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Multigenerational study of the hepatic effects exerted by the consumption of nonylphenol- and 4-octylphenol-contaminated drinking water in Sprague–Dawley rats☆

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Abstract

Our multigenerational study evaluates the hepatic effects of the xenoestrogens nonylphenol (NP), and 4-octylphenol (4OP) on male and female rats when they are exposed uninterruptedly, from conception to adult age, to tap water containing 25 ppm of NP or 4OP. Our results showed that these compounds did not induce any change in liver/body weight ratio (relative liver weight, RLW). In the morphological analysis we did not find evident signs of cytotoxicity. The most relevant findings were the presence of both an increase in the apoptotic index and in the percentage of binuclear hepatocytes in livers from exposed animals. Additionally, our study revealed the presence of hepatocellular glycogenosis (mainly in 4OP-exposed rats): the type of glycogen accumulated was in aggregates (gamma-glycogen), a non-functional form of glycogen. This study demonstrates that, at levels close to those described in the environment, NP and 4OP are capable of inducing a number of hepatic effects, potentially related with adaptive, and/or metabolic alterations of liver tissue.

Keywords: Nonylphenol (NP); 4-Octylphenol (4OP); Xenoestrogens; Rat liver; Apoptosis; Glycogen accumulation

1. Introduction

Alkylphenol ethoxylates (APEOs), especially nonylphenol and octylphenol ethoxylates, are widely used as non-ionic surfactants. They have been added to detergents, toiletries, herbicides, and many other every-day use products (annual global production over 300,000 tonnes). APEOs enter the hydrologic cycle after use; during wastewater treatment they are degraded into alkylphenols (APs) (Chapin et al., 1999). APs, such as nonylphenol (NP) and 4-octylphenol (4OP), have been described in sewage effluent, groundwater and surface and drinking waters (Ahel et al., 1996; Blackburn et al., 1999; Céspedes et al., 2005; Lavado et al., 2005).

As major research studies have indicated, many environmental chemicals, among them APs, are considered as xenoestrogens and endocrine disruptors because of their capacity to disrupt reproductive development in wildlife and humans (White et al., 1994; Blake and Boockfor, 1997; Lee, 1998; Laws et al., 2000; Blake et al., 2004). To date, research has focused on their estrogenicity and potentially adverse effects on the reproductive system, using *in vitro* and *in vivo* systems, while neglecting their potential impact on other tissues. In fact, there is only limited information concerning the effects of these environmental pollutants in sexually modulated (not differentiated) tissues, such as the liver (Lee et al., 1996; Zumbado et al., 2002).

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The liver is a target tissue for sex steroid hormones. A specific receptor for a number of steroid hormones exists, among them the estrogen receptor (ER) (Aten et al., 1978). As established previously by others, environmental contaminants with estrogenic activity bind and activate ER (Hall and Korach, 2002). Currently there is enough information about the cellular responses involving ER and environmental contaminants with estrogenic activity (xenoestrogens) (Gaido et al., 1997).

It is well known that natural and synthetic estrogens exert a number of toxic effects on liver tissue, including the development of liver adenomas and hepatocarcinomas (Vickers and Lucier, 1993). In fact, estrogens are considered primary liver mitogens (Columbano et al., 1991; Columbano and Shinozuka, 1996), and various synthetic and natural steroids have been classified as hepatocarcinogens by the International Agency for Research on Cancer (IARC, 2004). Our group has described that alkylphenols NP and 4OP, similarly to estrogens, exert a number of effects on rat liver. Among those effects, they induce cell proliferation and spindle disturbances and they are capable of modulating the expression of putative membrane receptors for estrogens (Zumbado et al., 2002). These effects are also exerted by synthetic estrogens, such as ethynilestradiol and diethylstilbestrol (Ochi, 1999; Zumbado et al., 2002). Our results give rise to questions concerning the potential hepatotoxicity of these environmental estrogens. However, these results were obtained in short-term studies with high doses of APs. Many authors express some concern about the capacity of short-term rodent assays because these assays may do not possess enough sensitivity and, as in the case of APs, human and wildlife would never be exposed to environmental contaminants at the doses and route of administration employed in them.

With respect to long-term studies, although currently there is very limited information, APs do not seem to exert toxic effects on rat liver. However, we must take into account that, among the long-term studies published in mammals (Chapin et al., 1999; Tyl et al., 1999, 2006; Blake et al., 2004), only in the study developed by Blake and co-investigators the main route of exposure to APs in the environment (water) was employed and, on the other hand, the authors did not study hepatic effects in depth. The present study was designed to fill in this gap.

