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Mono-(2-ethylhexyl) phthalate (MEHP) affects intercellular junctions of Sertoli cell: A potential role of oxidative stress

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ABSTRACT

We analyzed the potential role of oxidative stress induced by mono (2-ethylhexyl) phthalate (MEHP) in adherent cell junction protein expression of prepubertal rat Sertoli cells (SC) *in vitro*. Five-day SC cultures were treated with MEHP (200 μM) for 24 h and compared to cells in basal conditions. Western blot and immunofluorescent (IF) analyses showed that MEHP induced increase of *N*-cadherin and catenin expression, modifying its distribution. Concomitantly, Cx-43 expression decreased significantly and delocalization of the IF signal for tight junction proteins (occludin, claudin-11 and ZO-1) occurred. Indicative of oxidative stress, MEHP induced in SC an increase of lipoperoxides, a decrease in glutathione (GSH) levels and a concomitant increase in Glutathione *S*-Transferases (GST) activity. Antioxidant *N*-acetyl-cysteine (1 mM) treatment prevented GSH decrease and *N*-cadherin and α-catenin up-regulation induced by MEHP. Our data suggest that oxidative stress signaling is a mechanism involved in adherent cell junctions disruption induced by MEHP in SC cultures.

1. Introduction

Phthalates are synthetic chemicals widely used as plasticizers since they provide flexibility in plastics. Human population gets exposed to phthalates through direct contact or general environmental contamination [1]. Most studies focused their effects on the reproductive tract, and these compounds are classified as endocrine disruptors [2]. In utero exposure to phthalate leads to male reproductive disorders in rats [3]. Prepubertal rat exposure to di-(2-ethylhexyl) phthalate (DEHP) demonstrated several alterations of seminiferous tubules and a reduction in testosterone production by Leydig cells [4–6].

Sertoli and Leydig cells are the major cell targets of phthalates [7]. Detachment of germ cells from seminiferous epithelium and increase in germ cell apoptosis in young peripubertal rodents after exposure to mono (2-ethylhexyl) phthalate (MEHP)/DEHP were also demonstrated [8,9].

Sertoli cells are polarized epithelial cells that interact with one another and with germ cells via a large, unique complex of specialized cell junctions (adherens, tight and gap) essential for spermatogenesis [10,11]. Tight junctions (TJ) are established between adjacent Sertoli cells and are an integral part of the blood testis-barrier (BTB), a structure that divides seminiferous tubules into distinct basal and adluminal compartments. The TJ structure, is a transmembrane region including occludin, the claudin multigene family, and junctional adhesion molecules plus a peripheral region expressing tight junction protein 1 (TJP1), formerly known as zonula occludens-1 (ZO-1) [12,13].

A gap junction (GJ) consists of two hemichannels (connexon) formed by a hexameric assembly of protein subunits (connexins) that provide direct intercellular communication pathways allowing rapid exchange of ions and metabolites. In testis, the predominant GJ protein is connexin 43 (Cx43), localized between Sertoli cells, Sertoli/germ cells or adjacent germ cells [14]. Data on Sertoli cell-specific knockout of Cx43 showed that expression of this molecule in Sertoli cells is an absolute requirement for normal testicular development and spermatogenesis [15,16].

In the testis, cadherin/catenin complexes assembled as functional adherens junctions (AJ) are distributed at the site of basal ectoplasmic specialization (ES) and at inter-Sertoli cell junctions

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where they are involved in the BTB structure [17]. AJ are also found at the apical ES, localized between Sertoli cells and elongated spermatids. In these junctions, classical cadherins (*e.g.*, *N*-cadherin) bind directly to β-catenin *via* its intracellular domain and associate with α-catenin which in turn links to the actin cytoskeleton network. p120 catenin binds directly to classical cadherins at the juxtamembrane region of the cytoplasmic domain, but does not associate with α-catenin [11].

In vivo experiments identified Sertoli cells as the primary target for phthalate-induced injury. In fact, this toxicant induces cytoplasmic vacuolization in this cell type, early alterations in cell function and disruption of Sertoli-spermatogenic cell physical interaction [9,18].

Any disruption of Sertoli cell junctions interferes with germ cell migration, inducing premature release of immature germ cells [19], a phenotype commonly observed after phthalate injury [8,20].

