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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

sample serially diluted from 1/2 to 1/500). The CTs from each sample were referred to the relative curve to estimate the mRNA content/sample and the values obtained were normalized for procedural losses using *18s ribosomal* mRNA as the normalizing unit. To determine the relative abundance of the mRNAs of interest, we used the SYBR GreenER qPCR SuperMix system (Invitrogen). Primers for amplification were designed using the PrimerSelect tool of DNASTAR 11 software (Madison, WI, USA) or the NCBI online Primer-Blast program (Table 1). PCR reactions were performed in a total volume of 10 μl, each reaction containing 1 μl of diluted cDNA or a reference cDNA sample, 5 μl of SYBR GreenER qPCR SuperMix and 4 μl of primers mix (300 nM of each gene specific primer). The PCR conditions used were 95 °C for 5 min, followed by 40 cycles of 15 s at 95 °C and 60 s at 60 °C. To confirm the formation of a single SYBR Green-labeled PCR amplicon, the PCR reaction was followed by a three-step melting curve analysis consisting of 15 s at 95 °C, 60 s at 60 °C, ramping up to 95 °C at 0.5 °C s^{-1}, detection every 0.5 s and finishing 15 s at 95 °C, as recommended by the manufacturer. Experiments were performed three times. Data were normalized to ribosomal 18s mRNA in each sample.

2.6. Chromatin immunoprecipitation (ChIP) assay

To assess the recruitment of AhR to the CYP1A1 and CYP1B1 gene promoters, we performed ChIP assays using chromatin extracted from the ovarian tissue from female Sprague-Dawley rats. The ChIP procedure has been described previously (Lomniczi et al., 2013, 2015), and was carried out with minimal modifications. Ovarian tissue (20 mg) was homogenized in ice-cold PBS containing a protease inhibitor cocktail (1 mM phenylmethylsulfonyl fluoride, 7 μg ml^{-1} aprotinin, 0.7 μg ml^{-1} pepstatin A, 0.5 μg ml^{-1} leupeptin), a phosphatase inhibitor cocktail (1 mM β-glycerophosphate, 1 mM sodium pyrophosphate and 1 mM sodium fluoride), and a histone deacetylase inhibitor (20 mM sodium butyrate). Cross-linking was performed by incubating the homogenates in 1% formaldehyde for 10 min at room temperature. After two additional washing steps in PBS, homogenates were lysed with 200 μl SDS buffer (0.5% SDS, 50 mM Tris–HCl, 10 mM EDTA) containing protease, phosphatase and HDAC inhibitors, and sonicated for 40 s to yield chromatin fragments of ~500 bp using a Fisher Scientific FB 705 sonicator. Size fragmentation was confirmed by agarose gel electrophoresis. The sonicated chromatin was clarified by centrifugation at 16,200*g* for 10 min at 4 °C, brought up to 1 ml in Chip dilution buffer (16.7 mM Tris-HCl, pH 8.1, 150 mM NaCl, 1.2 mM EDTA, 1.1% Triton X-100 and 0.01% SDS) containing protease, phosphatase and HDAC inhibitors and stored at −80 °C for subsequent immunoprecipitation. For this step, chromatin was pre-cleared with Protein A/G beads (Dynabeads, Invitrogen) for 1 h at 4 °C. Aliquots of chromatin (100 μl) were then incubated with 5-μg antibody (rabbit anti-AhR polyclonal, BML-SA550, Enzo Life Sciences, Farmingdale, NY, USA). Antibody–chromatin complexes and 25 μl of protein A or G beads solution (Dynabeads) were incubated at 4 °C overnight with gentle agitation. Immunocomplexes were washed sequentially with low-salt wash buffer (20 mM Tris–HCl, pH 8.1, 150 mM NaCl, 2 mM EDTA, 1% Triton X-100 and 0.1% SDS), high-salt wash buffer (20 mM Tris–HCl, pH 8.1, 500 mM NaCl, 2 mM EDTA, 1% Triton X-100 and 0.1% SDS), LiCl buffer (10 mM Tris–HCl, pH 8.1, 250 M LiCl, 1% Nonidet P-40, 1% sodium deoxycholate and 1 mM EDTA) and TE buffer (10 mM Tris–HCl, pH 8.0 and 1 mM EDTA). Then, immunocomplexes were eluted with 100 μl of 0.1 M $NaHCO_3$ and 1% SDS at 65 °C for 45 min. Cross-linking was reversed by adding 4 μl of 5 M NaCl and incubating at 95 °C for 30 min. DNA was recovered by using the ChIP DNA Clean and Concentrator columns (Zymo Research) and stored at −80 °C until subsequent PCR analysis. All chemicals were purchased from Sigma–Aldrich (St Louis, MO, USA). Genomic regions of interest were amplified by qPCR. Table 1 shows the primer sequences (Eurofins MWG Operon, Huntsville, AL, USA) used to detect the DNA fragment of interest in the immunoprecipitated (IP) DNA. For semi quantitative detection, PCR reactions were performed using SYBR Green ER (Invitrogen) with each IP DNA or input samples. The thermocycling conditions used were 95 °C for 5 min, followed by 40 cycles of 15 s at 95 °C and 60 s at 60 °C. Data are expressed as percent recruitment relative to chromatin input. Experiments were performed three times.

2.7. Cytotoxicity assays

To study whether the 3MC treatments, at 0.1 and 1 mg/kg, cause systemic toxicity, the bone marrow of each animal was used to evaluate genetic damage and clastogenic effects using two methodologies: the micronucleus (MN) test and the comet assay. To this end, both femurs were extracted to obtain a suspension of bone marrow cells by flushing with PBS using a 1-ml syringe with a 21-G needle. For the MN test, we proceeded as previously described (Schmid, 1975; Cebral et al., 2011). Briefly, cell suspensions were spread on slides, and smears were air-dried, fixed in methanol, and stained in May-Grünwald/Giemsa. The presence of micronucleated polychromatic erythrocytes was visually scored as MN per 1000 cells, using an Olympus CX21 microscope. Comet slides were prepared as previously described (Olive and Banáth, 2006). Briefly, bone marrow cells were prepared using low melting agarose and immersed in a lysing solution containing 2.5 M NaCl, 100 mM EDTA, 10 mM Tris-base, 1% Triton-X100 (v/v) and 10% (w/v) dimethylsulfoxide, pH 10, at 4 °C overnight under conditions of light deprivation. After the incubation period, slides were immersed in an alkaline solution containing 300 mM NaOH and 1 mM EDTA, pH > 13, at 4 °C for 20 min. Then, slides were electrophoresed at 4 °C at 0.9 V/cm (300 mA) for 20 min. After electrophoresis, slides were washed three times in neutralization buffer (0.4 M buffer Tris-base, pH 7.5) for 5 min each. Slides were dehydrated with methanol for 5 min to fix cells and stained with 10 μM acridine orange. The alkaline comet assay was also used to quantify the DNA breaks in oocytes as

Table 1
Primers used for real time-PCR and chromatin immunoprecipitation (ChIP) Assay.