As cited previously, the major source of exposure to APs are contaminated freshwaters. In fact, the European Union Council has recently included APs and their derivatives in the list of substances to be evaluated in order to obtain toxicological information necessary in environmental risk assessment studies (Regulation 648/2004/EC). These facts led us to establish an experimental *in vivo* model that could mimic the real conditions of exposure to these contaminants (chronic exposure to low doses of APs through APs-contaminated drinking water). This model would allow us to evaluate the possibility that these environmental contaminants could exert hepatotoxic effects either in wildlife or humans. To provide as many opportunities as possible to observe such toxic effects, our study maximized chronic exposure to NP and 4OP: three generations of pups exposed from conception to adult age were produced. This experimental model implies that animals are exposed *in utero* to APs because these chemicals can be accumulated in maternal livers (Daidoji et al., 2003) and could reach rat foetuses through the placenta, as demonstrated previously by others (Haavisto et al., 2003). The dose selected throughout this experiment (25 ppm) has been described in the environment (Cantero et al., 2006) and it is close to that capable of inducing alterations in the male reproductive system in Fischer rats (Blake et al., 2004).

2. Materials and methods

2.1. Animals

Sprague–Dawley rats were used throughout these experiments. The animals were purchased from Charles River Inc. (St. Aubin les Elbeuf, France). Animals were housed in polycarbonate cages (model PC10147HT, Allentown Inc., Allentown, NJ, USA) and they had free access to laboratory chow (Safe Standard Diet A04, Usine d'Alimentation Rationnelle, Lille, France) and tap water. Water quality was monitored prior to the study to ascertain that contaminants were below levels defined for drinking water in the Spanish and European Community Legislation (RD 140/2003 and RD 1744/2003, and Directive 1998/83/EC, respectively). Laboratory chow was monitored for contaminants by the vendor. Animals were maintained on a 12:12 h light–dark cycle and housed in an animal room where temperature (22–24 °C) and humidity (65–75%) were controlled.

2.2. Chemicals

NP ($C_9H_{19}C_6H_4OH$; CASRN 25154-52-3), 4OP ($CH_3(CH_2)_7C_6H_4OH$; CASRN 1806-26-4), ethynilestradiol (EE_2; (17α)-19-Norpregna-1,3,5(10)-trien-20-yne-3,17-diol), and, unless otherwise indicated, the rest of the products cited in this work were purchased from Sigma–Aldrich Co. (St. Louis, MO, USA). In order to dose appropriately the chemical contaminants in water we dissolved APs in 100% ethanol and subsequently we added them to tap water (1 l) in a polypropylene carboy (Nalgene7, Nalge Europe Ltd., Neerijse, Belgium) to prepare the 25 ppm solution. The mixtures were then stirred for 24 h. Similarly, ethanol without APs was also added to tap water in control groups.

2.3. Preliminary studies

Because of the high hydrophobic character of APs (log K_{ow} of 4.5 and 4.48 for NP and 4OP, respectively) (Ferguson et al., 2001) and in order to be sure that chemicals were absorbed sufficiently by laboratory animals we decided to evaluate the biodisponibility reached, in our experimental conditions, by the synthetic estrogen EE_2, that presents similar hydrophobic properties to those of APs (log K_{ow} 4.2). In these preliminary studies four inmature rats (21-day-old) (2 males and 2 females) were exposed for 4 months to tap water containing 25 ppm of EE_2 (previously dissolved in 10 μl ethanol). Similarly, another group of four rats were exposed to appropriate vehicle during the same period (control group). EE_2-exposed rats showed a number of adverse effects (data not shown): food and water consumption was decreased in both, males and females; there were evident differences in terminal body weight (decreased) and in liver/body weight ratio (relative liver weight, RLW) (increased), if we compare them with the animals of the control group. Despite the fact that these animals were mated at 3 months old, they were not capable of procreating. Likewise, EE_2-treated animals showed an impaired physical appearance (roughened coat and loss of long hair). The results obtained with the groups of rats exposed to EE_2 allow us to ensure that EE_2 was sufficiently absorbed and to assume that APs tested throughout this work would be absorbed and would reach enough biodisponibility to be capable of exerting some effect in body organs of exposed rats.

2.4. Study design

Twelve immature animals (6 males and 6 females), 21-day-old, were used as parental (P) generation animals in this study. Two males and 2 females per chemical compound (NP, 4OP, or vehicle) were exposed to tap water containing 25 ppm of NP, 4OP, or vehicle. At the age of 3 months animals from P were mated (1:1). The litter of each P couple was culled to around 10 pups (with

equal sex ratio when possible) on postnatal day (PND) 4. Animals from P were sacrificed and discharged when their litters (F_1 generation) reached 21 days of age. Animals from P were not the subject of this study.

Again at the age of 3 months animals from F_1 were mated, and their litters were culled to around 10 pups (F_2). Similarly, animals from F_2 were mated and their litters also were culled to around 10 pups. Finally, another generation was obtained (F_3).

Each animal of F_1, F_2 and F_3 (42 animals each generation) was exposed for approximately 180 ± 15 days (≈6 months), uninterruptedly (including mating, gestation, and lactation) to the contaminated drinking water. Body weights, and food and water consumption were recorded weekly, and clinical signs were recorded at least once a day.

As previously mentioned, when animals from each generation reached around 6 months of age, they were sacrificed. At termination of each generation-group, the animals (126) were weighed and killed by cervical dislocation. Livers were quickly removed and weighed. Livers were excised in three blocks, each measuring about 1 cm in length and 3 mm in thickness. The blocks were removed from the right, middle, and left hepatic lobes of the liver in order to be used for structural (fixed in 10% neutral phosphate-buffered formalin for 24 h), ultra-structural (fixed in 2.5% glutaraldehyde), and TUNEL technique analysis (fixed in 10% neutral phosphate-buffered formalin for 24 h). The rest of the liver was stored at −70 °C to be employed in biochemical studies.

