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Effect of di-(2-ethylhexyl) phthalate on N-cadherin and catenin protein expression in rat testis

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Abstract

This study investigated the effect of DEHP exposure on N-cadherin and α-, β- and p120-catenin immunoreactivities in the rat testis. DEHP was administered by daily gavage to 25-day-old male Sprague–Dawley rats at a dose of 2 g DEHP/5 ml corn oil/kg body weight for 2 days or 7 days. Control rats were treated with corn oil vehicle under the same conditions. Animals were killed at 24 h after the last treatment. Another group of rats treated with DEHP or corn oil vehicle (control group) for 2 days were held for 30 days without treatment to observe recovery. Testes were analyzed for histopathology, TUNEL staining, immunofluorescence (IF) and Western blot analyses. Animals exposed to DEHP for 2 days or 7 days showed severe alterations of seminiferous tubules characterized by germ cell sloughing. Animals from the longer term recovery group treated with DEHP showed foci of delayed spermatogenesis. A linear and continuous pattern of N-cadherin was observed in the basal compartment of the seminiferous tubules. A similar pattern but with higher IF intensity was observed for N-cadherin in rats treated with DEHP for 2 days or 7 days, compared to control animals. The α-, β- and p120-catenins were detected in the basal compartment of seminiferous tubules in similar localization and IF pattern for DEHP and control groups. A significant increase in testicular N-cadherin and α-catenin levels was detected by Western blot analysis in DEHP-exposed versus control rats. No variations in N-cadherin or catenin expression were detected in the recovery groups. These findings demonstrate that DEHP induces an up-regulation of N-cadherin and α-catenin expression and may perturb cell–cell adhesion phenomena in the seminiferous tubule.

Keywords: Testis; Sertoli cell toxicant; Di-(2-ethylhexyl) phthalate (DEHP); N-cadherin; Catenins (α, β and p120)

1. Introduction

Spermatogenesis is an elaborate process involving interactions between supporting somatic cells (Sertoli cells) and germ cells in the seminiferous tubules. These adhesive interactions contribute to germ cell maturation [1,2] and may be sensitive to endocrine or local (paracrine) effects via multiple signal transduction pathways [3,4].

Cell–cell adhesion is essential for the establishment and maintenance of normal tissue architecture. One of the most important and ubiquitous types of adhesive interaction is mediated by the cadherins, which belong to the family of calcium-dependent cell adhesion glycoproteins [5]. The cadherin superfamily of proteins can be divided into subfamilies that include classic or type I cadherins, highly related classic type II cadherins, desmosomal cadherins, protocadherins, and the seven-pass transmembrane or Flamingo cadherins [6]. Type I cadherins are the most extensively studied class and are single-span transmembrane proteins located primarily within adherens junctions [7,8]. This subfamily includes neural cadherin (N-cadherin), placental cadherin (P-cadherin) and epithelial cadherin (E-cadherin) subtypes, among other members. The molecules are composed of three domains (intracellular, transmembrane, extracellular) [9] and are mainly involved in homotypic cell adhesion; however, some studies report that interactions between cadherins can be more promiscuous [10].

The physiological regulation of cadherin-mediated adhesion is thought to be controlled by the catenins associated with the cadherin cytoplasmic domain. The carboxy-half region of the cadherin cytoplasmic domain binds to β-catenin, which in turn

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associates with α-catenin and the actin cytoskeleton [11]. Lee et al. [12] used Sertoli–germ cell co-cultures from normal rats and described N-cadherin within the seminiferous tubules as being largely actin-linked but also associated with vimentin of intermediate filaments via adaptor molecules. These interactions are essential for the normal activity of the adhesion system because β-catenin has an essential role in the structural organization and function of cadherin. Another catenin, p120, a member of the Armadillo/β-catenin gene family, also binds to cadherin at the juxtamembrane portion of the cadherin cytoplasmic domain; this is different from the β-catenin binding region and regulates the structural integrity and function of the cadherin complex [13]. Structural studies have provided new insights into the mechanisms underlying cadherin-mediated cell interactions and catenin-mediated cellular signaling [14,15].

The distribution of several cadherins and catenins were studied in the rat testis [2,16–22]. Byers et al. [16] reported N-cadherin, α- and β-catenins in the seminiferous epithelium and E-cadherin in fetal Leydig cells during rat development. Our group using in vitro systems detected N-cadherin in Sertoli cells of prepubertal rats [17] and in Leydig cells of adult rats [18]. Johnson et al. [19] reported multiple cadherins in rat testis and showed that different cadherins have a unique protein distribution during testicular development. Mulholland et al. [20] employed pan-cadherin and β-catenin antibodies to demonstrate co-localization of cadherin and β-catenin in the area of the inter-Sertoli cell junction complex in the testis of adult rats. Based on immunoelectron microscopy observations these authors also suggested that cadherins are not concentrated at the ectoplasmic specialization actin site but rather at the desmosome-like junctions. Also, Johnson and Boekelheide [2] described the spatial and temporal expression of four classic cadherins (N-cadherin, 6, 11 and L4 cadherins) in normal rat testis during development. They also localized N-cadherin at the basal inter-Sertoli cell junctions and spermatocyte-associated junctions and at the head of elongated spermatids in the testis of adult rats. These authors also observed p120-catenin during the development of rat testis and suggested that N-cadherin and p120 might be components of the desmosome-like junctions [2,21]. Lee et al. [22] used Sertoli–germ cell co-cultures demonstrating that N-cadherin is associated with β- and α-catenins and that the N-cadherin complex is linked to the actin cytoskeleton.

