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The Effects of Di(2-Ethylhexyl)Phthalate Exposure and Selenium Nutrition on Sertoli Cell Vimentin Structure and Germ-Cell Apoptosis in Rat Testis

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Abstract This study aimed to investigate the effects of di(2-ethylhexyl)phthalate (DEHP) on Sertoli-cell vimentin filaments and germ-cell apoptosis in testes of pubertal rats at different selenium (Se) status. Se deficiency was produced in 3-weeks old Sprague-Dawley rats by feeding them ≤0.05 Se mg/kg diet for 5 weeks, Se supplementation group was on 1 mg Se/kg diet, and DEHP was applied at 1000 mg/kg dose by gavage during the last 10 days of the feeding period. The diet with excess Se did not cause any appreciable alteration in vimentin staining and apoptosis of germ cells, but Se deficiency caused a mild decrease in the intensity of vimentin immunoreactivity and enhanced germ-cell apoptosis significantly (approximately 3-fold, $p < 0.0033$). DEHP exposure caused disruption and collapse of vimentin filaments and significantly induced apoptotic death of germ cells (approximately 8-fold, $p < 0.0033$). In DEHP-exposed Se-deficient animals, compared with the control, collapse of vimentin filaments was more prominent; there was serious damage to the seminiferous epithelium; and a high increment (approximately 25-fold, $p < 0.0033$) in apoptotic germ cells was observed. Thus, Se deficiency exacerbated the toxicity of DEHP on Sertoli cells and spermatogenesis, whereas Se supplementation provided protection. These results put forward the critical role of Se in the modulation of redox status of testicular cells and emphasize the importance of Se status for reproductive health.

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Di(2-ethylhexyl)phthalate (DEHP) is one of the most commonly used plasticizers in consumer products, biomedical devices, and food-packaging materials (Blount et al. 2000). DEHP is an endocrine disrupter and a peroxisome proliferator (PP). It is rapidly metabolized mainly to mono(2-ethylhexyl)phthalate (MEHP), a derivative that is even more toxic than the parent compound (Erkekoglu et al. 2010a; Kluwe 1982; Koch et al. 2006). Both DEHP and MEHP have been shown to induce testicular damage, decrease fertility, and decrease sperm motility in both developing and adult animals and thus are reproductive and developmental toxicants in rodents (Erkekoglu et al. 2011a; Kwack et al. 2009; Lyche et al. 2009; Noriega et al. 2009). DEHP is a member of the phthalate family, the inevitable environmental exposure to which has been suspected to contribute to the increasing incidence of a range of reproductive defects in humans, including cryptorchidism and hypospadias in newborn boys and testicular cancer and decreased sperm quality in adult men (Virtanen et al. 2005). In fact, "testicular dysgenesis syndrome" has been shown to develop in male rats exposed to phthalates in utero (Frederiksen et al. 2007). Although available evidence is limited, such concerns are increasing, particularly for the developing reproductive system of male infants and children (Durmaz et al. 2010).

One of the primary targets of phthalates in testis is Sertoli cells, which are the supportive cells of the seminiferous epithelium that provide an appropriate hormonal and nutritional environment necessary for the differentiation of immature spermatogenic cells to spermatozoa. Sertoli cells possess a highly organized and quite active cytoskeleton with three major components: microfilaments, microtubules, and intermediate filamentsŭwhich consist of actin, tubulin, and vimentin, respectively. Actin makes up the microfilament network, and tubulin comprises the

microtubule cytoskeleton. The extensive intermediate filament of the cytoskeleton in Sertoli cells comprises primarily the type III family member vimentin (MW 55 to 58 kDa) (Franke et al. 1979; Vogl et al. 1993). Disruption of vimentin filaments and apoptosis in testicular cells was reported to be induced by various testicular toxicants, including DEHP and MEHP (Alam et al. 2010; Richburg et al. 1999; Tay et al. 2007).

Several mechanisms have been proposed for the testicular toxicity of DEHP, and recent evidence supports the view that oxidative stress is one of the underlying mechanisms (Erkekoglu et al. 2010a, b, 2011b; Fan et al. 2010). Oxidative stress is the imbalance between formation of reactive oxygen species (ROS) and antioxidant defense mechanisms. Oxidative stress and thus ROS play an important role in the modulation of several important physiological functions but also account for changes that can be detrimental to cells; thus, ROS contribute to cellular damage, apoptosis, and cell death (Dröge 2002). Oxidative stress has also been linked to apoptosis in germ cells and to male infertility (Kaushal & Bansal 2007). The essential trace element selenium (Se), is the critical component of cellular antioxidant defense and is involved in modulation of intracellular redox equilibrium with its several forms of cellular selenoproteins (Oberley et al. 2000). Se partakes in the fundamental biological processes ranging from cellular antioxidant defense to the protection and repair of DNA and apoptosis (Fischer et al. 2006; Ganther 1999). Se is also essential for the production of normal spermatozoa and thus plays a critical role in reproduction (Flohé 2007). Any alteration in the physiological concentrations of Se leads to abnormal spermatogenesis and, hence, male infertility (Olson et al. 2004).