	Specie	Gene	Accesion #	Oligonucleotide sequences
Real time-PCR	Rat	*Cyp1a1*	NM_012540.2	Sense 5′-GTTTGGGGAGGTTACTGGTTCTGG-3′ Antisense 5′-GATGTGGCCCTTCTCAAATGTCCT-3′
		Cyp1b1	NM_012940.2	Sense 5′-CCGCCGAAAAGAAGGCGAC-3′ Antisense 5′-CCACAACCTGGTCCAACTCG-3′
		Rn18s	NR_046237.1	Sense 5′-CGGCTACCACATCCAAGGAA-3′ Antisense 5′-GGGCCTCGAAAGAGTCCTGT-3′
ChIP assay	Rat	*Cyp1a1*	NM_012540.2	Sense 5′-CTACAGGGAGCCGCGAGAGGAATC-3′ Antisense 5′-CCAGAGGTCACAGGGCAGGAAG-3′
		Cyp1b1	NM_012940.2	Sense 5′-AGGGCGTGCTCTTGGTTGC-3′ Antisense 5′-CGCCGGCCGGGACTGTTC-3′

Table 2
Effect of the daily treatment with 3-methylcholanthrene (3MC) and/or α-naphthoflavone (αNF, 80 mg/kg), or vehicle (Ctrl), on different end point in immature rats. 3MC and vehicle treatments started at 22 days of age whereas αNF started at 21 days of age. All treatments were completed at 40 days of age. Each point represents the mean ± S.E.M. for eight to ten animals per group.

	Body weight (g)		Ovarian weight (mg)	Vaginal opening (days)	Second estrus (days)
	Day 22	Day 36			
Control	45 ± 1	129 ± 3	39 ± 2	33 ± 1	41 ± 1
3MC (0,1 mg/kg)	48 ± 1	124 ± 2	33 ± 1	33 ± 1	44 ± 1
3MC (0.1 mg/kg) + αNF (80 mg/kg)	50 ± 1	135 ± 3	38 ± 3	32 ± 1	40 ± 2
3MC (1 mg/kg)	46 ± 1	129 ± 1	39 ± 2	34 ± 1	45 ± 2
3MC (1 mg/kg) + αNF (80 mg/kg)	46 ± 1	122 ± 3	33 ± 1	33 ± 1	41 ± 1
αNF (80 mg/kg)	42 ± 1	128 ± 2	35 ± 3	34 ± 1	44 ± 2

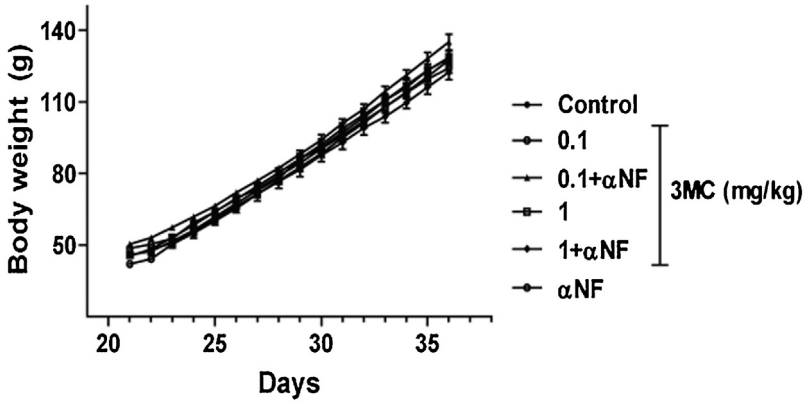

Fig. 1. Pattern of changes in body weight in immature rats treated with 3-methylcholathrene (3MC) and/or α-naphthoflavone (αNF), or vehicle (Ctrl). 3MC and vehicle treatments started at 22 days of age whereas αNF treatment started at 21 days of age. All treatments were completed at 40 days of age. Each point represents the mean ± S.E.M. for eight to ten animals per group.

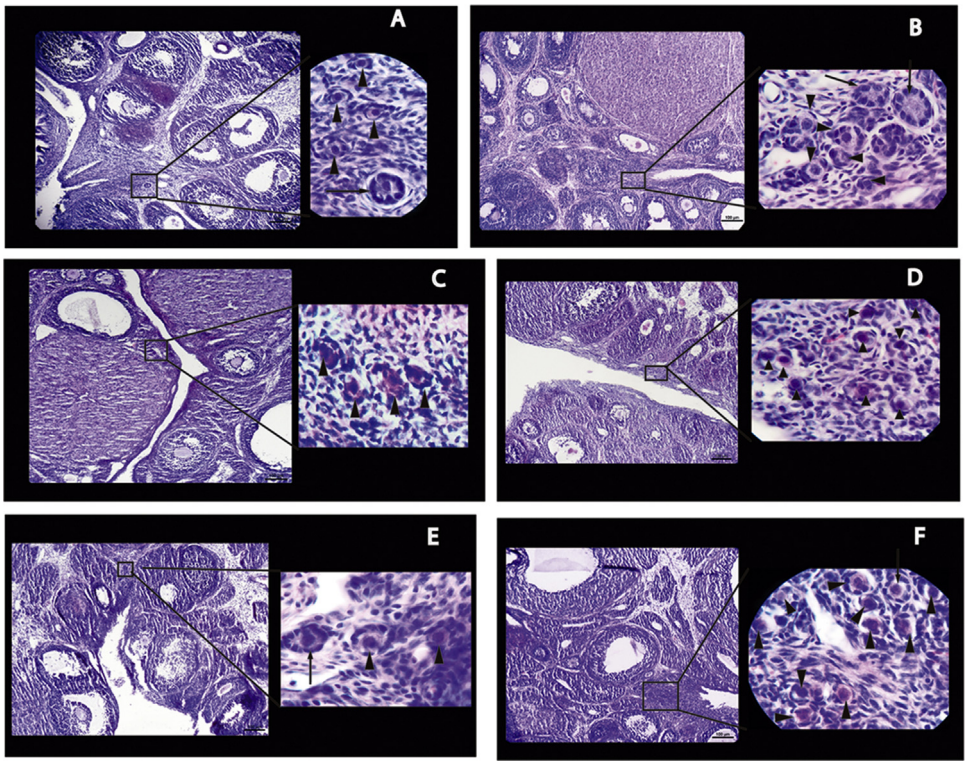

Fig. 2. Effect of daily treatments with 3-methylcholathrene (3MC) and/or α-naphthoflavone (αNF), or vehicle (Ctrl) on the number of small follicles. 3MC and vehicle treatments started at 22 days of age whereas αNF treatment started at 21 days of age. All treatments were completed at 40 days of age. (A-F) Histological appearance of ovarian sections from each group: Ctrl (A), αNF 80 mg/kg (B), 3MC 0.1 mg/kg (C), 3MC 0.1 mg/kg plus αNF (D), 3MC 1 mg/kg (E), or 3MC 1 mg/kg plus αNF (F). Arrow head: primordial follicle (Po), Arrow: primary follicle (Pi), PA: preantral follicle. Magnification: ×100 and ×1000.