Di-(2-ethylhexyl) phthalate (DEHP), a well known reproductive toxicant, is one of the plasticizers of the phthalate esters most commonly utilized as an additive in the manufacture of plastics such as polyvinyl chloride (PVC), and in dispersions and varnishes. DEHP is ubiquitously distributed in the environment and poses a potential risk for human exposure through several ways (reviewed in Refs. [23,24]). It is currently the only phthalate plasticizer used in PVC medical devices. Testicular injury by DEHP in animal models is characterized by marked degeneration of seminiferous tubules resulting in testicular atrophy [25–27]. The precise mechanisms responsible for the loss of germ cells are still incompletely understood [28]; however, a recent review [29] highlighted the participation of peroxisome proliferator-activated receptor (PPAR) subtypes as potential mediators of phthalate-induced effects on the male reproductive tract. Young rats appear more sensitive to DEHP than adult rats [30]. DEHP in rats is rapidly hydrolyzed in the gut to the major metabolite, mono-(2-ethylhexyl) phthalate (MEHP) which has been shown to cause testicular atrophy [31].

The intricate regulation and cellular interactions that occur in the testis provide multiple distinct targets by which toxicants can disrupt spermatogenesis. In light of the reports mentioned above, we studied the expression of classic N-cadherin and its associated proteins (α-, β- and p120-catenins) in the testis of prepubertal rats treated with DEHP.

2. Material and methods

2.1. Animals

Prepubertal (25-day-old) male Sprague–Dawley rats were used. Rats were kept at 22 °C with 14 h light, 10 h dark schedule and were fed standard food pellets and water ad libitum. They were handled in compliance with protocols of the National Institute of Health Guide for the Care and Use of Laboratory Animals. Rats were housed in stainless steel cages with six animals per group. Rats were euthanized by cervical dislocation (prepubertal rats) or decapitation (post-pubertal rats).

2.2. Antibodies

Mouse monoclonal antibodies against N-cadherin (clone 32), β-catenin (clone 14), α-catenin (clone 5), p120-catenin (clone 98), vimentin (clone RV202) were purchased from BD Pharmingen (San Diego, CA). Rabbit polyclonal antibody against actin was obtained from Sigma Chemical Co. (St. Louis, MO). Antibody against digoxigenin conjugated with alkaline phosphatase and other reagents for TUNEL were purchased from Roche Molecular Biochemicals GmbH (Mannheim, Germany). The following secondary antibodies were also used: biotinylated horse monoclonal antibody against mouse IgG and goat monoclonal antibody against rabbit IgG, horse anti-mouse IgG-fluorescein isothiocyanate (FITC) (Vector Laboratories, Burlingame, CA).

2.3. Experimental design

DEHP (Sigma Chemical Co.) was administered by daily gavage at a dose of 2 g per 5 ml corn oil per kg body weight for 2 days or 7 days. The control rats received corn oil vehicle only under the same regimen (5 ml per kg body weight) as rats exposed to DEHP. Animals were euthanized at 24 h after the last dosing for evaluation and assessment of the testis. Another group of rats treated with DEHP for 2 days and given free access to tap water and rat chow were held for 30 days without treatment to assess recovery (recovery group). Control and DEHP-exposed rats of the recovery group (57 days old) were killed 30 days after the last treatment. For comparison normal rats of the same age were used as additional controls. Rats were weighed and euthanized; and both testes removed, weighed and assessed by the following assays.

2.4. Histopathology and transmission electron microscopy

One testis of each rat was fixed in Bouin's solution, dehydrated and embedded in paraffin. Sections (8 μm thick) were stained with hematoxylin and eosin and analyzed by light microscopy. Histopathology of the liver from rats treated with DEHP showed no tissue damage (data not shown). For ultrastructural analysis, the testes were fixed by perfusion with 5% glutaraldehyde buffered with 0.1 M sodium cacodylate, pH 7.4, post-fixed with osmium tetroxide and embedded in plastic. Ultrathin sections (~80 nm) were stained with uranyl acetate and lead citrate and examined with a Zeiss EM-9A.

2.5. Apoptosis

Apoptotic cell death was evaluated by TUNEL (TdT-mediated dUTP-digoxigenin Nick End Labeling). Briefly, paraffin tissue sections (3 μm thick) of

the testis were used. Tissue sections were deparaffinated and hydrated in 10 mM sodium citrate buffer, pH 6.0, heated by microwave (5 min at 370 W) and quickly cooled in phosphate-buffered saline (PBS). Sections were permeabilized with 0.1% Triton X-100 in 0.1% sodium citrate for 5 min at 4 °C. Non-specific labeling was blocked by pre-incubating sections with 2% blocking reagent (Roche Molecular Biochemicals GmbH). Sections were then incubated for 10 min with TdT buffer (Roche Molecular Biochemicals). DNA 3′-end labeling was done by incubating the sections with digoxigenin-dideoxy-uridine triphosphate (dig-ddUTP) containing TdT (0.4 U/ml TdT, 4 μM dig-ddUTP in TdT buffer) for 1 h at 37 °C in a humidified chamber. After washing, sections were incubated with 2% blocking reagent in 100 mM maleic acid, 150 mM NaCl, pH 7.5, for 30 min at room temperature followed by incubation with alkaline phosphatase conjugated anti-digoxigenin antibodies (1:2000) for 2 h at room temperature. The sections were rinsed and equilibrated in alkaline phosphatase buffer (100 mM Tris–HCl, 100 mM NaCl, 50 mM $MgSO_4$, pH 9.5) containing 1 mM levamisole (Sigma Chemical Co.). Color was allowed to develop by adding nitroblue tetrazolium and 5-Br-4-Cl-3-indolyl-phosphate for 60–90 min. The reaction was stopped by washing the sections with 10 mM Tris–HCl, 1 mM EDTA, pH 8. For the negative control, TdT was replaced with the same volume of reaction buffer. Light counterstain was done with eosin.

TUNEL positive germ cells were quantified in each tissue section by counting the number of TUNEL positive cells in each round cross-section of the seminiferous tubule. The apoptotic index (AI) reflects the percentage of seminiferous tubule cross-sections with more than three TUNEL positive germ cells.