2.5. Tissue preparations for light microscopic examination

Tissue sections fixed in formalin were transferred to 70% ethanol and embedded in paraffin, and they were cut 4–5 μm thick and stained with hematoxylin and eosin (H/E) and periodic acid Schiff in accordance with standard procedures.

2.6. Tissue preparations for electron microscopic examination

The other block of the liver fixed in 2.5% glutaraldehyde was cleared in propylene oxide, and it was impregnated and embedded in epon resin. Thin sections were mounted on copper grids, stained with 4% uranyl acetate and lead citrate, and examined in a Zeiss EM 109 electron microscope.

2.7. TdT-mediated dUTP-biotin nick end labelling (TUNEL) assay

TUNEL assay (terminal deoxynucleotidyl transferase-mediated dUTP nick end labelling) was used to detect apoptosis (Finnberg et al., 2000). The TUNEL (*In Situ* Cell Death Detection, AP kit Roche Diagnostics, Indianapolis, IN, USA) was applied following the steps recommended in the manufacturer's instructions. After the final washing in PBS, the sections were then counterstained with Harris haematoxylin, dehydrated in graded alcohols, cleared in xylene and mounted in synthetic resins. The mean values of all cells examined from each specimen were used to determine apoptotic index, defined as the percentage of positive cells among all the cells counted.

2.8. Protein measurement

Proteins were measured as described by Lowry et al. (1951) using Bovine Serum Albumin (BSA) as standard.

2.9. Glycogen determination

Small liver pieces (0.5 g) were separated for determination of the glycogen content of the liver using the anthrone reagent method (Seifter et al., 1950) according to Muriel and Deheza (2003) on a Beckman DU7400 spectrophotometer (Beckman Instruments Inc., CA, USA).

2.10. Evaluation of oxidative stress in liver tissue

2.10.1. Measurements of lipid peroxidation in liver

A piece of liver (approximately 0.5 g) was processed to obtain a homogenate and for measuring malondialdehyde levels (MDA), as described by Boada et al. (1999) (modified from Esterbauer and Cheeseman, 1990), on a Beckman DU7400 spectrophotometer (Beckman Instruments Inc., CA, USA).

2.10.2. Measurement of reduced and oxidized Glutathione (GSH/GSSG)

Another piece of liver (approximately 0.5 g) was processed to obtain a homogenate for measuring GSH/GSSG levels, as described by Zumbado et al. (2002), modified from Ellman and Lysco (1979).

2.11. Analysis of data

Results were analyzed statistically using multivariate analysis of variance (SPSS v 13.0; Chicago, IL, USA). Post hoc analysis employed was the two-tailed Student's-Newman–Keuls test. If significant effects were seen the data were further evaluated using the Bonferroni multiple comparison test. Results are expressed as mean ± S.E.M. p Values <0.05 were considered significant.

3. Results

3.1. Effects of APs in the drinking water on water and food consumption

Water and food intake did not vary significantly between APs-exposed- and control groups during the study. Additionally, there were not statistically significant differences between NP- and 4OP-exposed groups. Only, as expected, there were important differences between male and female rats from any experimental group (data not shown).

3.2. Relative liver weight (RLW)

Our results show clearly that at these doses APs-exposure was not capable of inducing any statistically significant change in RLW from male and female rats of any generation (see Table 1). However, male and female rats exposed to NP showed, although without statistical significance, increasing values of RLW in F_1 and F_2 groups, while in F3 were the animals exposed to 4OP those who showed the highest RLW values.

3.3. Histological and biochemical evaluation of liver tissue

Structural and ultrastructural studies showed a normal liver structure in control rats from every experimental group (Fig. 1a). Nevertheless, the results were very different in rats receiving, NP- or 4OP-contaminated water. APs exposure to animals induced a number of effects in liver tissue and the difference between the effects exerted by these two chemicals was mainly quantitative, not qualitative.

Although APs-exposure did not induce an evident hepatotoxicity, livers from animals of any APs-exposed group showed a slight lipidic degeneration (Fig. 1b) and increased levels of apoptotic cells. The presence of apoptotic figures was studied by H/E and by TUNEL technique. Interestingly, NP was the AP that induced the highest increase of apoptosis both in male and female rats from F_1. On the contrary, in male and female rats from F_2 and F_3 4OP was the chemical that induced a more evident increase of apoptosis. It should be highlighted that the highest values of apoptotic index were found in 4OP-exposed female rats from F_3 (Table 1 and Fig. 2a–c). Because