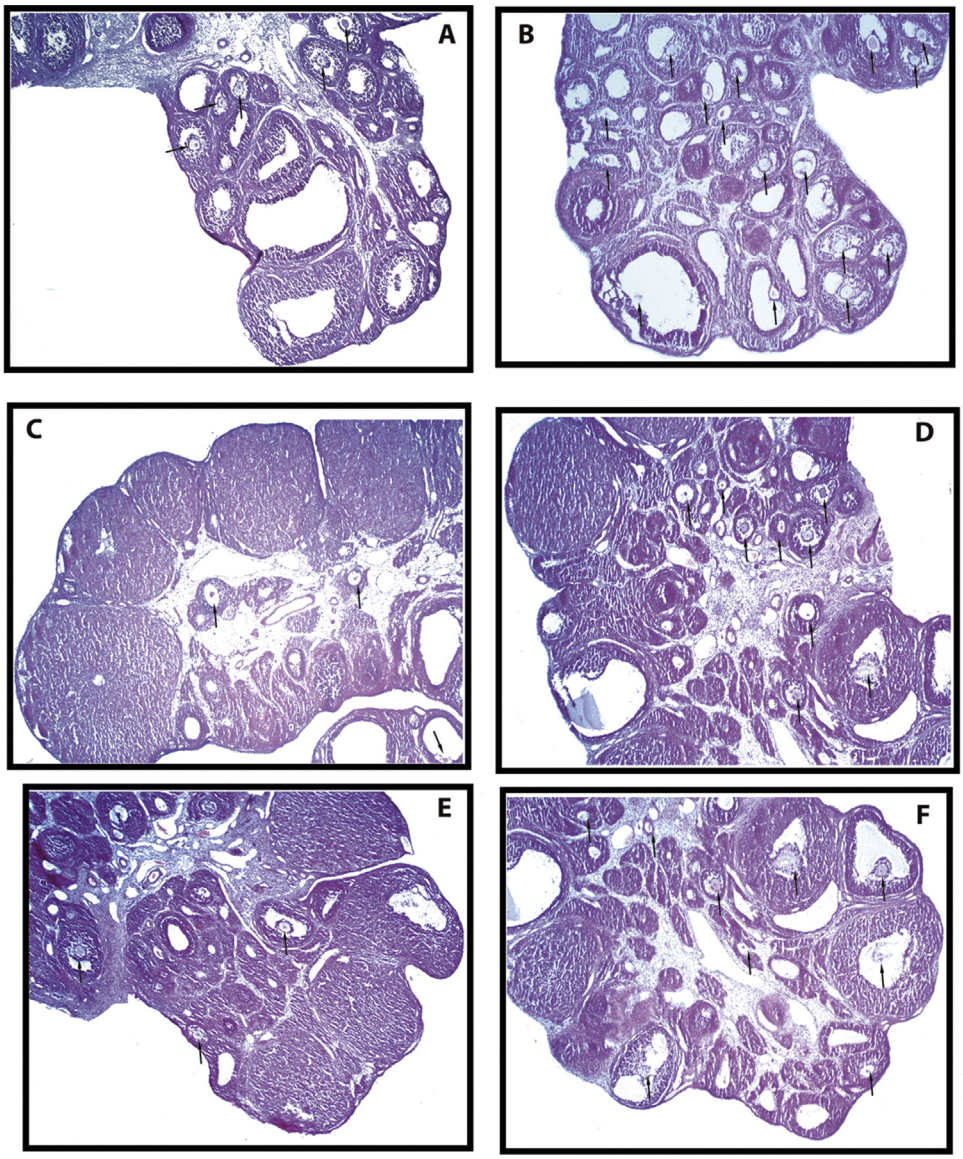

Fig. 3. Effect of daily treatments with 3-methylcholanthrene (3MC) and/or α-naphthoflavone (αNF), or vehicle (Ctrl) on the number of antral follicles. 3MC and vehicle treatments started at 22 days of age whereas αNF started at 21 days of age. All treatments were completed at 40 days of age. (A–F) Histological appearance of ovarian sections from each group: Ctrl (A), αNF 80 mg/kg (B), 3MC 0.1 mg/kg (C), 3MC 0.1 mg/kg plus αNF (D), 3MC 1 mg/kg (E), or 3MC 1 mg/kg plus αNF (F). A: antral follicle. Magnification: ×40.

a measure of possible genetic damage in germ cells (Berthelot-Ricou et al., 2011; Einaudi et al., 2014). The presence of comets was observed by fluorescence microscope (Nikon Eclipse E200 at 200× magnification with photo camera Nikon DS-Fi1). At least 150 cells were analyzed, with two slides scored per animal. DNA damage in the cells was analyzed by measuring the length of DNA migration and the percentage of migrated DNA, using the software CASP 1.2.2 (Comet Assay Software Project).

2.8. Statistical analysis

All data are expressed as means ± S.E.M. Statistical analysis was performed by two-way ANOVA with Bonferroni post-test. Levene's test and a modified Shapiro–Wilk test were used to assess homogeneity of variances and normal distribution, respectively. Differences between groups were considered significant when $P < 0.05$.

3. Results

3.1. Body and ovarian weight, vaginal opening and estrous cycle

Since some aryl hydrocarbons may cause a reduction in body weight as a result of their toxic effects, body weight was daily monitored throughout the treatment. Adding the doses administered over the 20 days of dosing, the mean of total quantity received by the animals was 0.185 ± 0.005 mg and 1.80 ± 0.07 mg for the doses of 0.1 and 1 mg/kg with ranges of 0.166–0.198 and 1.51–2.02, respectively. The body weight gain of control animals was between 144% and 210%, with a mean of 184% throughout the experiment. No differences were found between the controls and the 3MC-treated groups (Table 2). Likewise, since the variation in ovarian weight may be an indicator of the ovarian function, ovarian weight was recorded on the day of death. We found no differences between rats treated with 3MC, 3MC plus αNF and controls