Table 1
Body and relative testicular weights of testes from rats treated with corn oil (control groups) or rats treated with DEHP (2 g/kg) administered for 2 days or 7 days

Treatment (days)	Body weight (g)		Relative testicular weight (g)	
	Control	DEHP	Control	DEHP
2	61.4 ± 9.2	60.3 ± 4.0	0.36 ± 0.03	0.25 ± 0.03***
7	71.5 ± 4.2	62.2 ± 9.9*	0.42 ± 0.05	0.27 ± 0.02***
2 (Recovery group)	190.5 ± 9.7	198.2 ± 12.0	0.57 ± 0.13	0.50 ± 0.13

Recovery group: rats from control (treated with corn oil) or experimental group (treated with DEHP) for 2 days and held for 30 days without treatment. Rats were treated as described in Section 2. Data are expressed as the mean ± S.D. ($n = 10$).
* $p < 0.05$ DEHP-treated vs. control rats.
*** $p < 0.001$ DEHP-treated vs. control rats.

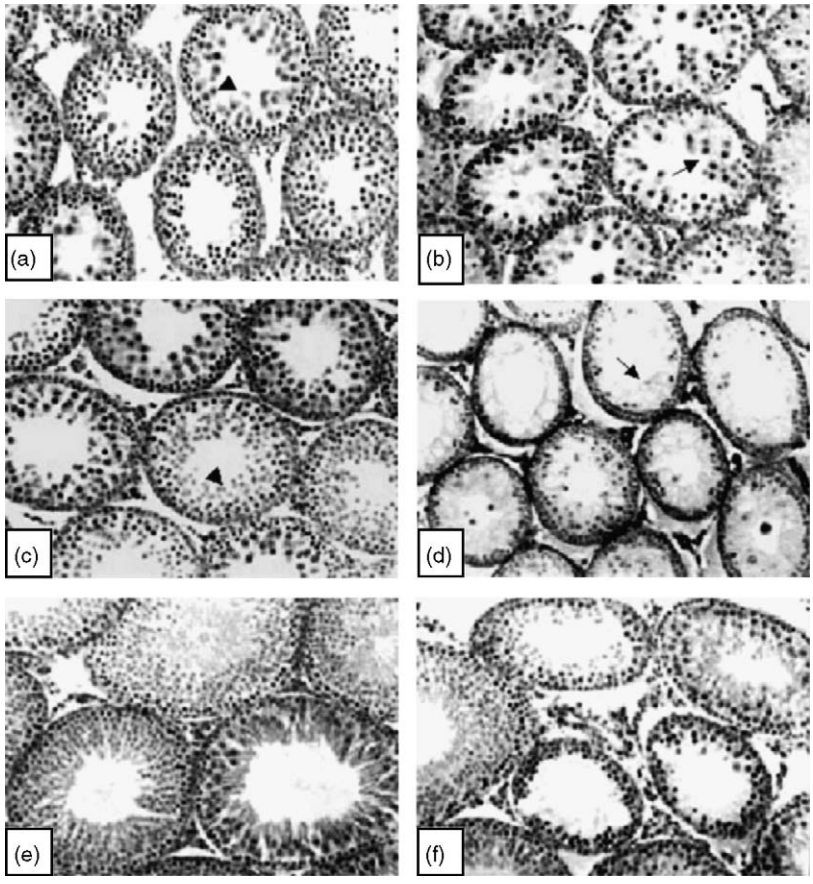

Fig. 1. Photomicrographs of testis sections from control groups treated with corn oil for 2 days (a) or 7 days (c), showing normal testicular morphology and spermatogenesis (arrowhead). Testis sections of rats treated with DEHP for 2 days (b) or 7 days (d), showing disruption of seminiferous tubules with sloughing of germ cells (b) and testicular atrophy due to severe sloughing of seminiferous tubules (d) (arrow). Testis sections from control rats treated with corn oil for 2 days and held for 30 days without treatment (recovery group) showing normal spermatogenesis (e). Delayed spermatogenesis observed in rats treated with DEHP for 2 days and held for 30 days without treatment (recovery group) (f); ×20. Rats were treated as described in Section 2.

Two testicular cross-sections per rat, with a minimum of at least 100 tubule cross-sections, were analyzed; data are shown as the average ± S.D. of three to five rats.

2.6. Immunofluorescence

To study the distribution of N-cadherin and catenins, the testes were removed and frozen at −70 °C. Cryostat sections (8 μm) were fixed with methanol for 15 min. After blocking with 5% normal horse serum in 3% bovine serum albumin (BSA) in PBS (room temperature for 30 min) sections were treated with avidin/biotin blocking solution (Vector Laboratories). Then, slides were incubated with primary antibodies against N-cadherin, α-, β- and p120-catenins diluted in PBS (1:5) overnight at 4 °C. After three washes (5 min each), sections were incubated with a secondary antibody horse anti-mouse FITC diluted 1:50 for 45 min. For negative control, sections were either incubated with the normal IgG isotype or the first antibody was omitted. Sections were then mounted in buffered glycerine and observed using an Axiophot fluorescent microscope with epi-illumination (Carl Zeiss Inc., Oberkochen, Germany).

2.7. Western blot analysis

Testes from prepubertal and adults rats were detunicated, weighed and homogenized in three volumes of ice-cold RIPA buffer, pH 7.4 (50 mM Tris–HCl, 150 mM NaCl, 1% NP-40, 0.5% desoxycholate, 0.1% SDS, 50 mM NaF, 2 mM $NaVO_4$), with protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 10 μg/ml of leupeptin, pepstatin A and aprotinin). The homogenate was passed through a 21-gauge needle to shear the DNA and centrifuged at 13,500 × *g* for 30 min at 4 °C. Supernatant proteins were measured by the Bradford assay (Bio-Rad, Hercules, CA, USA). Extracts were dissolved with sample buffer (500 μM Tris–HCl, pH 6.8, 10% SDS, 30% glycerol, 0.5% β-mercaptoethanol and 0.5% bromophenol blue), boiled for 5 min and immediately placed on ice. Equal amounts of proteins (∼50 μg/lane) under reducing conditions were resolved on 10% SDS-polyacrylamide gel (SDS-PAGE, 10% acrylamide–bisacrylamide). Proteins were electroblotted at 150 V for 60 min to polyvinylidene difluoride membranes (Immobilon-P, Millipore Co., Bedford, MA). Transfer was monitored by Ponceau red staining. The membranes were blocked with 5% non-fat dry milk in PBS-Tris containing 0.1% Tween 20 for 90 min. For immunostaining the following primary antibodies were used: mouse anti-N-cadherin (1:1000), mouse anti-β-catenin (1:300), mouse anti-α-catenin (1:250), mouse anti-p120-catenin (1:1000) and rabbit anti-actin (1:3000). Blots were washed and incubated with an appropriate second biotinylated antibody, such as horse anti-mouse IgG (1:6000) and goat anti-rabbit (1:6000). Then the reaction was enhanced with horseradish peroxidase-conjugated (Chemicon International Inc., Temecula, CA) and blots were developed by enhanced chemiluminescence. Prestained protein standards (Bio-Rad) with a molecular weight range of approximately 199.7–6.7 kDa were used. Autoradiographic band intensities were quantified by densitometry. As negative control, normal IgG primary antibody was omitted. Western blots were performed at least three times with similar results. This method was also used for testes of rats from the recovery group treated with DEHP or corn oil for 2 days.