Table 1
Histological and biochemical parameters evaluated

Generation	Sex	T	RLW	Binucleated cells (%)	TUNEL (%)	MDA (nmol/g tissue)	Glycogen (mg/g tissue)
F1	Male	C	3.31 ± 0.27	3.04 ± 0.71	2.05 ± 0.66	23.08 ± 7.06	24.81 ± 7.06
		NP	3.27 ± 0.20	3.33 ± 0.78	2.88 ± 0.75*	22.02 ± 6.13	26.93 ± 6.12
		4OP	3.21 ± 0.18	3.25 ± 0.90	2.21 ± 0.71	18.97 ± 5.57	29.54 ± 5.56
	Female	C	3.83 ± 0.35	3.12 ± 0.67	2.12 ± 1.87	23.17 ± 11.33	23.10 ± 8.53
		NP	3.85 ± 0.32	3.31 ± 0.58	2.52 ± 0.55	21.07 ± 11.83	25.80 ± 6.71
		4OP	3.77 ± 0.27	3.21 ± 0.95	2.35 ± 0.83	25.45 ± 4.86	25.07 ± 5.70
F2	Male	C	3.02 ± 0.30	3.45 ± 0.84	1.87 ± 0.54	28.72 ± 2.54	24.09 ± 6.90
		NP	3.11 ± 0.23	3.90 ± 0.71	2.23 ± 0.47	31.55 ± 5.67	24.53 ± 7.22
		4OP	3.04 ± 0.12	3.73 ± 0.68	2.91 ± 0.75*	27.92 ± 5.26	32.88 ± 8.63*
	Female	C	3.22 ± 0.39	3.31 ± 0.45	2.01 ± 0.47	25.87 ± 9.43	29.84 ± 4.87
		NP	3.26 ± 0.35	3.62 ± 0.71	2.37 ± 0.62	33.94 ± 5.29	31.62 ± 11.27
		4OP	3.25 ± 0.23	3.53 ± 0.51	2.86 ± 0.66*	29.63 ± 4.27	25.68 ± 4.60
F3	Male	C	3.09 ± 0.18	3.06 ± 0.36	2.31 ± 0.36	31.55 ± 3.07	34.05 ± 6.02
		NP	3.12 ± 0.29	3.91 ± 0.77*	3.06 ± 0.84	35.23 ± 5.20	37.33 ± 7.10
		4OP	3.04 ± 0.31	3.88 ± 1.02	3.16 ± 1.02*	30.83 ± 7.60	49.88 ± 6.02*,#
	Female	C	3.14 ± 0.46	3.27 ± 0.44	2.30 ± 0.54	30.09 ± 2.00	29.00 ± 4.73
		NP	3.20 ± 0.34	4.13 ± 0.84*	3.17 ± 0.57	31.90 ± 2.44	36.40 ± 9.50
		4OP	3.26 ± 0.28	3.74 ± 0.76	3.31 ± 0.78*,#	39.16 ± 1.83*,#	37.20 ± 8.10

T: treatment; C: vehicle; NP: nonylphenol; 4OP: 4-octylphenol. Results are means ± S.E.M. for each group. RLW: relative liver weight; TUNEL: terminal deoxynucleotidyl transferase-mediated dUTP nick end labelling; MDA: malondialdehyde.

* $p < 0.05$. APs-exposed-group *vs*. its respective control group.

# $p < 0.05$. APs-exposed-group *vs*. its respective group from the other two generations.

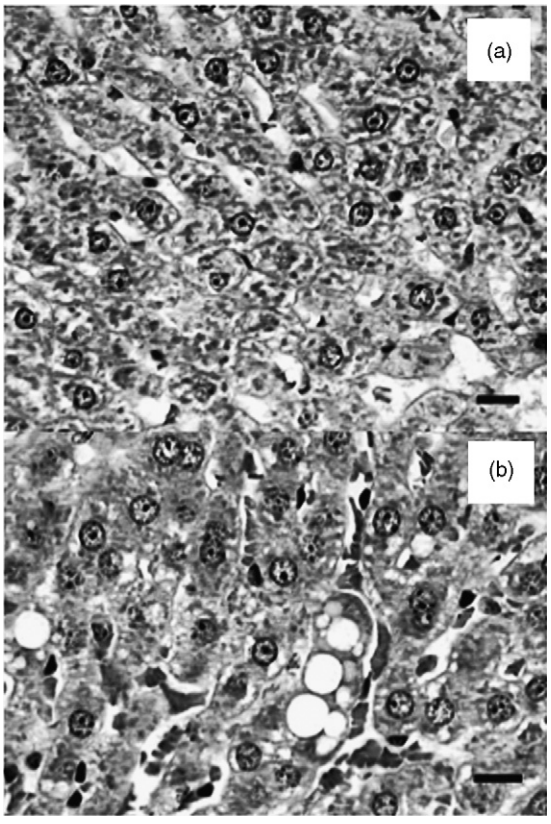

Fig. 1. Representative photomicrographs of liver sections from exposed and not exposed rats to alkylphenols. (a) Photomicrograph of liver from control rat. There is a relatively uniform cytoplasmic volume, nuclear size, and staining density, and the presence of binucleate and apoptotic cells is slight (H&E 40×; bar 20 μm). (b) Photomicrograph of liver from male of F_2 exposed to 4OP showing lipidic degeneration (H&E 40×; bar 20 μm).

of the well known relationship between apoptosis and oxidative stress we evaluated the presence of by-products of lipid peroxidation processes (MDA) and GSH/GSSG levels in livers from animals chronically exposed to APs. These chemicals did not seem to induce oxidative stress in liver tissue, in our experimental conditions, as established by the fact that GSH/GSSG levels were not affected by APs-exposure (data not shown). However, although long-term APs-exposure does not seem to increase lipid peroxidation processes, female rats from F_3 exposed to 4OP showed increasing values of MDA. In this case, similarly to that described for apoptotic index, 4OP-exposed female animals from F_3 showed higher levels of MDA than both animals from their respective groups in F_1 and F_2 and from their respective control group (see Table 1).