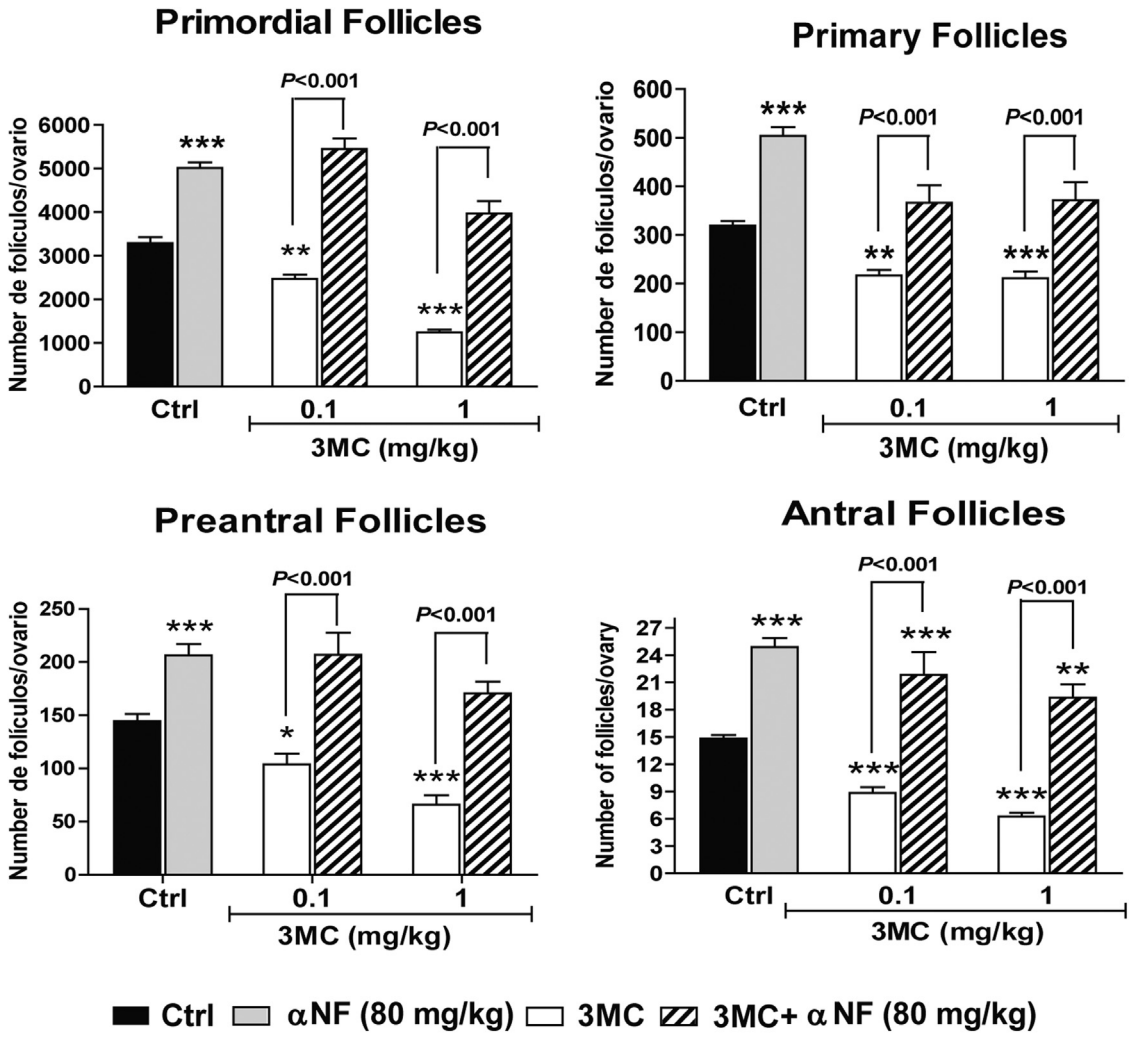

Fig. 4. Effect of daily treatments with 3-methylcholanthrene (3MC) and/or α-naphthoflavone (αNF), or vehicle (Ctrl) on the follicular growth. 3MC and vehicle treatments started at 22 days of age whereas αNF started at 21 days of age. All treatments were completed at 40 days of age. (A–D) Quantitative morphometric analysis of ovarian sections for the different stages of follicles classified as primordial (A), primary (B), preantral (C) and antral (D) follicles as indicated in Section 2. Values are expressed as the number of follicles per ovary. Data represent the mean ± S.E.M. for 8–10 ovaries from different animals with the same treatment, and each value represents the mean of 10 sections. $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ versus Ctrl (two-way ANOVA and Bonferroni post-test).

(Table 2). We also found no differences in the day of vaginal opening or in the establishment of the second estrous cycle between the different groups of rats (Table 2). It is important to note that the values of body weight shown in Table 2 and Fig. 1 represent values recorded between 21 and 36 days of age, because the first rat to reach estrus in the second cycle did so at 36 days of age.

3.2. Follicular growth

Histological examination of the different growing follicles during sexual maturity is an excellent method to assess toxicological action on the ovarian tissue. Figs. 2 and 3 show photomicrographs of the small and antral follicles, respectively, from ovarian sections of each treatment, whereas Fig. 4 shows the quantitative results obtained with histological evaluation, as described in Materials and methods. The response profile observed in all types of follicles after 3MC administration was similar. At both the 0.1 and 1.0 mg/kg doses, the rats treated with 3MC exhibited a lower number of primordial (2470 ± 96, 1245 ± 60, respectively), primary (217 ± 11, 211 ± 14, respectively), preantral (104 ± 10, 71 ± 8, respectively) and antral (8.8 ± 0.6, 6.2 ± 0.4, respectively) follicles than control animals (Po: 2901 ± 174, Pi 327 ± 8, PA: 149 ± 11, A: 14.9 ± 0.5). αNF treatment induced a significant increase in the number of follicles at all stages analyzed (Po: 4856 ± 155, Pi: 497.2 ± 39, PA: 206 ± 16, A: 23.6 ± 1.3) as compared to those of control animals. Moreover, the αNF treatment totally prevented the 3MC-induced decrease of all types of follicles and rats treated with 3MC plus αNF exhibited greater number of follicles in most of the stages analyzed when compared with controls.