Apropos to this knowledge and information, this study was designed to investigate the effects of DEHP on the expression pattern of vimentin filaments in Sertoli cells and on germ-cell apoptosis in rats at different Se status.

Materials and Methods

Chemicals and Reagents

All animal feed (A03/R03 base) was supplied by Scientific Animal Food and Engineering Laboratories (Augy, France). Antimouse vimentin primary antibody and 3,3′-diaminobenzidine (DAB) were obtained from Zymed (San Francisco, CA). Secondary antibody (Histostain Plus Broad Spectrum) and streptavidin-peroxidase complex (Histostain Plus Broad Spectrum) were supplied from Invitrogen (Carlsbad, CA). Entellan, Cell-Death Detection Kit (AP), terminal deoxynucleotidyl transferase (TdT)-mediated deoxyuridine triphosphate nick end-labeling (TUNEL) dilution buffer, and fast red tablets were obtained from Roche Applied Science (Mannheim, Germany). All other chemicals were from Sigma-Aldrich (St. Louis, MO).

Animals and Treatment

Male Sprague Dawley (SD) rats, 3 weeks old and supplied by the Experimental Animals Laboratory of Hacettepe University, were used in the study. The animals were divided randomly in six groups of six of each, and each group was housed in plastic cages with stainless-steel grid tops. The cages were placed in a room with controlled temperature (23°C) and humidity (50%) under a 12:12-h light-to-dark cycle. Body weights (bw) were monitored weekly and again just before the first dose of DEHP treatment. The feeding period was 5 weeks. Animals were treated humanely and with regard to alleviation of suffering, and the study was approved by Hacettepe University Ethical Committee.

Experimental Groups

(1) the control group (C) was fed a regular diet (0.15 mg/kg Se); (2) the Se-supplemented group (SeS) was fed Se-supplemented diet (1 mg/kg Se); (3) the Se-deficient group (SeD) was fed Se-deficient diet ($\leq$0.05 mg/kg Se); (4) the DEHP-treated group (DEHP) was fed a regular diet (0.15 mg/kg Se) and received 1000 mg/kg DEHP by intragastric gavage (i.g.) during the last 10 days of treatment; (5) the Se-supplemented DEHP Group (DEHP/SeS) was fed Se-supplemented diet (1 mg/kg Se) and received 1000 mg/kg DEHP by i.g. during the last 10 days of treatment; and (6) the Se-deficient DEHP group (DEHP/SeD) was fed Se-deficient diet ($\leq$0.05 mg/kg Se) and received 1000 mg/kg DEHP by i.g. during the last 10 days of treatment.

DEHP was dissolved in corn oil, and the animals in the C, SeS, and SeD groups received an equivalent amount of the vehicle by i.g. during the last 10 days of treatment. Animals were allowed to access ad libitum feed and drinking water throughout the experiments. Twenty-four hours after the last dose of DEHP treatment, overnight-fasted animals were weighed and killed by decapitation while under thiopental anesthesia. Testis tissues were removed and used for the detection of vimentin structure by immunohistochemistry and germ-cell apoptosis by TUNEL assay.

Vimentin Immunohistochemistry

Sertoli cells were immunolabelled with vimentin by immunoperoxidase technique. Samples from testis were rapidly fixed in Bouin's fixative solution, dehydrated

through graded alcohols, embedded in paraffin, and 5-μm sections were cut. For the immunolabelling of vimentin, sections were incubated at 60°C overnight and then cleared in xylene for 15 min. After rehydrating through a decreasing series of alcohols, sections were washed with 0.01 M phosphate buffer saline (PBS) at pH 7.4 for 5 min. Heat-mediated antigen retrieval was performed in 10 mM citrate buffer (pH 6.0) by microwave for 15 min at 600 W. Sections were cooled and washed with PBS three times for 5 min. Endogenous peroxidase was blocked by incubation in 10% H_2O_2 in PBS for 10 min at 4°C. Unspecific binding was blocked using rat serum at a dilution of 1:10 for 30 min at room temperature. Then sections were incubated with antimouse vimentin primary antibody for 1 h at room temperature. After washing 3 times for 5 min with PBS, sections were incubated with biotinylated secondary antibody for 30 min and then with streptavidin-peroxidase complex for 10 min at 37°C. After the washing in PBS, peroxidase activity was revealed by incubation with DAB for 5 min and counterstaining with Mayer's hematoxylin. After washing with tap water, sections were dehydrated through graded alcohols and cleared in xylene before mounting with Entellan. Negative-control experiments were performed by omitting incubation with the primary antibody. All slides were examined and photographed by using Leica DM6000B microscope (Wetzlar-Germany) with a DC490 digital camera (Leica). Immunohistochemical vimentin staining was semiquantitatively evaluated based on the labeling index of the percentage of Sertoli cells. Sertoli cells showing vimentin labeling in their cytoplasm as cytoplasmic extensions in addition to perinuclear labeling were scored as positive vimentin labeling. The immunoreactivity scores were evaluated in 20 cross-sections of seminiferous tubules in each sample. Labeling indices were grouped into five immunoreactivity scores: 0 (no reactivity), 1 (10% positive Sertoli cells), 2 (10% to 50% positive Sertoli cells), 3 (50% to 90% positive Sertoli cells), and 4 (90% positive Sertoli cells).