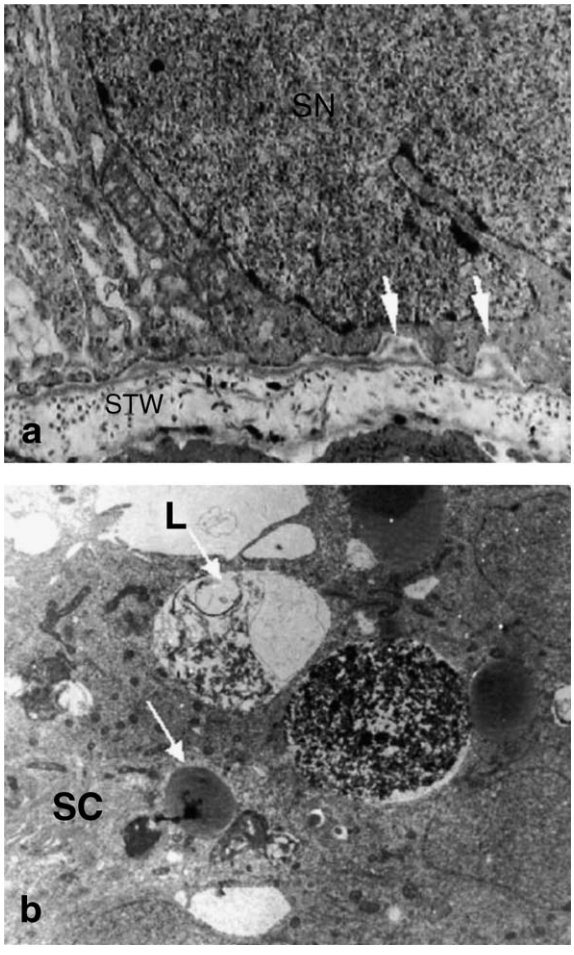

Fig. 2. Electron microscopy of testis sections from rats treated with DEHP for 2 days (a) Duplication of the basal lamina observed at the seminiferous tubular wall (arrows); ×10,800. (b) Vacuolization of Sertoli cell and increase of lipid droplets (arrows) was frequently seen; ×2800. SN: Sertoli cell nucleus; STW: seminiferous tubular wall; L: lumen; SC: Sertoli cell cytoplasm.

2.8. Statistical analysis

Data were analyzed by the non-parametric Mann–Whitney *U*-test for comparison of body weight, testicular weight and apoptotic index, and by one-way analysis of variance and the Student–Newman–Keuls multiple comparison test for analysis densitometry, using an Instat program. Data were expressed as the mean and the standard deviation. A difference of $p < 0.05$ was considered significant.

3. Results

3.1. Effect of DEHP on body and testis weight

Relative testicular weights in rats treated with DEHP for 2 days or 7 days were significantly lower than vehicle control rats (Table 1); however, the body weight of rats treated with DEHP for 2 days, and the DEHP-exposed rats from the longer term recovery group, was not significantly different from their respective control groups except that rats treated with DEHP for 7 days had a lower body weight than the control group. Testicular weights in the DEHP recovery group were not different from the corresponding controls.

Table 2
Apoptotic index using TUNEL assay in testes of rats treated with corn oil (control groups) or rats treated with DEHP (2 g/kg) administered for 2 days or 7 days

Treatment (days)	Apoptotic index	
	Control	DEHP
2	1.8 ± 0.5	15.5 ± 3.4***
7	2.0 ± 0.8	2.2 ± 1.3
2 (Recovery group)	3.1 ± 0.3	3.2 ± 0.9

Recovery group: rats from control (treated with corn oil) and experimental group (treated with DEHP) for 2 days and held for 30 days without treatment. Rats were treated as described in Section 2. Data are expressed as the mean ± S.D.
*** $p < 0.001$ rats treated with DEHP vs. control groups.

3.2. Effect of DEHP on testis morphology

By light microscopy, testes from prepubertal normal (untreated) and control (vehicle) rats showed normal morphology and spermatogenesis (Fig. 1a and c). In rats treated with DEHP, various degrees of spermatogenic cell damage and cell sloughing were observed within the seminiferous tubules. In seminiferous tubules with severe cell sloughing only Sertoli cells and spermatogonia were attached to the tubular wall (Fig. 1b and d). Testes of the exposed rats in the recovery group presented seminiferous epithelium with some signs of delayed spermatogenesis (Fig. 1f) compared to testes of the corresponding control group (Fig. 1e) and age-matched adult normal rats.