3.3. Ovulation rate

To study the effect of these treatments on the ovulation rate, the number of ovulated oocytes within oviducts was counted and expressed per rat. The daily treatment with 0.1 and 1 mg/kg of 3MC significantly decreased the ovulation rate since the number of oocytes was lower (6.8 ± 0.6, $P < 0.05$; 5.6 ± 1.1, $P < 0.01$, respectively) than that of controls (10.6 ± 0.7) (Fig. 5). The treatment with αNF completely prevented the 3MC-induced effect when the dose was 0.1 mg/kg (14.1 ± 1.3; $P < 0.001$ vs 3MC), and partially, when

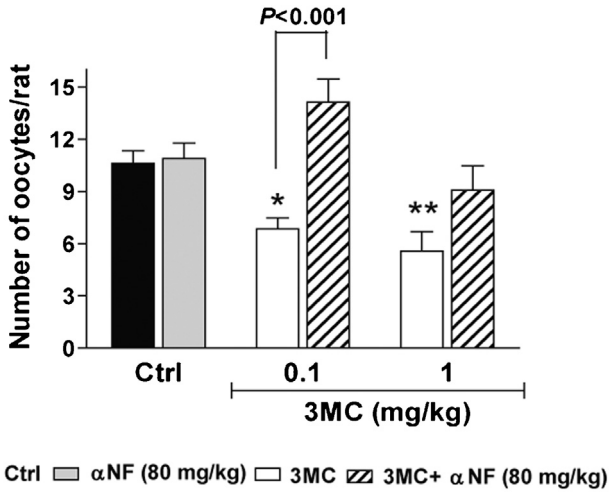

Fig. 5. Effect of daily treatments with 3-methylcholanthrene (3MC) and/or α-naphthoflavone (αNF), or vehicle (Ctrl) on the ovulation rate, determined as the number of oocytes present within oviducts and expressed per rat. 3MC and vehicle treatments started at 22 days of age whereas αNF started at 21 days of age. All treatments were completed at 40 days of age. Each bar represents the mean ± S.E.M. of 8–10 animals, whose values were obtained as the sum of both ovaries from each animal. $^{*}P < 0.05$ and $^{**}P < 0.01$ versus Ctrl (two-way ANOVA and Bonferroni post-test).

the dose was 1 mg/kg (9.1 ± 1.4), although the latter was not significant. The αNF treatment had no effect on the ovulation rate since the number of oocytes in these animals was similar (10.9 ± 0.9) to that of controls.

3.4. Expression of *Cyp1a1* and *Cyp1b1*

Considering that many PAHs act through AhR and that this transcription factor regulates many functions, including metabolizing enzymes and xenobiotic detoxifications, we also studied the levels of two gene targets of AhR, *Cyp1a1* and *Cyp1b1*. To this end, we first measured the levels of mRNA of both enzymes in the ovarian tissue from all the animals. Both doses of 3MC significantly increased the expression of both mRNAs (8–9 and 2-fold for CYP1A1 and CYP1B1, respectively) compared with controls, and αNF treatment prevented these increases (Fig. 6, upper panel). Further, when the animals were treated with αNF alone, the mRNA expression of CYP1A1 was slightly increased compared with control animals. Then, and to study whether these changes were mediated by AhR, we performed the ChIP assay using a specific antibody to AhR protein, which allows recruiting the DNA fragments associated to AhR, and then measured the expression of the *Cyp1a1* and *Cyp1b1* promoters. The promoter of both enzymes were markedly increased in the 3MC-treated animals (3–4 and 2–3-fold for *Cyp1a1* and *Cyp1b1*, respectively) in comparison with those of control animals, and the daily treatment with αNF prevented these increases (Fig. 6, bottom panel). Also, the *Cyp1A1* promoter was increased in the animals treated with αNF alone compared with that of controls.

3.5. Cytotoxicity assays

Figs. 7 and 8 show the results obtained with the MN test and the comet assay in bone marrow cells after rats received the different treatments with 3MC and αNF. Both treatments with 3MC (0.1 and 1 mg/kg) induced the formation of MN (12 ± 1 and 20 ± 2, $P < 0.001$, respectively) and a higher tail DNA percentage (7.6 ± 0.7 and 9.6 ± 0.4, $P < 0.001$, respectively) in a dose-dependent manner when compared with control (MN: 3 ± 1; EC 1.5 ± 0.2). No differences were found in the MN number or in tail DNA percentage when the rats received αNF alone compared with controls. However, the animals that received αNF and 3MC exhibited values (MN: 2.8 ± 0.8, EC: 1.3 ± 0.1) similar to those found in control animals. A similar pattern of responsiveness was observed in oocytes. The treatment with 3MC induced an increase in the genetic damage (0.1 mg/kg: 18.7 ± 0.5; 1 mg/kg: 20.7 ± 0.6, $P < 0.001$) compared with controls (11.1 ± 0.3) and αNF prevented the 3MC-induced toxic action (9.5 ± 0.8 and 8.5 ± 0.7, respectively). However, when the animals received αNF alone, the percentage of basal genetic damage (7.9 ± 0.7, $P < 0.001$) was even lower than the one found in control animals (Fig. 9).

4. Discussion

Many works have demonstrated that a single or a daily dose of 3MC, a known carcinogen, like other PAHs, destroys immature follicles in rodents (Mattison and Thorgeirsson, 1978, 1979; Mattison, 1980; Borman et al., 2000). This effect seems to be mediated by AhR since the intrabursal injection of αNF prevents the 3MC-induced depletion of primordial follicles (Shiromizu and Mattison, 1985). In the present study, we investigated changes caused by daily exposure to 3MC during spontaneous sexual maturation by analyzing not only immature follicles but also the presence of mature follicles and the ovulation rate. We found that rats treated with either dose of 3MC exhibited a lower number of all types of follicles analyzed than the control animals. In addition, the ovulation rate was also inhibited. In some cases, these effects were dose dependent. These results confirm those previously obtained by other authors, although in most previous studies the doses were higher than those used in the present study. The doses applied in this study were based on those used by Borman et al. (2000), who used a range of doses between 0.0015 mg/kg and 60 mg/kg in rats. Our results show, for the first time, that repeated exposure to 3MC inhibits or destroys the formation of immature and mature follicles in parallel with reducing ovulation. It is worth mentioning that these effects were observed without changes in body and ovarian weight or in the day of vaginal opening. Few works have studied the effects of 3MC on the female ovarian function. Konstandi et al. (1997) observed that rats treated with 3MC, at a dose higher than that used in this study, exhibited a prolonged cycle and an increased incidence of constant diestrus and abnormal cycles. These results seem to contrast with ours but the biological model was different. Without considering the loss occurred due to the toxicokinetic process, those authors administered 25 mg/kg twice a week for a month to adult rats, which would represent a total dose of about 40–100 mg, depending on the age of the rats. In our studies, the rats were immature and the total doses of 3MC received ranged between 0.166 and 0.198 mg and between 1.51 and 2.02 mg, when the daily doses were 0.1 mg/kg and 1 mg/kg, respectively. Because the doses were so different, it is difficult to compare the results obtained in these two studies. In a recent work using neonatal mouse ovaries, Sobinoff et al. (2012) found that a high dose of 3MC (5 and 10 mg/kg) daily administered to mice for seven days induced a decrease in primordial follicle composition in parallel to an increase in activating follicle composition, and caused atresia in developing follicles. The authors suggested that the 3MC-induced follicular depletion occurs due to activation of primordial follicles. These data are partly consistent with our results since, although we did not study activating follicles, we found a reduction in the number of primordial follicles and developing follicles. This study shows, for the first time, that repetitive exposure to 3MC during the prepubertal stage, even at low doses, may interfere with sexual maturity in different