TUNEL Assay

Apoptosis of testicular germ cells was assessed by enzymatic labeling of DNA strand breaks using terminal deoxynucleotidyl transferase (TdT)-mediated deoxyuridine triphosphate nick end-labeling (TUNEL) assay with Cell Death Detection Kit according to the manufacturer's instructions. In this technique, the terminal TdT binds to the 3'-OH end and synthesizes a polynucleotide at the nick end. Then the biotinylated nucleotides interact with avidin-peroxidase, which can be detected histochemically.

Briefly, paraffin sections (5 μm) from testicular tissues were deparaffinized, rehydratated, and washed twice in PBS for 5 min. After incubation with the permeabilisation solution (0.1% Triton X-100 in 0.1% sodium citrate) for 8 min at 4°C and washing twice with PBS for 5 min, the labeling reaction was performed using 50 μl TUNEL reagent for each sample. After washing with PBS, slides were incubated with converter reagent for 30 min at 37°C. After the washing step, color development for localization of cells containing labeled DNA strand breaks was performed by incubating the slides with fast red substrate solution for 10 min. TUNEL-positive germ cells were identified by morphology and DAB substrate development and counted in 20 cross-sections of seminiferous tubules in each slide. All slides were examined and photographed by using a Leica DM6000B microscope with a Leica DC490 digital camera.

A positive control for detection of DNA fragmentation was included in each experiment by adding deoxyribonuclease I solution grade I (1500 U/ml in 50 mM Tris-HCl [pH 7.5]), 10 mM $MgCl_2$, and 1 mg/ml bovine serum albumin) and incubating for 30 min at 37°C before the TdT reaction. A negative control was also performed in which no TdT enzyme was present in the reaction mixture, and sections were incubated in label solution for 1 h at 37°C.

Statistical Analysis

All of the results for vimentin immunoreactivity scores and TUNEL assay are expressed as median and minimum–maximum values. Differences among the groups were evaluated with Kruskal–Wallis one-way analysis of variance. Mann–Whitney U-test with Bonferroni correction were used as multiple comparison. All statistical analyses were made using a Statistical Package for Social Sciences Program (SPSS) version 17.0, and $p<0.0033$ (0.05/15) was considered statistically significant.

Results

Vimentin Immunohistochemistry

The immunoreactivity of vimentin in Sertoli cells is shown in Fig. 1, and the immunoreactivity scores for vimentin are presented in Fig. 2. In the control group, the epithelia of the seminiferous tubules were intact (Fig. 1A, B). Vimentin-positive Sertoli cells were seen as extending along the whole thickness of the seminiferous epithelium. The immunoreactivity of vimentin was intense in the basal region of the cytoplasm housing the nucleus. Thin vimentin-positive apical extensions of Sertoli cells were evident between germ cells.

In the Se-supplementation group, the intensity and staining of vimentin was much like that of the control group, and vimentin filaments were observed as thin