3.3. Ultrastructural analysis

Transmission electron microscopy revealed no morphological changes in the testes of 2-day control rats (data not shown). In the testes 2-day DEHP-exposed rats we frequently observed vacuolization of Sertoli cells and mitochondrial alterations. In these

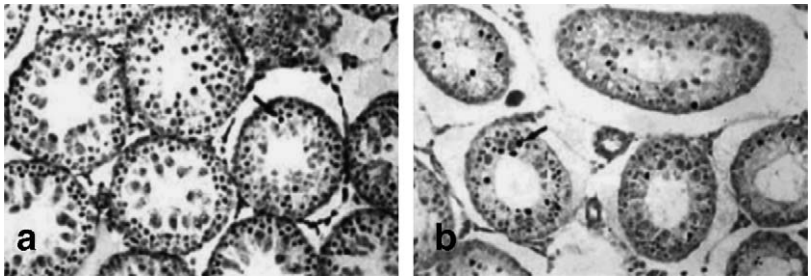

Fig. 3. TUNEL technique performed on testis sections. (a) Rats treated with corn oil for 2 days show normal levels of germ cell apoptosis. (b) An increased number of apoptotic germ cells is observed in rats treated with DEHP for 2 days. Arrows indicate apoptotic cells; ×40. Rats were treated as described in Section 2.

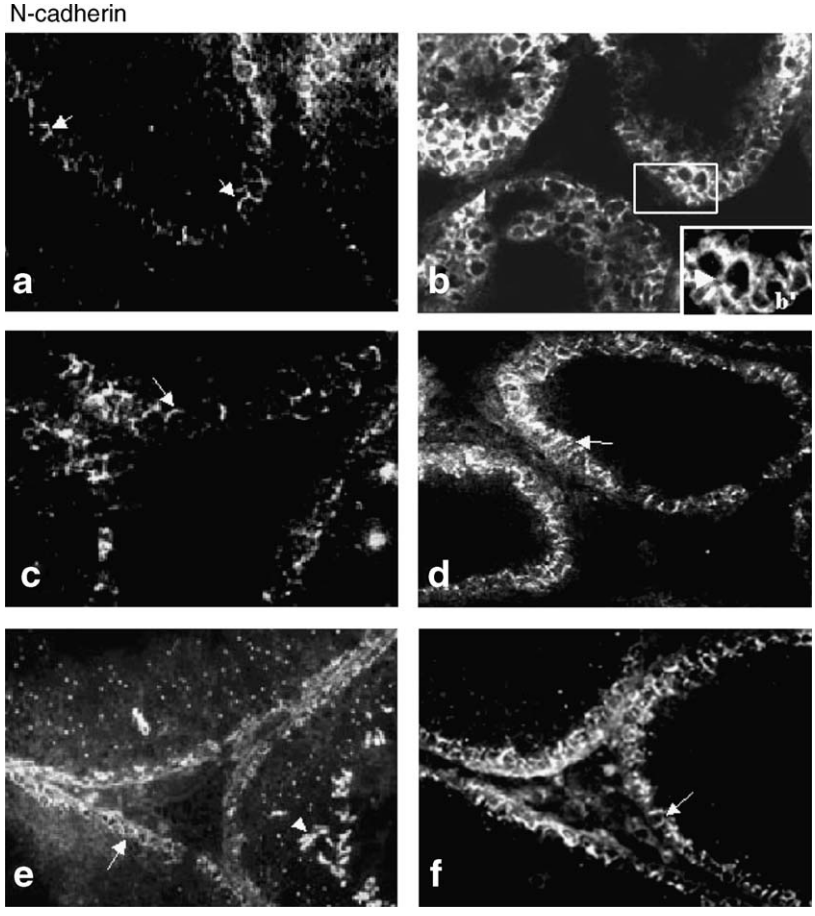

Fig. 4. Immunofluorescent (IF) localization of N-cadherin in the testis of rats treated with corn oil for 2 days (a) or 7 days (c) and rats treated with DEHP for 2 days (b) or 7 days (d). A linear and continuous IF was localized at basal Sertoli–germ cell junctions (arrows). Bright IF for N-cadherin was observed in rats treated with DEHP for 2 days (b) or 7 days (d). (b′) The Sertoli–germ cell junctions (arrowhead). In testes of rats from control (e) and experimental (f) recovery groups, N-cadherin was detected as a linear IF at basal Sertoli–germ cell junctions and patches of IF were associated with smaller cells in the adluminal compartment, probably round and elongated spermatids (arrowhead). (a–f) ×40 and (b′) ×60.

cells, an increase of lipid droplets and basal lamina duplication at the seminiferous tubular wall were also detected (Fig. 2a and b). Leydig cells appeared normal (data not shown).

3.4. Effect of DEHP on apoptosis in seminiferous tubules

Control rats showed basal levels of TUNEL-positive apoptotic germ cells. The AI revealed by TUNEL increased (∼15%) in 2-day DEHP-exposed rats relative to the corresponding control group (∼1.7%). In contrast, the AI was similar in the 7-day DEHP and control groups (Table 2). Spermatocytes from 2-day DEHP and control rats were identified as the predominant cell type undergoing apoptosis in the adluminal compartment of seminiferous tubules (Fig. 3a and b). Occasionally, a few spermatogonia localized in the basal compartment were also apoptotic. In the testes of 2-day DEHP rats held 30 days for recovery, the AI (∼3%) was similar to that observed in control and age-matched normal rats (Table 2).

3.5. Effect of DEHP on testicular N-cadherin and catenin immunostaining

N-cadherin immunofluorescence (IF) was localized in the basal compartment, most likely to Sertoli–Sertoli and Sertoli–germ cell junctions, in a continuous line. This was the case for testes of 2-day (Fig. 4a) and 7-day (Fig. 4c) control rats and 2-day (Fig. 4b) and 7-day (Fig. 4d) DEHP-exposed rats. Brighter IF was observed at basal sites in rats treated with DEHP for 2 days or 7 days relative to corresponding vehicle controls. In some areas, α-, β- and p120-catenins were expressed as patches of linear IF (Fig. 5) with similar localizations as N-cadherin. We observed no differences in the IF intensity for catenin immunoreactivity in rats treated with DEHP for 2 days or 7 days compared to controls groups (Fig. 5). Testes of normal rats presented N-cadherin and catenin patterns that were similar to 2-day or 7-day control rats (data not shown).