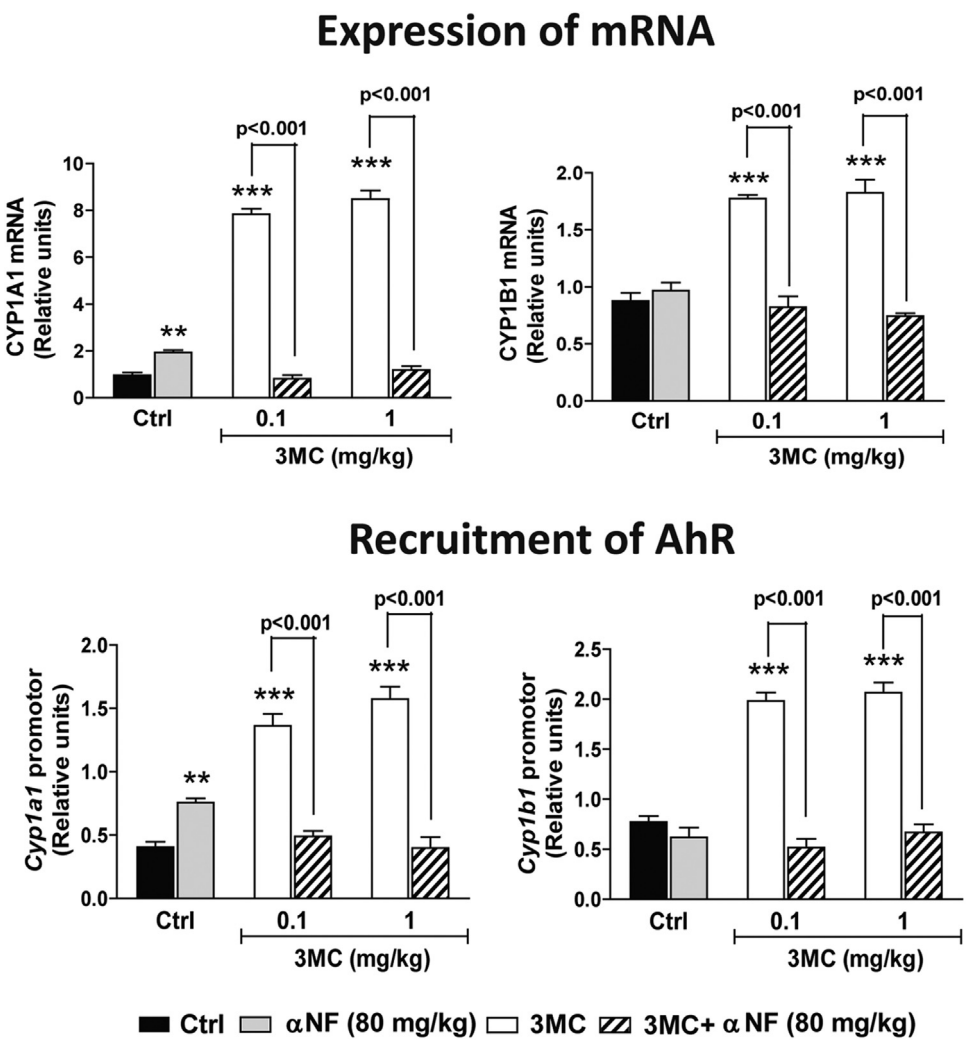

Fig. 6. Effect of daily treatment with 3-methylcholanthrene (3MC) and/or α-naphthoflavone (αNF), or vehicle (Ctrl) on the expression of the mRNA of CYP1A1 and CYP1B1 by real time-PCR (upper panel), normalized to ribosomal 18s mRNA, and the expression of the promoter of *Cyp1a1* and *Cyp1b1* by chromatin immunoprecipitation assay (bottom panel), expressed as percent recruitment relative to chromatin input. 3MC and vehicle treatments started at 22 days of age whereas αNF started at 21 days of age. All treatments were completed at 40 days of age. Data represent the mean ± S.E.M for 8–10 ovaries from different animals with the same treatment. $^{**}P < 0.01$, $^{***}P < 0.001$ versus Ctrl (two-way ANOVA and Bonferroni post-test).

processes, such as follicular development and ovulation, and may destroy the follicular reserve.

To understand more about the inhibitory action of 3MC on the ovarian function, especially its mechanism of action, rats were exposed to 3MC simultaneously with αNF, an antagonist of AhR, as previously described in different systems (Gasiewicz and Rucci, 1991; Gasiewicz et al., 1996; Lu et al., 1996; Zhang et al., 2003; Fukuda et al., 2007). Some studies have shown that a daily treatment with a high dose, such as 80 mg/kg of αNF, could enhance follicular growth in rats (Thompson et al., 2005; Barreiro et al., 2011). Further, Thompson et al. (2005) showed that this dose was effective to block completely the 4-vinylcyclohexene diepoxide-induced follicle loss and caspase-3 activity. Thus, in the present work, we assessed whether this dose of αNF prevented the action of 3MC. Rats that received αNF exhibited a higher number of follicles at all stages analyzed, including primordial follicles, as we found previously (Barreiro et al., 2011). Moreover, the daily treatment with αNF completely prevented the inhibitory action on all types of follicles analyzed and partially prevented it on the ovulation rate. These results are consistent with other findings, where an acute or repetitive treatment with αNF blocked the inhibitory effect of a PAH. Mattison and Thorgeirsson (1979) found that primordial oocytes from different mouse strains were destroyed by the injection of DMBA, BaP and 3MC, three different PAHs commonly found in cigarette smoke, which are also AhR ligands, and that this toxic action was blocked by simultaneous treatment with αNF. It is not surprising that αNF treatment increases the number of primordial follicles. Thompson et al. (2005) found that following 15 daily doses of αNF (80 mg/kg) the number of primordial and primary follicles is greater than in control rats and that this treatment protects against the 4-vinylcyclohexene diepoxide-induced follicle loss in a dose-dependent manner. By using intraovarian injection, Shiromizu and Mattison (1985) found that different PAHs, including 3MC, destroyed small oocytes in mice while an intraperitoneal administration of αNF (80 mg/kg) inhibited this destruction action. Unlike our studies, these authors did not show the individual increase in each type of follicle, especially antral or larger growing follicles. We found that the αNF treatment caused the same response in the different types of follicles, immature or larger growing follicles. Among the different synthetic flavones used in experimental studies as inhibitors of the AhR-mediated activity, αNF, at 80 mg/kg, is the reference by its typical behavior as AhR antagonist in *in vivo* studies. The fact that rats daily treated with