An increased presence of binucleate hepatocytes turned out to be evident. Although without statistical significance, male and female rats from F_1 and F_2 exposed to NP showed higher presence of binucleate hepatocytes than both their respective control groups and the 4OP-exposed groups. In addition, in the last generation (F_3), the increasing presence of binucleate hepatocytes reached statistical significance in male and female rats exposed to NP if we compare them with their respective control groups, being this result specially impressive in female animals (Table 1 and Fig. 3a–c).

Furthermore, the histological study of the livers from APs-exposed animals showed an evident glycogen accumulation with hepatocytes showing cytoplasmic vacuolation which give them a "ground-glass" appearance (Fig. 4a and b). The type and extent of the glycogen accumulated varied in relation with the sex, generation and APs-exposure group evaluated. Livers of the male and female animals exposed to 4OP showed the

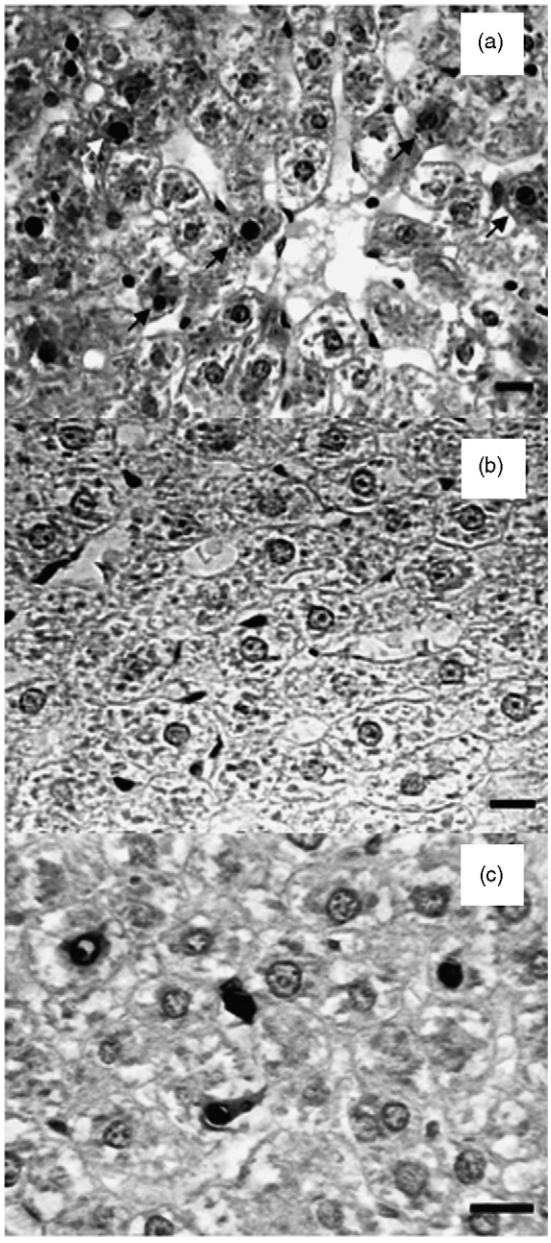

Fig. 2. Two representative photomicrographs showing the evident increase of apoptotic figures. (a) Photomicrograph of liver from male rat exposed to NP. Note the presence of apoptotic cells (arrows) (H&E 40×; bar 20 μm). (b) Photomicrograph of liver from control rat. Note the absence of apoptotic cells (40×; bar 20 μm). (c) Photomicrograph of histological detection of apoptotic cells by TUNEL of a liver from male rat exposed to 4OP (40×; bar 20 μm).