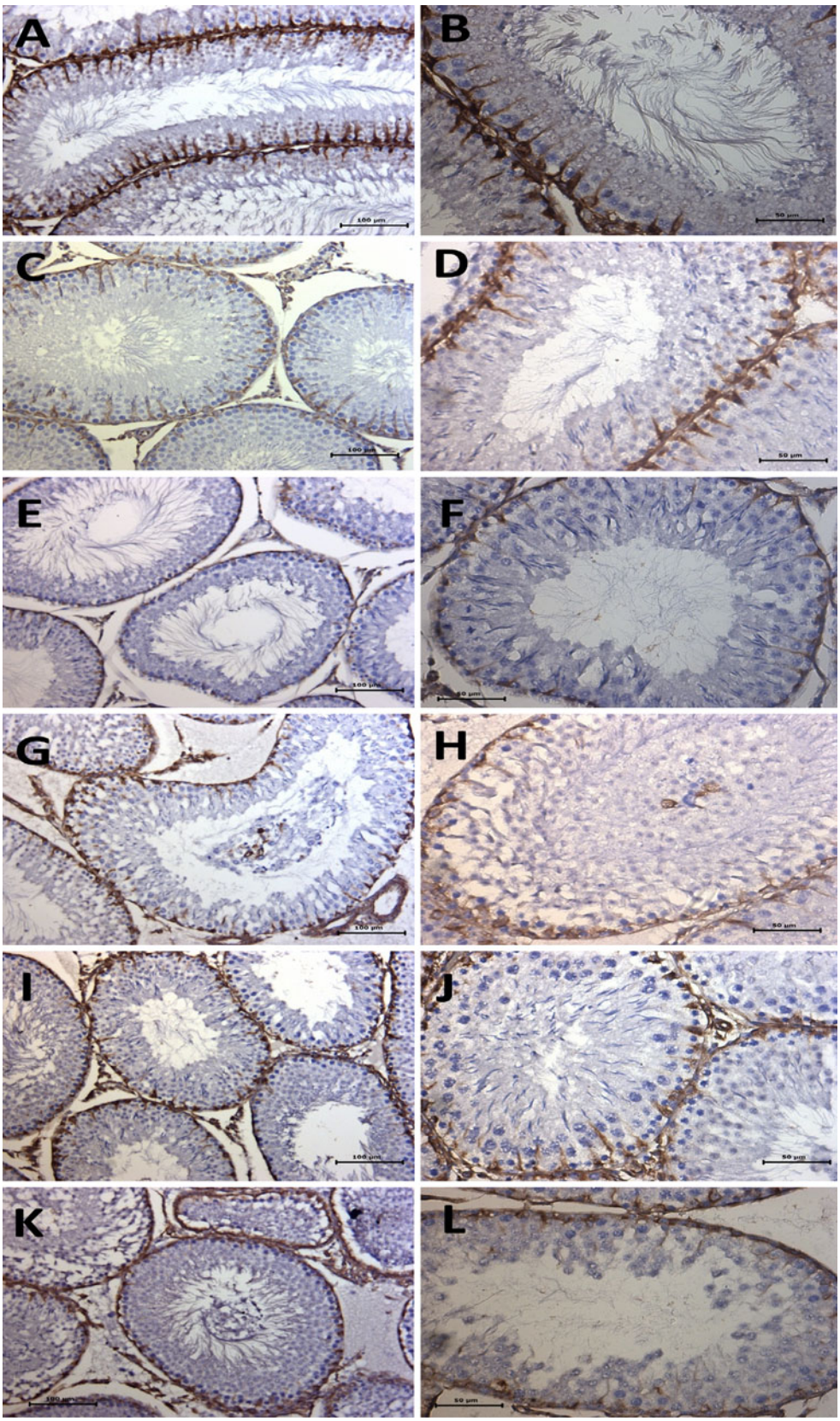

Fig. 1 Light micrographs showing vimentin immunoreactivity in Sertoli cells in the study groups. Original magnification ×200 for A, C, E, G, I, and K and ×400 for B, D, F, H, J, and L. The feeding period was 5 weeks for all study groups. **A**, **B** C group fed a regular diet (0.15 mg/kg Se). **C**, **D** SeS group fed an Se-supplemented diet (1 mg/kg Se). **E**, **F** SeD group fed an Se-deficient diet (≤0.05 mg/kg Se). **G**, **H** DEHP group fed a regular diet (0.15 mg/kg Se) and 1000 mg/kg i.g. DEHP for the last 10 days. **I** and **J** DEHP/SeS group fed an Se-supplemented diet (1 mg/kg Se) and 1000 mg/kg i.g. DEHP for the last 10 days. **K** and **L** DEHP/SeD group fed an Se-deficient diet (≤0.05 mg/kg Se) and 1000 mg/kg i.g. DEHP for the last 10 days