In control (Fig. 4e) and DEHP-exposed (Fig. 4f) recovery groups, we observed N-cadherin IF at basal cell junctions and punctate spermatocyte-associated junctions. N-cadherin was also detected in elongated spermatid heads in a stage-specific manner. Catenin (α, β and p120) immunoreactivity in these rats was restricted to the basal compartment at the Sertoli–germ cell junctions (Fig. 5e and f). No staining of peritubular or interstitial cells was observed. No immunoreactivity could be detected in testis sections incubated with the normal IgG isotype or without the first antibody (data not shown).

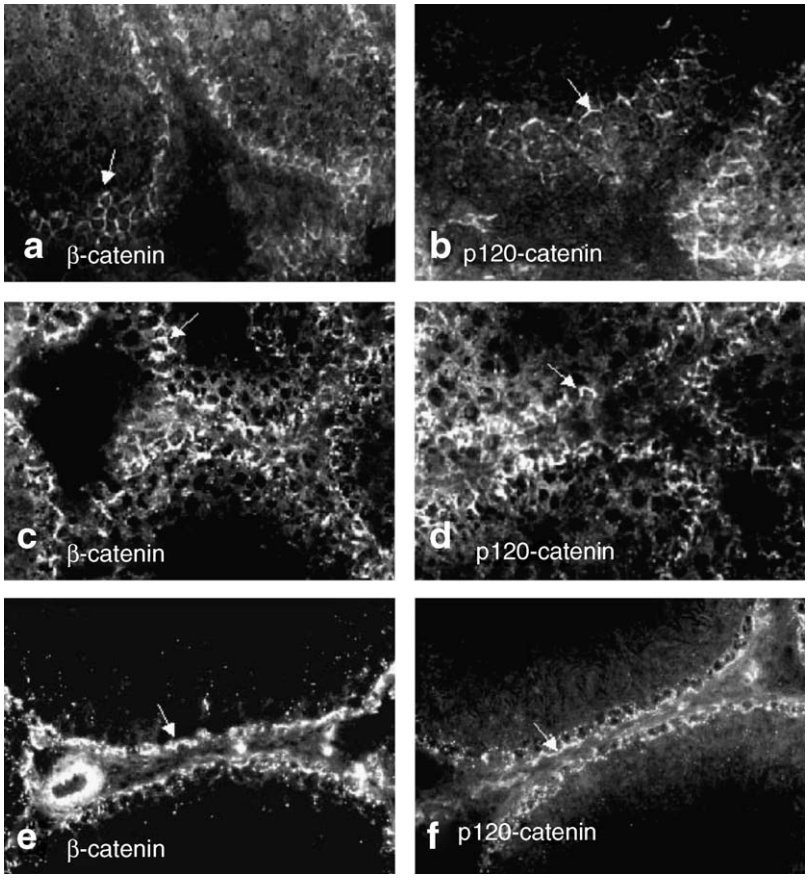

Fig. 5. Patches of linear immunofluorescent (IF) with similar intensity and distribution was observed at the Sertoli–germ cell junctions (arrows) in controls (corn oil) and rats treated with DEHP for 2 days or 7 days. IF of β-catenin (a) and p120-catenin (b) in testes of rats treated with corn oil for 2 days or 7 days, respectively. IF of β-catenin (c) and p120-catenin (d) is also shown in rats treated with DEHP for 2 days or 7 days, respectively. IF of β-catenin (e) and p120-catenin (f) showed similar localization in the testis of rats from the experimental recovery group; ×40.