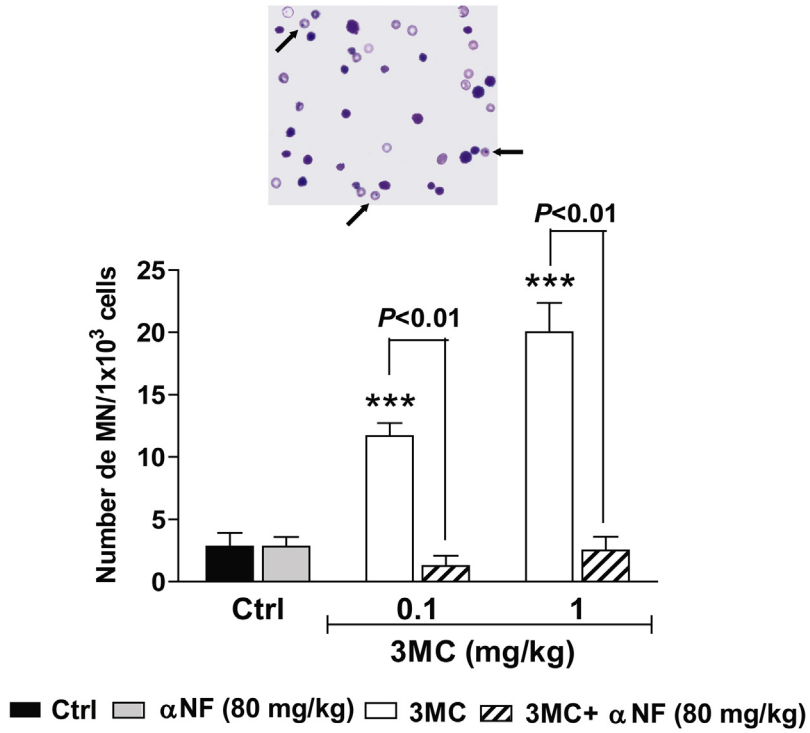

Fig. 7. Effect of daily treatments with 3-methylcholanthrene (3MC) and/or α-naphthoflavone (αNF), or vehicle (Ctrl) on the formation of micronucleus (MN) polychromatic erythrocytes observed in bone marrow cells. 3MC and vehicle treatments started at 22 days of age whereas αNF started at 21 days of age. All treatments were completed at 40 days of age. (A, B) Representative image of MN in a smear of bone marrow cells. (C) Quantitative results of the MN test. The presence of MN polychromatic erythrocytes was visually scored as MN per 1000 cells and each bar represents the mean ± S.E.M. of 8–10 animals, with the same treatment. Magnification: ×400.

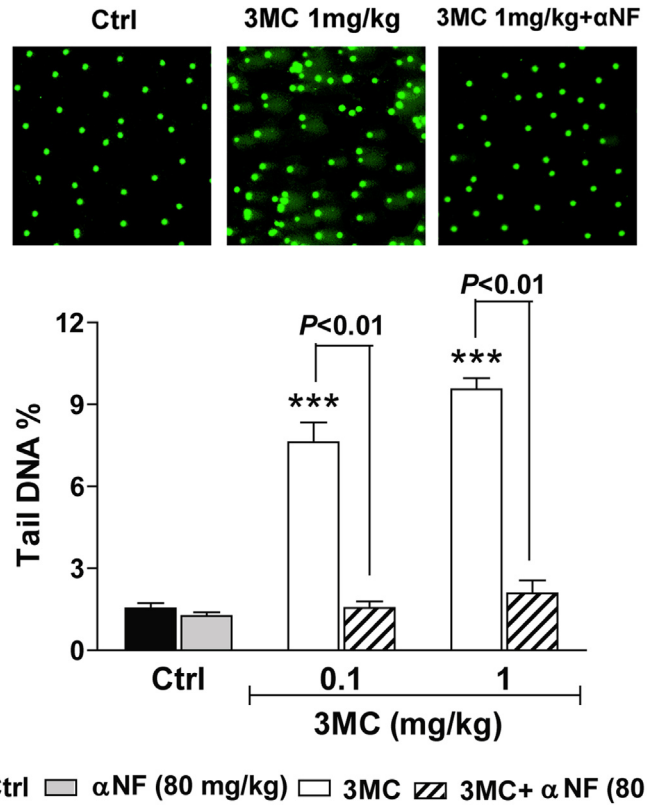

Fig. 8. Effect of daily treatments with 3-methylcholanthrene (3MC) and/or α-naphthoflavone (αNF), or vehicle (Ctrl) on comet formation analyzed in bone marrow cells. 3MC and vehicle treatments started at 22 days of age whereas αNF started at 21 days of age. All treatments were completed at 40 days of age. (A, B) Representative images of bone marrow cells from some groups. (C) Quantitative results of the comet assay. At least 150 cells were analyzed per animal. DNA damage in the cells was analyzed by measuring the length of DNA migration and the percentage of migrated DNA using the software CASP 1.2.2 (Comet Assay Software Project). Magnification: ×100.