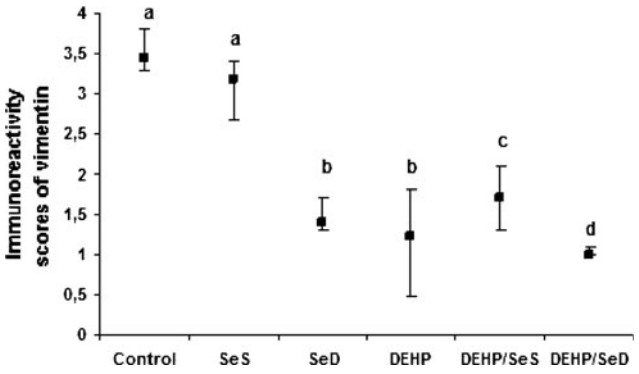

Fig. 2 Immunoreactivity scores of vimentin in the study groups. The immunoreactivity scores were evaluated in 20 cross-sections of seminiferous tubules in each sample. Labeling indices were grouped into five immunoreactivity scores: 0 (no reactivity), 1 (10% positive Sertoli cells), 2 (10% to 50% positive Sertoli cells), 3 (50% to 90% positive Sertoli cells), and 4 (90% positive Sertoli cells). All results are given as median and minimum–maximum values of $n = 6$ animals. Bars not sharing the same superscript letters are significantly different from each other ($p < 0.0033$). Experimental groups were fed for 5 weeks as follows: C = regular diet (0.15 mg/kg Se); SeS = Se-supplemented diet (1 mg/kg Se); SeD = Se-deficient diet (≤0.05 mg/kg Se); DEHP = regular diet (0.15 mg/kg Se) and 1000 mg/kg i.g. DEHP for the last 10 days; DEHP/SeS = Se-supplemented diet (1 mg/kg Se) and 1000 mg/kg i.g. DEHP for the last 10 days; and DEHP/SeD = Se-deficient diet (≤0.05 mg/kg Se) and 1000 mg/kg, i.g. DEHP for the last 10 days

projections extending from the basal region to the lumen (Fig. 1C, D). The difference between the control and the SeS group's vimentin immunoreactivity was not statistically significant (Fig. 2). In the SeD group, most of the seminiferous tubules were intact. The immunoreactivity of vimentin was seen at the basal part of the Sertoli cells, whereas typical vimentin-positive apical extensions were rarely noted compared with the control group (Fig. 1E, F); the immunoreactivity score was significantly (approximately 60%) lower than that of the control (Fig. 2).

In the DEHP group, seminiferous epithelium was disrupted, and loss of spermatogenetic cells were evident at the basal half of the epithelium (Fig. 1G, H). The immunoreactivity of vimentin was prominent at the basal part of Sertoli cells, but vimentin-positive apical extensions were rare and shorter. Moreover, there were gaps between spermatogenetic cells and Sertoli cells. Sloughed germ cells were seen in the lumen of seminiferous tubules. The Sertoli cells in this group showed significantly lower immunoreactivity scores (approximately 65%) for vimentin compared with those of the control group (Fig. 2).

In the DEHP/SeS group, vimentin-positive projections of Sertoli cells between spermatogenetic cells and thus Se supplementation was found to be partially protective against DEHP-induced alteration of vimentin expression (Fig. 1I, J). Sertoli cells showed significantly greater immunoreactivity scores for vimentin (approximately 40%) compared with those of the DEHP group (Fig. 2).

In the DEHP/SeD group, however, the integrity of seminiferous tubules was severely disrupted. The vimentin-positive apical extensions of Sertoli cells were not observed. The immunoreactivity of vimentin was confined to the basal part of the Sertoli cells around the nucleus (Fig. 1K, L). Thus, the disruption of vimentin filaments in the DEHP/SeD group, which ultimately resulted in detachment of germ cells from Sertoli cells, was more evident than those seen in the DEHP group. Sertoli cells in the seminiferous tubules of the DEHP/SeD group showed significantly lower immunoreactivity scores for vimentin (approximately 20%) compared with those of the DEHP group (Fig. 2).

TUNEL Assay

TUNEL-positive cells per each experimental group are presented in Fig. 3, and the micrographs of the TUNEL assay are given in Fig. 4. Although Se supplementation did not alter the apoptotic behavior of the cells, Se deficiency enhanced germ-cell apoptosis significantly compared with the control (approximately 3-fold, $p < 0.0033$). DEHP treatment also caused an approximately 8-fold increase ($p < 0.0033$) of apoptosis in germ cells compared with that of the control group. In the DEHP/SeS group, an approximately 3-fold decrease of apoptotic (TUNEL-positive) cell numbers was observed compared with the DEHP group,