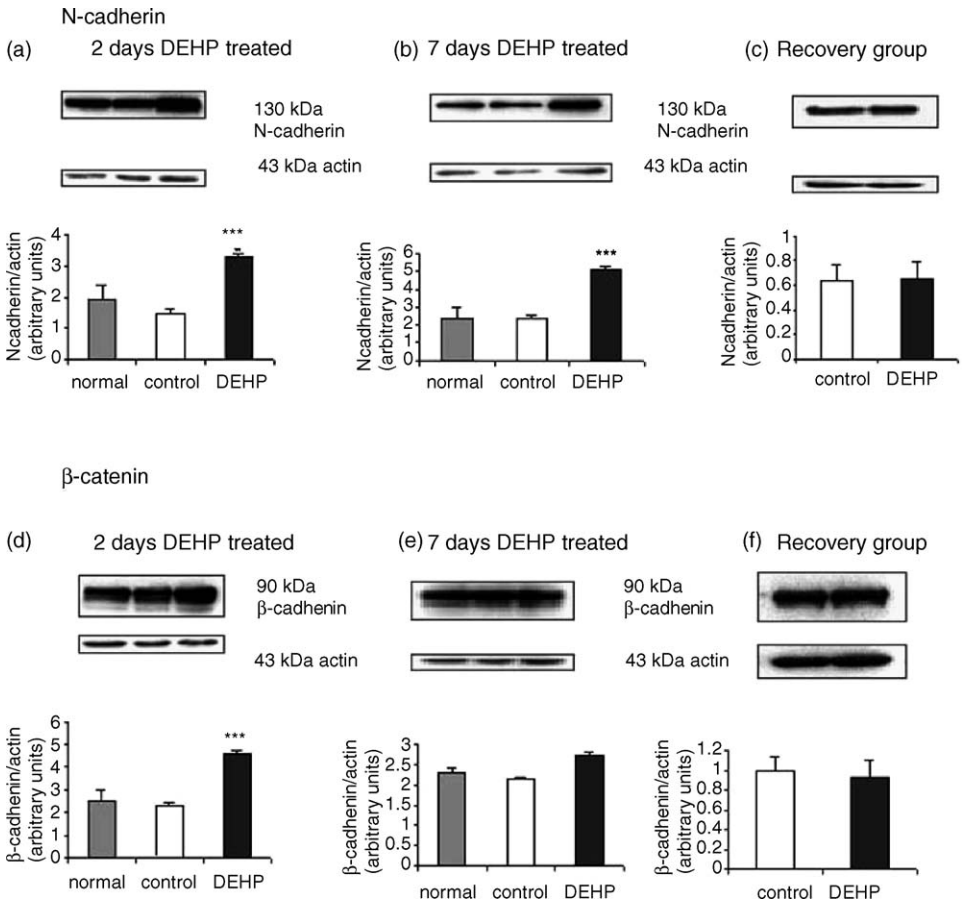

Fig. 6. Effect of DEHP on N-cadherin and β-catenin expression in testis of rats. Western blot performed with testis extracts (50 μg protein), reacted with antibodies to N-cadherin and β-catenin, found the expected molecular sizes. We detected a significant increase in the expression of N-cadherin in the testis of rats treated with DEHP for 2 days (a) or 7 days (b) compared to normal and control groups treated with corn oil for 2 days (a) or 7 days (b) $^{***}p < 0.001$. Increased expression of β-catenin in testis of rats treated with DEHP for 2 days (d) vs. rats treated with corn oil for 2 days (control group) (d) and normal rats $^{***}p < 0.001$. No difference in β-catenin expression was observed between testes of rats treated with DEHP for 7 days (e) in experimental, control and normal groups. No difference was detected in N-cadherin (c) and β-catenin (f) expression in control vs. experimental rats in the recovery groups. Each band was quantitated by densitometry using the Scion Image program and expressed as arbitrary units (mean ± S.D.) (N-cadherin/actin; β-catenin/actin). The upper panel shows one representative experiment of three. The lower panel shows pooled data from three independent experiments.

3.6. Effect of DEHP on expression of N-cadherin, α-, β- and p120-catenin immunoblotting

Western blot analysis was performed on the testis of 2-day control and DEHP-exposed rats and normal (untreated) rats at 27 days of age (Fig. 6a) as well as 7-day control and DEHP-exposed rats and normal rats at 33 days of age (Fig. 6b). A significant increase of N-cadherin expression was observed in the DEHP-exposed rats versus the corresponding control group. No variations in the expression of N-cadherin were found between experimental and control rats in the longer term recovery group (Fig. 6c).

Western blot analysis revealed a significant increase in β-catenin expression in the testes of rats treated with DEHP for 2 days compared to control rats, while a slight increase was detected in the testes of rats treated with DEHP for 7 days (Fig. 6d and e). A significant increase of α-catenin expression was observed in DEHP-exposed rats treated for 2 days or 7 days compared to corresponding control groups (Fig. 7d); however, an increase of p120-catenin expression was observed in the testes of 2-day DEHP-exposed rats (Fig. 7a). No changes in the expression of β-catenin (Fig. 6e) or p120-catenin (Fig. 7b) were found in the 7-day DEHP-exposed rats versus control or normal counterparts. We detected no variations of α-catenin (Fig. 7f), β-catenin (Fig. 6f) or p120-catenin (Fig. 7c) levels in testes of rats from the recovery group compared to control groups.

4. Discussion

Results of the present study show that DEHP induces an up-regulation of N-cadherin and α-catenin immunoreactivity in the rat seminiferous tubules, concomitantly with severe testicular pathology characterized by germinal cell sloughing. To our knowledge these results are the first reported on the effect of DEHP to the expression of N-cadherin and catenins (α, β and p120) in the testis of prepubertal rats.

Despite numerous studies on testis histopathology of rats treated with DEHP, very little is known about the alterations

