hydrocarbon(s); ARNT, aryl hydrocarbon nuclear translocator; αNF, α-naphthoflavone; BaP, benzo[a]pyrene; DMBA, dimethylbenz[a]anthracene; 3MC, 3-methylcholanthrene; AhR, aryl hydrocarbon receptor; Po, primordial follicle; Pi, primary follicle; PA, preantral follicle; A, antral follicle; VO, vaginal opening; MN, micronucleus; CYP, cytochrome P450 enzymes; AhRKO, AhR-deficient mice.

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ligand-activated transcription factor and the first protein involved in a signaling cascade of events that induce the expression of several genes involved in different processes, including those coding for xenobiotic-metabolizing enzymes (Hahn, 2002; Shimada, 2006).

Some reproductive processes including fertility, embryonic development, maintenance of pregnancy and ovarian function, need activation of AhR (Hernández-Ochoa et al., 2009). The presence of AhR has been demonstrated in the ovary of different species, such as rats (Chaffin et al., 2000) and humans (Khorram et al., 2002). It is present in ovarian follicles, oocytes, and granulosa and theca cells (Davis et al., 2000). Alteration in AhR functionality leads to poor follicular development, exhibiting less number of antral follicles and slow growth (Benedict et al., 2000, 2003; Barnett et al., 2007a; Hernández-Ochoa et al., 2010). It has also been noted that follicles from AhR-deficient (AhRKO) mice show decreased granulosa cell proliferation, reduced levels of cell cycle regulators, lower levels of estrogen, FSH and LH receptors, and estradiol as well (Barnett et al., 2007a,b).

Many PAHs are known potent carcinogens (Boffetta et al., 1997) as well as inducers of metabolizing enzymes (Shimada, 2006; Chahin et al., 2013). Some of them, including 3MC, are well recognized as ovotoxic since they destroy both primordial and primary follicles in mice and rats (Mattison, 1980; Shiromizu and Mattison, 1985; Borman et al., 2000). Exposure to PAHs has been linked to premature menopause in women who smoke (Mattison and Thorgeirsson, 1979; Shiromizu and Mattison, 1985).

The synthetic flavonoid, α-naphthoflavone (αNF) is an AhR antagonist and its action has been studied in different biological systems (Gasiewicz and Rucci, 1991; Gasiewicz et al., 1996; Lu et al., 1996; Zhang et al., 2003). However, only a few works have studied the *in vivo* action of flavonoids on follicular growth in rodents. Mattison and Thorgeirsson (1979) and Thompson et al. (2005) used αNF to prevent the toxic PAH-induced effect on the ovarian function. Shiromizu and Mattison (1985) found that the intraovarian injection of 3MC destroys small oocytes in mice and that intraperitoneal administration of αNF inhibits this toxic effect. In previous works, we found that αNF increases the number of developing follicles and the ovulation rate in rats stimulated with gonadotropin to induce ovulation in comparison with control animals (Barreiro et al., 2011). Therefore, the aim of the present work was to investigate the effect of daily exposure to low doses of 3MC on sexual maturity of rats and the ability of αNF to prevent this action. In addition, and since 3MC is considered a potent carcinogen (Sims, 1967; Cavalieri et al., 1978; Boffetta et al., 1997), we also studied whether daily administration of 3MC causes DNA injury in our biological model.

2. Materials and methods

2.1. Animals

Immature female Sprague–Dawley rats aged 20 days were purchased from the School of Veterinarian Sciences of Buenos Aires University, Argentina. Animals were maintained under controlled conditions of light (12 h light/12 h darkness), temperature (22 °C) and humidity, with free access to food and water. All animals were handled according to the Guiding Principles for the Care and Use of Research Animals, and all protocols were approved by the Institutional Committee of the School of Medicine of Buenos Aires University, Argentina (CICUAL) by Resolution 1928/14.

2.2. Experimental design

At 21 days of age, female rats were weighed, randomly distributed into different experimental groups, and daily dosed by intraperitoneal injections of 3MC (0.1 or 1 mg/kg) and/or αNF (80 mg/kg). Corn oil vehicle (2.0 ml/kg) was used as vehicle. Dosing with 3MC and vehicle started at 22 days of age, whereas, to insure αNF action, dosing with αNF started at 21 days of age. All treatments were completed at 40 days of age. All rats were inspected daily to record the body weight, vaginal opening (VO) and estrous cycle. From the day of VO, vaginal smears were examined daily to identify the period of estrous cycle. All animals (eight to ten per group) were killed by decapitation on the afternoon of the second estrous cycle. Both ovaries and femurs were obtained and used in the following studies.

2.3. Ovulation rate

After euthanasia, ovaries and their accompanying oviducts were immediately removed and oviducts were flushed with hyaluronidase (1 mg/ml in saline) using a 30-gauge needle. The number of oocytes from both oviducts was counted by means of a stereoscopic microscope (Faletti et al., 1995). Results are expressed as number of oocytes per rat.

2.4. Ovarian histology and follicle counting

After assessing the ovulation rate and weighing both ovaries, one ovary was fixed in neutral buffered formalin, while the other was frozen at −80 °C to be used in other studies. Ovaries fixed in formalin were then fixed in 70% ethanol. The tissues were dehydrated by a graded series of ethanol concentrations, xylene: ethanol (1:1), xylene (100%) and finally paraffin. Then, the tissues were serially sectioned at 6-μm thickness, mounted onto glass slides, and some of them stained with hematoxylin and eosin according to standard protocols and analyzed using a Olympus CX21 microscope. Ovarian follicles were analyzed every ten sections and only follicles containing an oocyte were counted to avoid double counting the follicles. Ten sections from the same ovary and eight to ten ovaries from different animals with the same treatment were analyzed in each group. Follicles were counted and classified as primordial (Po), primary (Pi), preantral (PA), or antral (A), as described previously (Myers et al., 2004; Barreiro et al., 2011). Primordial follicles were characterized as oocytes surrounded by a single layer of flattened granulosa cells. Primary follicles were characterized as oocytes surrounded by a single layer of cuboidal granulosa cells. Preantral follicles were characterized as oocytes surrounded by two or more layers of cuboidal granulosa cells with no visible antrum. Antral follicles were identified by the presence of an antrum. The abundance of each type of follicle was expressed per ovary.

2.5. RNA isolation and real time-Polymerase chain reaction (real time-PCR)

Total RNA was isolated from all frozen tissues using Quick-RNA™ MiniPrep (Zymo Research, Irvine, CA, USA) according to the manufacturer's instructions. To remove DNA contamination, RNA samples were treated by on-column deoxyribonuclease DNAse digestion according to the manufacturer's protocol. RNA concentrations were determined by spectrophotometric trace (Nanodrop, ThermoScientific, Wilmington, DE, USA). RNA (1000 ng) from rat ovary was reverse transcribed (RT) using the Omni RT Kit (Qiagen, Valencia, CA, USA) in the presence of random hexamer primers, as recommended by the manufacturer (Invitrogen, Carlsbad, CA, USA). All real-time PCR reactions were performed using a Quanttudio 12 K Real-Time PCR system. Threshold cycles (CTs) were detected by QuantStudio 12 K Flex software. Relative standard curves were constructed from serial dilutions of one reference sample cDNA (RT of 500 ng total RNA from one ovary