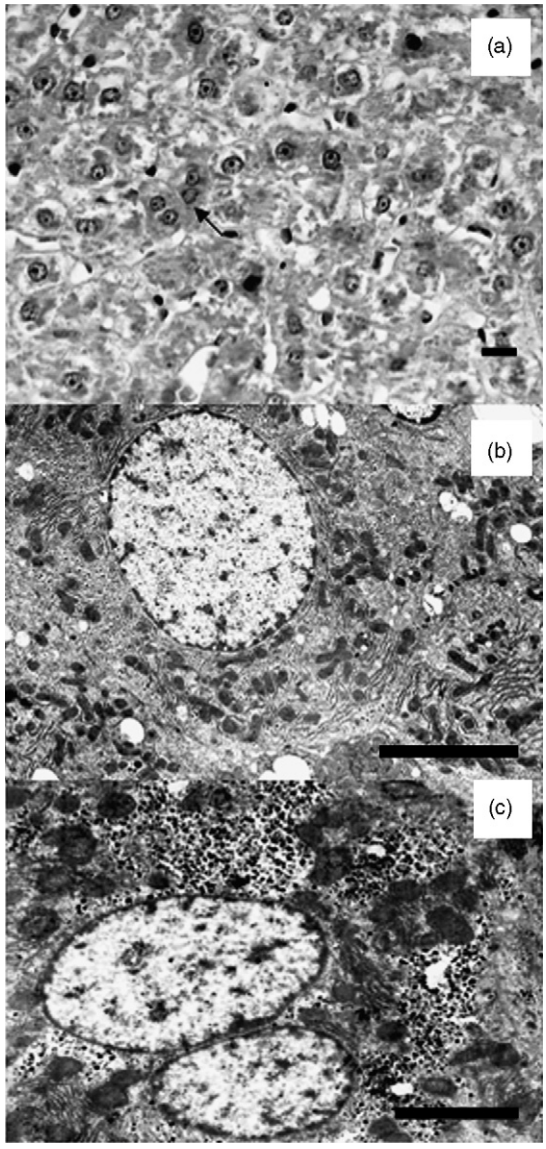

Fig. 3. Two representative photomicrographs showing an increased presence of binucleate cells in livers from APs-exposed animals. (a) Photomicrograph of liver from female rat from F_3 exposed to NP. There is a clear increase in the percentage of binucleate cells (arrows) (H&E 40×; bar 20 μm). (b) Electron micrograph of liver from control rat showing the nucleous of a liver cell (bar 5 μm). (c) Electron micrograph of liver from female rat from F_3 exposed to NP showing binucleate hepatocyte (detail) (bar 5 μm).

greatest cytoplasmic accumulation of glycogen and the type of glycogen accumulated showed a pattern of amorphous masses (γ-glycogen) (Table 1 and Fig. 5a). Although these findings were present in any rat liver exposed to 4OP, they were more evident in 4OP-exposed males from F_3. On the contrary, livers from animals exposed to NP showed a moderate cytoplasmic accumulation of glycogen, and glycogen was mainly accumulated in monoparticulate forms (α- and β-glycogen) (Table 1 and Fig. 5b), although in some livers from F_2 and F_3 NP-exposed rats the presence of γ-glycogen could also be observed, but at very low proportions. Biochemical evaluation of the liver cells glycogen content reinforced such results due to the fact that APs seem to increase the levels of glycogen as compared with control groups. Only in the case of 4OP-exposed male animals from F_2 and F_3 a statistically significant increase of glycogen exists (Table 1).

4. Discussion

The present study is focused on the toxic effects induced in rat liver by long-term exposure to environmentally persistent xenoestrogens NP and 4OP. Although, up to now, no author has described in depth the hepatic effects of APs in long-term studies (Chapin et al., 1999; Tyl et al., 1999, 2006), a relation-

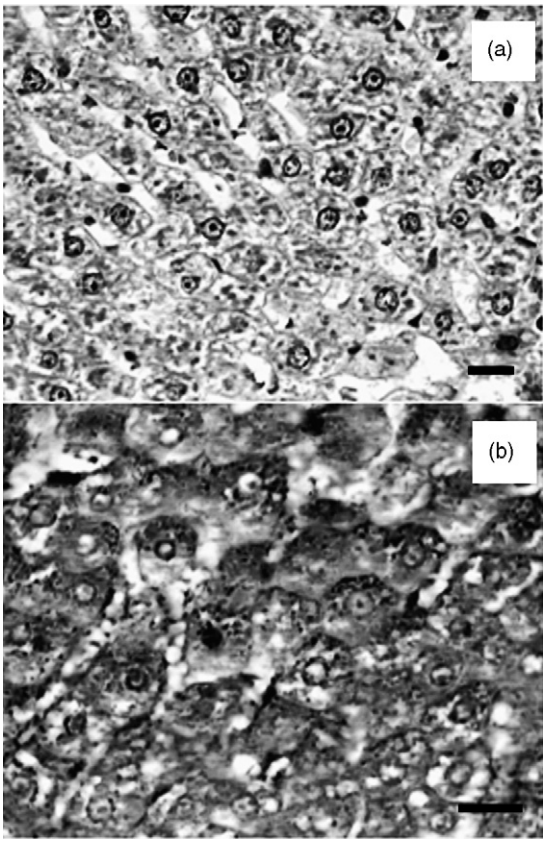

Fig. 4. Two representative photomicrographs showing hepatocellular glycogenosis (a) Photomicrograph of liver from male rat from F_2 exposed to 4OP (H&E 40×; bar 20 μm). (b) Photomicrograph of liver from female rat from F_3 exposed to 4OP. Note the "ground-glass" appearance of hepatocytes (PAS 40×; bar 20 μm).