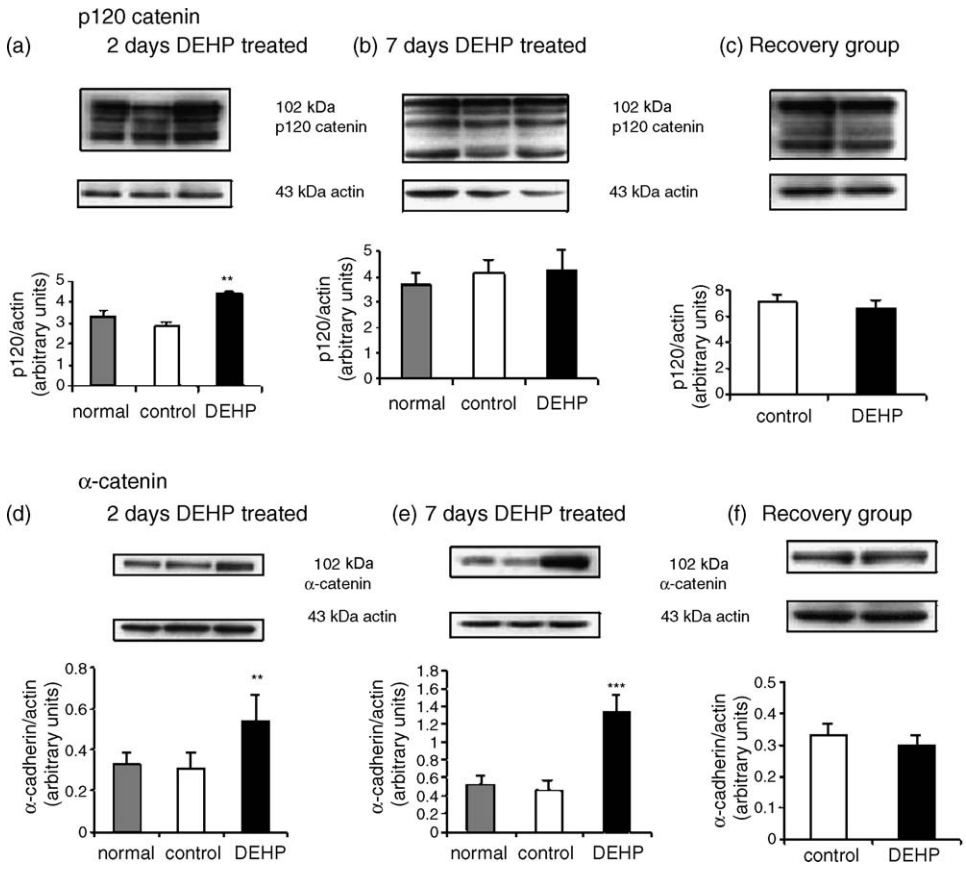

Fig. 7. Effect of DEHP on p120- and α-catenin expression in rats testis. Western blot of testis extracts (50 μg protein) reacted with antibodies to p120- and α-catenin and detected the expected molecular sizes. We observed a significant increase in p120-catenin expression in the testis of rats treated with DEHP for 2 days (a) vs. normal and control rats treated with corn oil for 2 days (a) $^{**}p<0.01$. No change in the expression of p120-catenin was detected in rats treated with DEHP or with corn oil for 7 days (b). Increased expression of α-catenin was observed in the testis of rats treated with DEHP for 2 days (d) or 7 days (e) compared to normal and control rats treated with corn oil for 2 days (d) or 7 days (e) $^{***}p<0.001$. We found no difference in the expression of p120-catenin (c) and α-catenin (f) in testes of control and experimental rats in the recovery groups. Each band was quantitated by densitometry using the Scion Image program and expressed as arbitrary units (mean ± S.D.) (p120/actin; α-catenin/actin). The upper panel shows one representative experiment of three. The lower panel shows pooled data from three independent experiments.

induced by this toxicant on cell–cell adhesion molecules. Cadherins are an important system involved in the adherens junctions between Sertoli and germ cells that promotes germ cell survival. Our results show that DEHP induced a decrease in relative testicular weight compared to control groups, similar to results described by others [32,33]. In rats treated with DEHP for 2 days or 7 days, testicular atrophy with different degrees of germ cell sloughing, spermatocytes in the lumen of seminiferous tubules and vacuolization of Sertoli cells were frequently observed, which corroborates the description of Boekelheide [30] and Park et al. [33]. Electron microscopy of testes of rats treated with DEHP for 2 days showed disorganization of seminiferous epithelium with shedding and degeneration of spermatocytes. Duplication of the basal lamina and Leydig cells with normal morphology was also observed. These alterations were similar to the report by Creasy et al. [34] in the testis of prepubertal rats treated with another toxicant in the same family, di-*n*-pentyl phthalate. We observed delayed spermatogenesis in the testes of rats treated with DEHP in the recovery group, similar to previous reports on prepubertal rats [35].

TUNEL staining revealed a significant increase in germ cell apoptosis in testes of rats treated with DEHP for 2 days versus 7 days. Park et al. [33] also reported a peak AI on day 3 followed by a gradual AI decline on day 7 in the testis of prepubertal rats treated with DEHP. Our TUNEL results and those of other authors [36] suggest that apoptosis is an important mechanism of germ cell loss in this model.

Testicular N-cadherin was localized to the basal compartment in a linear and continuous IF pattern at Sertoli–germ cell junctions, similar to previous results in pubertal [2] and adult [20,22] rats. Although N-cadherin IF intensity in DEHP-exposed rats was brighter than controls at 2 days or 7 days exposure, the localization pattern was not altered. Fiorini et al. [37] reported that some testicular toxicants do not affect N-cadherin localization while others induce cadherin cell redistribution in a cultured Ser/W3 Sertoli cell line. Richburg et al. [28] reported a redistribution of another cadherin (Flamingo 1) in Sertoli cells of prepubertal rats treated with the DEHP metabolite MEHP. We also detected α-, β- and p120-catenin immunoreactivity in the testes of DEHP-exposed rats, similar to controls and with the

same general immunolocalization as described for N-cadherin. This result is similar to that described by others for β- and p120-catenins in the testis of normal young rats [21] and for α- and β-catenins in the testis of rats during development [16].

An increase in testicular N-cadherin, α-, β- and p120-catenins was detected by immunoblotting in rats treated with DEHP for 2 days. In rats exposed to DEHP for 7 days, N-cadherin and α-catenin increased in contrast to the normal β- and p120-catenin levels observed. Our morphological results also showed a disruption of Sertoli–spermatocyte junctions in the adluminal compartment in rats treated with DEHP. It is known that in this compartment cell adhesion interactions are more labile, and Sertoli–germ cell junctions more susceptible to disrupting factors. N-cadherin and catenin levels were normal in the longer term recovery group, and spermatogenesis was almost complete. This association supports the notion that normal N-cadherin and associated protein function is needed for normal cell organization in the seminiferous epithelium. Other studies using different toxicants reported no variations [19] or reduced expression of N-cadherin [37] in rat Sertoli cells. A transient up-regulation of N-cadherin and catenins (α, β and p120), similar to our results in rats treated with DEHP for 2 days, was observed in rats treated with the male contraceptive 1-(2,4-dichlorobenzyl) indazole-3-carbohydrazide (AF-2364) [22,38], also known as Adjudin [12]. Those studies suggest that AF-2364 induced germ cell loss and probably functional alterations of ectoplasmic specialization in Sertoli–spermatid interactions without affecting the adherens junctions between Sertoli cells and spermatogonia or primary spermatocytes. Although our results do not allow us to pinpoint a precise mechanism by which the toxicant may exert its effect on the seminiferous tubules, it is tempting to speculate that in our experimental model the up-regulation of N-cadherin and α-catenin is a mechanism to compensate for the dissociating effects of DEHP on Sertoli–germ cell junctions by avoiding germ cell sloughing from seminiferous tubules. We cannot disregard that an up-regulation of N-cadherin observed 12 or 24 h after DEHP exposure (data not shown) could itself be responsible for germ cell sloughing. The up-regulation of N-cadherin and α-catenin could reflect a change in the tyrosine kinase signal transduction pathway [39] or malignant properties [40]. Recent evidence of up-regulation of N-cadherin together with a loss of E-cadherin has been reported in invasive tumor cell lines and tissues from breast and prostate [41].

In conclusion, we demonstrated that DEHP induces severe damage of the testis with increased germ cell apoptosis in prepubertal rats. This effect is simultaneous with up-regulation of N-cadherin and catenin associated protein cell expression, suggesting that a deregulation of cell adhesion molecules involved in Sertoli–germ cell interactions is associated with germ cell sloughing of the seminiferous epithelium.

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