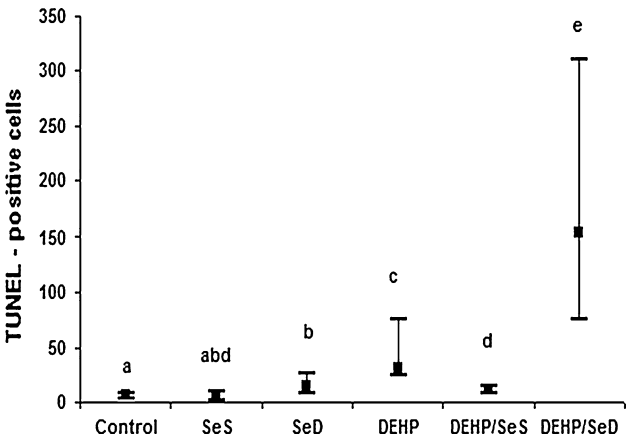

Fig. 3 Germ-cell apoptosis in the study groups. All results were given as median and minimum–maximum values of $n = 6$ animals. Bars not sharing the same superscript letters are significantly different *from* each other ($p < 0.0033$). Experimental groups were fed for 5 weeks as follows: C = regular diet (0.15 mg/kg Se); SeS = Se-supplemented diet (1 mg/kg Se); SeD = Se-deficient diet (≤0.05 mg/kg Se); DEHP = regular diet (0.15 mg/kg Se) and 1000 mg/kg i.g. DEHP for the last 10 days; DEHP/SeS = Se-supplemented diet (1 mg/kg Se) and 1000 mg/kg i.g. DEHP for the last 10 days; and DEHP/SeD = Se-deficient diet (≤0.05 mg/kg Se) and 1000 mg/kg, i.g. DEHP for the last 10 days

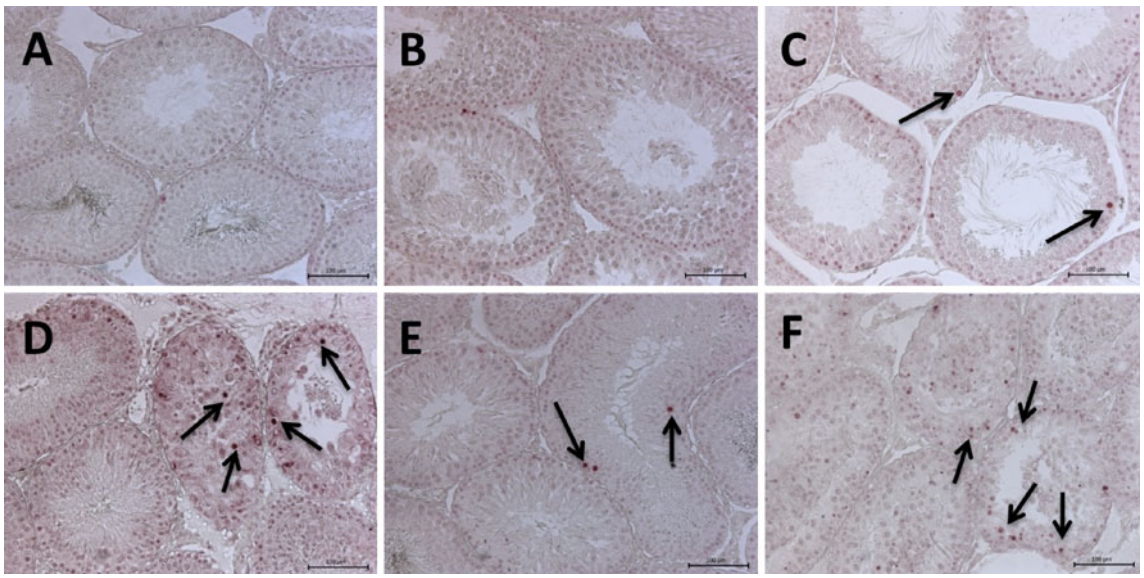

Fig. 4 Evaluation of apoptotic germ cells in seminiferous tubules using TUNEL staining. Original manification ×400. TUNEL-ositive (apoptotic) germ cells are indicated by arrows The number of apoptotic cells markedly increased in the SeD, DEHP, and DEHP/SeD groups. The feeding period was 5 weeks for all study groups. **A** C group fed a regular diet (0.15 mg/kg Se). **B** SeS group fed an Se-supplemented diet (1 mg/kg Se). **C** SeD group fed an Se-deficient diet (≤0.05 mg/kg Se). **D** DEHP group fed a regular diet (0.15 mg/kg Se) and 1000 mg/kg i.g. DEHP for the last 10 days. **E** DEHP/SeS group fed an Se-supplemented diet (1 mg/kg Se) and 1000 mg/kg i.g. DEHP for the last 10 days. **F** DEHP/SeD group fed an Se-deficient diet (≤0.05 mg/kg Se) and 1000 mg/kg i.g. DEHP for the last 10 days

indicating the protective effect of excess Se. In DEHP-exposed Se-deficient animals, apoptosis increased to a large extent compared with both the control (approximately 25-fold, p <0. 0.0033) and DEHP groups (approximately 9-fold, p <0. 0.0033).

Discussion

Sertoli cells possess a well-developed cytoskeleton with intermediate filaments of vimentin type surrounding the nucleus basally and radiating upward to the cell periphery to form thin columnar rows with tips (Franke et al. 1979). Thus, the essential structural components of the cell, i.e., vimentin filaments, play a role in positioning the Sertoli-cell nucleus and anchoring spermatogenetic cells to the seminiferous epithelium (Amlani and Vogl 1988). Spermatogenesis is fully dependent on Sertoli cells for maturation, differentiation, and survival; therefore, cooperation between Sertoli and spermatogenetic cells is essential for successful spermatogenesis (Bardin et al. 1988). Many reproductive toxicants have been shown to disturb Sertoli-cell junctional complexes, resulting in loss of intercellular contacts between Sertoli cells and germ cells, which may contribute to alteration of Sertoli-cell functions and germ-cell losses through apoptosis (Fiorini et al. 2004).