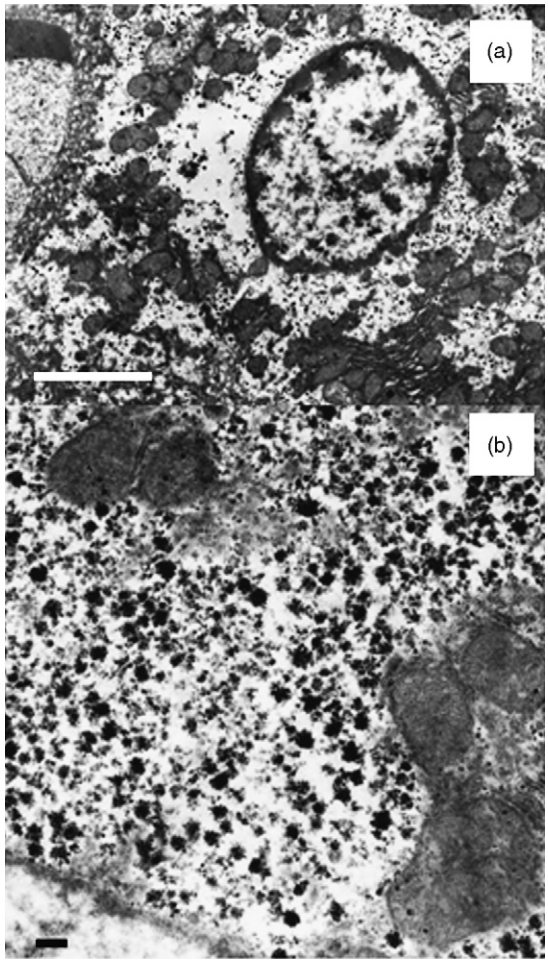

Fig. 5. Two representative electron micrographs showing the different types of glycogen (a) Electron micrograph of a liver from male rat from F_3 exposed to OP. Note the amorphous glycogen masses (γ-glycogen) (detail) (bar 5 μm). (b) Electron micrograph of a liver from male rat from F_2 exposed to NP. Note the electron-dense rosettes of glycogen (α-glycogen) (detail) (bar 1 μm).

ship between APs and liver has been reported previously by others. Liver monooxygenase enzymes, e.g. cytochrome P450, are implicated in the metabolization processes of these chemicals (Lee et al., 1996; Hanioka et al., 1999; Pedersen and Hill, 2000) and it is well known that APs can accumulate in liver tissue (Certa et al., 1996; Daidoji et al., 2003). Furthermore, NP and 4OP induce cellular proliferation and abnormal mitotic processes in immature male rat liver (Zumbado et al., 2002).

The results obtained by us in this study show that these compounds are not capable of inducing any change in RLW of rats when they were exposed at low doses for a long period of time. This result is in accordance with previously known data indicating that NP did not exert any effect on RLW when administered at low doses through diet in a multigenerational study. Nevertheless, in those studies males of F_0, F_1, and F_2 showed an increase in RLW when they were fed with diet containing high doses (650 or 2000 ppm) of NP (Chapin et al., 1999; Hossaini et al., 2003).

According to our study, chronic exposure to APs seems to exert a number of effects in liver tissue. Our results open the possibility that APs could act as metabolic disruptors, affecting glucose metabolism in rat liver. This metabolic effect has been described for other environmental estrogens, such as PCBs and hexachlorobenzene which are capable of altering gluconeogenic and lipogenic enzymes in rat liver (Boll et al., 1998; Mazzetti et al., 2004). Livers from animals exposed to NP or 4OP showed an increase in their intracellular glycogen content. This accumulation of glycogen gives rise to the characteristic image of hepatocytes with "ground-glass" appearance (Rybicka, 1996).

Although insulin is the major direct signal for glycogen deposition (Parkes and Grieninger, 1985), sex steroid hormones have been implicated in glucose metabolism and therefore in liver glycogen deposition (Carrington and Bailey, 1985; Tarnopolsky and Ruby, 2001). Furthermore, other estrogenic compounds, such as the mycoestrogen zearalenone, increase liver tissue glycogen content (Nogowsky et al., 1994) by means of an alteration of the Insulin/Glucagon ratio (Youssef et al., 2003). It seems clear that estrogens increase glycogen accumulation in mammalian liver, although the precise mechanism that induces such accumulation is not as yet well understood. One possibility is that such an effect could be an indirect action mediated by pancreatic hormones (insulin and glucagon) (Parkes and Grieninger, 1985). Another one could be the existence of sexual dimorphism in the adrenergic regulation of hepatic glycogenolysis (Studer, 1987). Although the capability of NP and 4OP to induce glycogen accumulation in liver tissue may be an estrogenic effect of

these compounds, it must be highlighted that glycogen accumulation by hepatocytes could be also related with adaptive responses in order to resist further insults rather than let cells undergo estrogenic or toxicity processes (Nayak et al., 1996). The fact that livers from F_2 and F_3 animals showed higher levels of glycogen point to the possibility that this effect is only an adaptive response. However, we must take into account that there is a close relationship between hepatocellular glycogenosis and hepatocarcinogenesis. Thus, the hepatocellular glycogenosis seems to be an excellent biological marker of early changes during hepatocarcinogenesis (Bannasch et al., 1980), and, as cited previously, estrogens are well known hepatocarcinogens. In conclusion, although our results do not indicate that these environmental estrogens exert any hepatocarcinogenic effect in rats, the possibility that APs could act as liver carcinogens should be explored in greater depth.