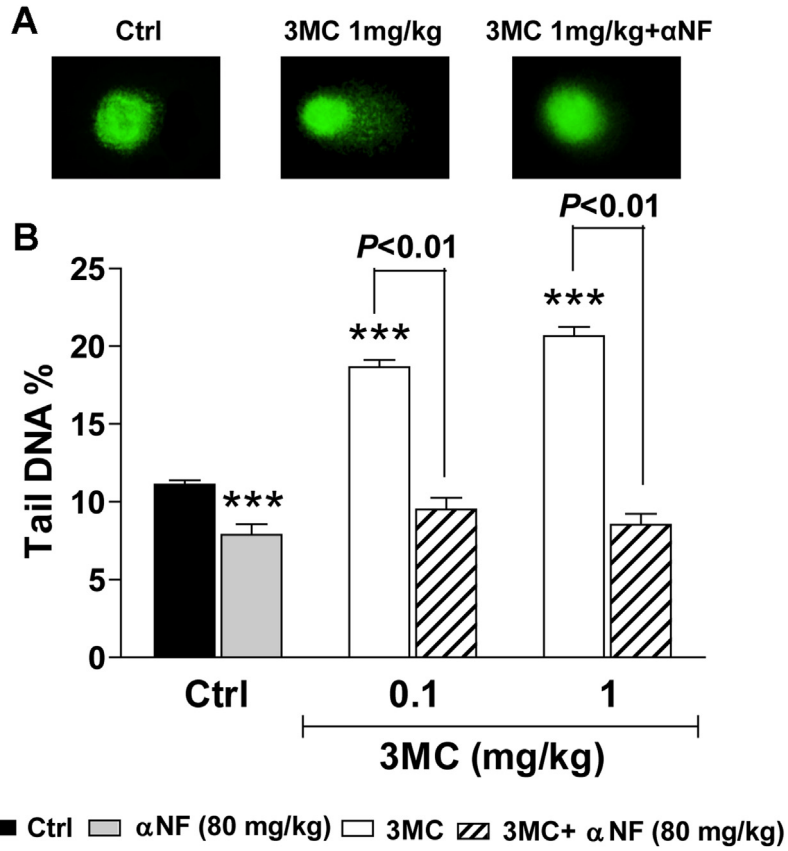

Fig. 9. Effect of daily treatments with 3-methylcholanthrene (3MC) and/or α-naphthoflavone (αNF), or vehicle (Ctrl) on comet formation analyzed in oocytes. 3MC and vehicle treatments started at 22 days of age whereas αNF started at 21 days of age. All treatments were completed at 40 days of age. (A) Representative images of oocytes from some groups. (B) Quantitative results of the comet assay. DNA damage in oocytes was analyzed by measuring the length of DNA migration and the percentage of migrated DNA using the software CASP 1.2.2 (Comet Assay Software Project). Magnification: ×100.

this isoflavone exhibited a higher number of follicles at all stages analyzed reinforces that observed by other authors, using AhRKO mice, about the physiological role of AhR in the generation of primordial follicles and follicular development (Robles et al., 2000; Benedict et al., 2000, 2003). Therefore, and if we assume that αNF acts as an AhR antagonist, the daily treatment with this isoflavone may be blocking an endogenous ligand of AhR that physiologically regulates follicular growth. However, further studies are necessary to understand the αNF action on the follicular dynamics, especially its stimulatory action on primordial follicles.

Considering that 3MC, like other PAHs, may be acting through the AhR pathway and that the activation of this transcription factor regulates a wide variety of gene targets, including metabolizing enzymes (Conney, 1982; Murray et al., 2014), we measured the expression of both *Cyp1a1* and *Cyp1b1*. We found that the mRNA levels of both enzymes were induced by the 3MC treatment and that αNF prevented these inductions. Using different biological models and doses of 3MC higher than those used in the present work, other authors also found 3MC-induced increases in the mRNA of different CYP in various tissues, including the ovary (Kondraganti et al., 2005; Sobinoff et al., 2012; Miao et al., 2014 Sobinoff et al., 2012; Miao et al., 2014). After verifying the induction of mRNA expression by 3MC, we used the ChIP assay to detect the AhR-bound promoter region of both *Cyp1a1* and *Cyp1b1*. We found an increase in the recruitment of AhR to the promoters of both *Cyp* in ovarian tissue after daily exposure to 3MC. This result is consistent with a previous study where Pansoy et al. (2010), using ChIP and DNA microarrays, detected numerous induced AhR-bound genomic regions, including the *Cyp1a1* and *Cyp1b1* genes, after exposure of a cell line of human breast cancer to 3MC. Thus, this work is the first to show that daily exposure to low doses of 3MC, in an animal model, affects the follicular pool and sexual maturity in parallel with an AhR-mediated induction of two important metabolizing enzymes, *Cyp1a1* and *Cyp1b1*, in ovarian tissue. These results suggest that the toxic effect of 3MC on the ovarian development may be mediated by an AhR-dependent mechanism, without excluding the contribution of other pathways. However, and despite that 3MC treatment induced these metabolizing enzymes, with these studies we cannot identify whether the toxic action observed was mediated by 3MC and/or its metabolites. In addition to reversing completely the 3MC action, our results also showed that αNF alone increased the expression of the *Cyp1a1* gene. Previous works found a considerable increase in CYP1A1 mRNA in rat lungs treated with αNF for four days (Sinal et al., 1999) and in tissues other than ovary from trouts fed with αNF for five days (Aluru et al., 2005). However, the mechanism involved in this process still needs to be elucidated. Thus, more experiments using other AhR antagonists are currently in progress in our laboratory.

Pleil et al. (2010) developed a methodology to measure the blood levels of mixtures of PAHs, including 3MC, which could be useful to assess chronic exposures to environmental pollutants. These authors measured the concentrations of PAHs, including 3MC, in humans exposed to diesel exhaust in a controlled chamber study for 2 h (Pleil et al., 2011). However, and to our knowledge, there are no reports documenting the concentrations of 3MC in blood of women smokers or experimental animals exposed to this PAH, or even in ovarian follicular fluid. However, Neal et al. (2007, 2008) measured BaP levels in the serum and follicular fluid in smoker and non-smoker women and found that in women

smokers the levels of BaP were higher in follicular fluid than in serum, and that these levels were significantly higher in women who did not conceive than in those that achieved a pregnancy by in vitro fertilization. Considering that the origin and behavior of 3MC may be similar to those of BaP, it seems reasonable to speculate that this PAH may be preferentially accumulated in the ovarian follicular fluid, a fact that could imply greater toxicity at ovarian level than at systemic level, especially if it is assumed that the doses used in our studies are not high. Thus, and considering that 3MC is a potent carcinogen, we assessed whether the 3MC treatment caused genetic damage at both systemic and local level. We found that both doses of 3MC induced the formation of MN and degradation of DNA in both oocytes and bone marrow cells. However, the αNF treatment again prevented the toxic action on these cells. Accumulated evidences have shown that 3MC is a potent carcinogen through the action of its oxidized metabolites (Sims, 1967, 1970; Cavalieri et al., 1978; Flesher et al., 1998; Shimada, 2006), some of which are generated by CYP enzymes. Some authors have stated that αNF is a selective inhibitor of CYP1A1/2 activity (Chang et al., 1994; Guengerich and Shimada, 1998; Patel and Bhat, 2004). Thus, it is likely that the ability of αNF to prevent the 3MC-induced genetic damage is mediated by inhibition of 3MC metabolism rather than by its action as an AhR antagonist, since it has been demonstrated that its active metabolites form different DNA adducts in different tissues (Stewart and Haski, 1984; Moorthy et al., 2002; Shimada et al., 2003; Xu et al., 2005; Mense et al., 2009; Meinl et al., 2013). However, we cannot discard other possibilities.

Thus, our findings suggest that (i) daily exposure to low doses of 3MC during the pubertal period destroys the primordial follicles and alters sexual maturity by interfering with follicular growth and ovulation; (ii) the 3MC action on follicular growth seems to be mediated by an AhR-dependent mechanism; (iii) a daily treatment with αNF has a clear stimulatory action on follicular growth; and (iv) this isoflavone prevents both the systemic and gonadal 3MC-induced toxicity, probably by the same or different mechanisms, protecting the organism from genetic damage.

Conflict of interests

The authors declare that there are no conflicts of interest.

Acknowledgments

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