In the present study, we observed that vimentin-positive apical extensions of Sertoli cells extended along the whole thickness of seminiferous epithelium and hence clearly delineated the Sertoli-cell boundaries in the testis of control animals fed on adequate Se diet. The diet with excess Se did not cause any appreciable alteration of vimentin staining in the SeS group compared with control, but Se deficiency caused a significant decrease in the intensity of vimentin immunoreactivity. In DEHP-treated animals, however, we observed significant disruption and collapse of vimentin filaments and disruption of seminiferous epithelium in Sertoli cells. The latter findings were in accordance with earlier reports, which put forward the Sertoli cell as one of the main targets of DEHP and its main metabolite, MEHP. In fact, Richburg and Boekelheide (1996) demonstrated histopathological disturbances and alterations of cytoplasmatic distribution of vimentin in Sertoli cells in testis of 28-day-old Fisher rats after a single oral dose (2000 mg/kg) of MEHP. Administration of MEHP to Wistar rats at a single oral dose (400 mg/kg bw) was toxic to Sertoli cells and caused detachment of germ cells (Dalgaard et al. 2000). Tay et al. (2007) reported vimentin disruption in MEHP-treated C57Bl/6 N mice and gradual disappearance of vimentin in Sertoli-cell cultures as time and dose increased.

Yet, the distinctive finding of our study was the effect of DEHP treatment on Se-deficient animals, in which much more prominent collapse of vimentin filaments and serious damage of seminiferous epithelium were observed, and almost no vimentin-positive extensions of Sertoli cells was noted. Thus, we observed for the first time that Se deficiency had an aggravating effect on DEHP toxicity in

testicular cells, although Se deficiency by itself caused only mild but significant changes in vimentin microfilaments in the SeD group. In both the DEHP and DEHP/SeD groups, vimentin collapse was associated with the detachment of germ cells from Sertoli cells; only the basal cytoplasm around the nucleus of Sertoli cells were vimentin positive, indicating that the collapse of vimentin filaments occurred toward the basal part of the cell. Furthermore, the lumen of the seminiferous tubules contained sloughed germ-cell residues and vimentin-positive membrane-like structures. In the DEHP/SeS group, in contrast, small vimentin-positive extensions of Sertoli cells were observed, as was some recovery in vimentin assembly of Sertoli cells, especially in unaffected tubules, indicating a protective effect of excess Se against DEHP toxicity.

Our data further showed that the collapse of the intermediate filaments and decreased expression of vimentin immunoreactivity observed in Sertoli cells of DEHP-treated rats were accompanied by a substantial increment (approximately 8-fold) in apoptotic germ cells. These findings are also in line with earlier reports demonstrating that vimentin disruption correlated with the loss of structural integrity of seminiferous epithelia and spermatogenic-cell apoptosis (Alam et al. 2010; Richburg & Boekelheide 1996; Tay et al. 2007). However, the correlation we observed in the DEHP/SeD group was high, with a high number of TUNEL-positive cells (approximately 25-fold compared with control) and substantial vimentin filament losses. Thus, the extent of the germ-cell apoptosis we observed in DEHP-exposed rats was overwhelming and emphasized the testicular toxicity of DEHP. Furthermore, the produced data emphasized the importance of Se nutrition on DEHP toxicity.

Because healthy Sertoli cells hold and nurture the spermatogenic cells, disruption of vimentin signifies the loss of the support mechanism for germ cells so that they can no longer survive and may eventually undergo apoptosis. Such events have been shown earlier to occur in concert with the failure of spermatogenesis after testosterone (T) withdrawal (Show et al. 2003), in cryptorchidism (Wang et al. 2002), and in cases of chemical insults (Alam et al. 2010; Allard et al. 1993; Dalgaard et al. 2001; Hall et al. 1991; Hess & Nakai 2000; Richburg & Boekelheide 1996), and vimentin disruption has been suggested as the cause of the increased TUNEL-positive cells; thus, the increase of programmed death rate of spermatogonia.