Unexpectedly, the type of glycogen accumulated in liver varied between experimental groups. In normal liver micrographs, glycogen appears as electron-dense rosettes, named alpha particles. These structures can also form agglomerates, the beta particles (Devos et al., 1983). Animals (males and females) from control groups from any generation and from NP- and 4OP-exposed groups from F_1 showed accumulation of glycogen, mainly in form of alpha and beta particles (the physiological forms of intracellular glycogen). However, livers from rats of F_2 and F_3 generation chronically exposed to APs, mainly OP, showed low-density amorphous glycogen masses, termed gamma-glycogen. Gamma-glycogen is formed from small particles of glycogen and, although its functional role is currently unknown, it is considered as the last state in the glycogenolysis processes. Nevertheless for many authors, this is a non-functional or degenerative form of glycogen (Herbst, 1976; Delorme and Hevor, 1985). The actual fact is that currently there exists very little information about the biochemical role exerted by alpha-, beta- or gamma-glycogen particles in glucose or glycogen metabolism. The differences in glycogen deposition between the experimental groups and the increasing presence of gamma-glycogen in 4OP-exposed animals from F_3 require further studies.

In spite of the absence of an increase in RLW of animals exposed to APs, their liver showed an increased percentage of binuclear hepatocytes. This result is consistent with those previously described by our group in short-term studies, in the sense that APs could induce cellular proliferation of liver cells (Zumbado et al., 2002). However, the increasing values of binucleate cells in the absence of hepatomegaly could indicate that in these livers there is not cellular proliferation but that endomitotic processes exist.

Morphological analysis showed the presence of an evident increase in apoptotic figures. The TUNEL method was carried out to show the evidence that apoptosis was enhanced in livers from animals chronically exposed to APs. With this method the apoptotic index was calculated. As cited in Section 3, APs-exposure, mainly to 4OP, induces a statistically significant increase in the apoptotic index. Apoptosis is a morphological and biochemical description of a physiological cell death mechanism that is commonly associated with programmed events that are necessary for the differentiation and development of individuals and organs (Aoki et al., 2004). Recently it has been reported that some endocrine-disrupting chemicals, among them NP, can cause apoptosis in fish cells (Hughes et al., 2000; Kwak et al., 2001) and in rat cells (Aoki et al., 2004). In recent years, many authors have described the ability of a variety of environmental toxicants to increase apoptosis and it is becoming evident that different noxious stimuli can exert their toxicity via apoptotic cell death (Robertson and Orrenius, 2000). However, the precise influence that an environmental contaminant could exert on apoptosis is currently unknown. Other xenoestrogens, such as polychlorinated biphenyls (PCBs) and dioxins are capable of inducing apoptosis (Narayanan et al., 1998; Robertson and Orrenius, 2000). Although Reactive Oxygen Species (ROS) have been related with the activation of apoptotic processes in liver (Fernández-Checa, 2003; Gómez-Lechón et al., 2003), in our study the existence of oxidative stress in liver cells exposed to APs does not seem to be related with the increasing values of apoptotic index. In any case, it should be remarked that livers of female animals from F_3 exposed to 4OP showed increasing levels of lipoperoxidation by-products, pointing to the possibility that APs could induce oxidative stress in liver tissue. These findings could to indicate that APs exert their toxic effects on liver, at least in part, by oxidative stress which could be the cause of the promotion of apoptotic cell death.

In spite of the differences in estrogenic potency between the two chemicals tested, in the sense that 4OP is more estrogenic on the reproductive system of male rats than NP (Bian et al., 2006), our results do not seem to confirm such circumstance. On the contrary, in the liver tissue, NP seems to be more estrogenic than 4OP as indicated by the fact that NP-exposed rats from F_1, F_2 and F_3 present more evident increasing values of binucleate cells (although with statistical significance only in animals from F_3) than 4OP-exposed animals from the same groups. In our opinion the different results found in this work could be due to the existence of differences in their toxicokinetical properties. For example, the fact that in the F_3-groups 4OP is the chemical that induces more evident histological and biochemical alterations could be related with the possibility that this compound could reach and be accumulated more easily in the liver than NP. Notwithstanding, in any case the evaluation of the differences in hepatic actions between these chemicals is not the overall objective of our work and, furthermore, there is not enough data that allows us to explain the differences in hepatic effects exerted by them.

To sum up, bearing in mind that this is a descriptive, not mechanistic, study, our findings may well indicate that low doses of APs do not seem to exert an evident estrogenic or proliferative effect on rat liver. Yet, on the contrary, our results point to the possibility that they could induce a number of cytotoxic effects such as the increase in apoptotic processes and lipidic degeneration. Furthermore, APs altering the glycogen metabolism inducing hepatocellular glycogenosis. Such effects could be related with both cytotoxic and adaptive tisular responses. The data cited above give rise to questions concerning the potential hepatotoxic effects of these chemicals at environmentally relevant doses (Cantero et al., 2006). In our opinion

such data should be considered for the risk evaluations for these compounds.

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