Spermatogenesis is an androgen-dependent process. Decreasing the intratesticular T levels in rats results in failure of spermatogenesis and the apoptotic death of germ cells (Kim et al. 2001; Zikrin et al. 1989). The loss of the Sertoli-cell intermediate filament cytoskeleton has also been suggested as one mechanism by which the Sertoli cell communicates an apoptotic signal to germ cells after T withdrawal (Show et al. 2003). These findings and considerations are also in line with those of our recent study (Erkekoglu et al. 2011a), which demonstrated a T-lowering effect of DEHP along with other hormonal alterations, including disturbed testicular histology and diminished motility in epididymal sperm. In fact, that article and the present study are part of the same project; therefore, the assessment of both data provides a broader picture of the effects of DEHP in rat testis and signifies its effects on Sertoli-cell structure and functions in addition to its effects on Leydig cells, i.e., decreasing T level.

Multiple mechanisms of action were suggested for phthalate effects in the reproductive system, including PP-activation or estrogen receptor–mediated mechanisms (Gazouli et al. 2002), dysregulation of gene-expression pattern (Borch et al. 2006; Fan et al. 2010), and effect of spermatogenesis by altering the activities of enzymes responsible for the maturation of sperm (Barlow et al. 2003). An ever-increasing possibility in this regard is the induction of oxidative stress. Earlier data demonstrated an increase of ROS generation and depletion in antioxidant defenses by DEHP treatment in rat testis (Kasahara et al. 2002, among others). Our recent studies on MA-10 Leydig (Erkekoglu et al. 2010b) and LNCaP human prostate cells (Erkekoglu et al. 2010a) produced comprehensive data suggesting that at least one of the mechanisms underlying the reproductive toxicity of DEHP is the induction of intracellular ROS. The data of Fan et al. (2010) also suggested oxidative stress as a new mechanism of MEHP action on Leydig cells steroidogenesis by way of CYP1A1-mediated ROS stress. All of these data have thus put forward the importance of the redox status of the testicular cells, in which Se has a critical role.

With its several forms of cellular selenoproteins, the essential trace element Se is primarily involved in the modulation of intracellular redox equilibrium and plays a crucial role in cellular antioxidant defense. Se is remarkably and preferentially maintained in testis and is essential for normal spermatogenesis (Behne et al. 1982). Glutathione peroxidase 4 (GPx4), which is necessary for the integrity of mature sperm and required for proper sperm stability, is the most abundantly present selenoprotein in rat testis (Flohé 2007);thus, it is vital for male fertility (Aitken & Baker 2006). Recently, Seiler et al. (2008) demonstrated that GPx4 is also a sensor of oxidative stress and controls a distinctive cell death–signaling pathway involving 12/15-lipoxygenase-derived lipid peroxides and apoptosis-inducing factor–mediated cell death. Decrease of GPx4 expression was shown to cause termination of spermatogenesis (Behne et al. 1982; Roveri et al. 1992). Taken together, decreased GPx4 activity may be used as a marker for germ-cell apoptosis in rats. In fact, in our DEHP/SeD

group rats, we observed a significant decrease in GPx4 activity along with a significantly high redox ratio (data not shown).

It is known that the seminiferous epithelium and mature sperm require a particularly efficient protection against oxidative stress (Tramer et al. 1988). Several antioxidants have been used to minimize the detrimental effects of oxidative stress-producing toxicants. Inhibition of Leydig-cell steroid production by ROS (Diemer et al. 2003) has been shown to be partially prevented by antioxidants (Hanukoglu 2006; Sen Gupta et al. 2004). Increased ROS generation with MEHP exposure in MA-10 Leydig cells has been inhibited by *N*-acetylcysteine (Fan et al. 2010). Moreover, in the above-mentioned in vitro studies (Erkekoglu et al. 2010a, 2011b), we demonstrated that Se supplementation was highly protective against ROS production, p53-inducing potential, cellular antioxidant status–modifying effect, as well as cytotoxicity and genotoxicity induced by DEHP and MEHP in both MA-10 Leydig and LNCaP cells, stressing the role of Se as an effective cellular redox regulator. Thus, the results of the present study are supported by our previous in vitro findings and the data of the above-mentioned in vivo study (Erkekoglu et al. 2011a) regarding the critical role of Se in the modulation of redox status in the testicular cells and its protective role in DEHP-induced toxicity on Sertoli cells.

In conclusion, the data presented herein demonstrate that the abundantly used phthalate derivative DEHP is a Sertoli-cell toxicant that causes disruption and collapse of vimentin-type intermediate filaments of these cells and induces apoptotic death of germ cells in pubertal rat testis. These findings are in the same line as several earlier reports; however, the distinctive nature of the present study is the presentation of the effects of Se nutrition on DEHP toxicity in rat testis. Our data show that Se deficiency exacerbates the toxicity of DEHP on Sertoli cells and spermatogenesis, whereas Se supplementation provides protection. Thus, the results of the present study emphasize once more the importance of the Se status for reproductive health; however, it should be kept in mind that Se is bimodal, i.e., pro-oxidant and antioxidant in nature depending on the dose (Steinbrenner & Sies 2009).

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