Some reports evaluated the effects of MEHP exposure on tight and gap junctions induced by MEHP in Sertoli cell cultures; however its effects on AJ have only been explored in *in vivo* experiments. We examined the *in vitro* effects of MEHP on expression and distribution of AJ proteins in Sertoli cell cultures and compared to tight and gap junction protein expression.

Extensive junction-re-structuring events in testis involve assembly and dis-assembly of AJ, a dynamic process regulated by many factors and signaling cascades [21,22]. Numerous reports showed that hormones such as testosterone, estrogens and FSH, interleukin 1-α (IL-1α), growth factors as well as free radicals are involved in cell junction dynamic regulation in testis [23,24]. Moreover, several toxicants (phthalates and others) may affect Sertoli cell junctions [25,26].

Induction of oxidative stress has been shown to be a common mechanism for environmental contaminants related to reproductive abnormalities in male rats [27]. Therefore, one of the possible mechanisms underlying the reproductive toxicity of phthalates might be induction of intracellular ROS causing activation of PPARα and alterations in intracellular enzymatic and non-enzymatic antioxidants, thereby contributing to disruption of spermatogenesis [28]. Glutathione and *N*-acetyl-cysteine are antioxidants that minimize oxidative stress and associated negative effects downstream.

We hypothesized that MEHP can alter Sertoli adherens junction protein expression and that this effect might be mediated by oxidative stress generated by exposure of cells to this toxicant. In fact, it is known that conditions of oxidative stress have been associated with elevation in levels of ROS and/or loss in activities of anti-oxidant enzymes [29,30] affecting Sertoli cell function [31]. Excesses in levels of ROS cause a state of oxidative stress detrimental to spermatogenesis and fertility [32].

2. Materials and methods

2.1. Sertoli cell isolation and culture

Twenty-day-old Sprague-Dawley rats were obtained from the Instituto de Biología y Medicina Experimental (IBYME-CONICET, Buenos Aires, Argentina). All experimental procedures were performed in accordance with the NIH guidelines for care and use of experimental animals, and approved by the Institutional Animal Care and Use Committee of IBYME and University of Buenos Aires (UBA). Animals were killed by CO_2 asphyxiation and the testes removed in order to isolate Sertoli cells as previously described [33]. Briefly, decapsulated testes were digested with 0.1% collagenase and 0.006% soybean trypsin inhibitor in Hanksí balanced salt solution (HBSS). Seminiferous tubules were collected, cut and submitted to 1 M glycine-2 mM EDTA (pH 7.4) treatment to remove peritubular cells. The washed tubular pellet was then digested again with collagenase to remove germ cells. The Sertoli cell suspension, collected by sedimentation, was resuspended in culture medium consisting of a 1:1 mixture of Ham's F-12 and Dulbeccoís modified Eagle medium, supplemented with 20 mM Hepes, 1.8 mg/ml sodium bicarbonate, 100 IU/ml penicillin, 2.5 μg/ml amphotericin B, 10 μg/ml transferrin, 5 μg/ml insulin, 5 μg/ml vitamin E and 4 ng/ml hydrocortisone. Sertoli cells were cultured in 6- or 24-multiwell plates or in 25 cm^2 tissue culture flask (initial density: 5 μg DNA/cm^2) at 34 °C in a mixture of 5% CO_2: 95% air. For immunofluorescence, Sertoli cells were cultured on glass chamber slides BD Falcon (BD Biosciences, San José, CA, USA) coated with laminin (6 μg/cm^2). Purity of Sertoli cell cultures reached 95% after 5 days of culture [34]. During the last 24 h of culture MEHP (200 μM) (Wako Chemicals Inc., VA, USA) or MEHP plus *N*-Acetyl-cysteine (NAC 1 mM) was added to Sertoli cells. MEHP was originally dissolved in dimethyl sulfoxide (DMSO) (0.77 M), and then in fresh media (4 mM solution). Aliquots were added to wells in order to obtain a 200 μM final concentration. To evaluate cultures in basal conditions DMSO without MEHP in equivalent concentration was added to the medium. At the end of the incubation period, cells were observed under a phase contrast microscope and viability was evaluated by an MTS assay (Cell Titer 96 Aqueous non-radioactive cell proliferation assay (MTS), Promega Corp., Madison, WI). Intracellular concentration of total glutathione (GSH), glutathione S-Transferases (GST) activity, lipid peroxidation assay, immunofluorescence and Western blot (Wb) techniques were performed in Sertoli cell cultures.

Table 1
Antibodies obtained from different vendors and their usage for different experiments in this study.

Target	Vendor	Host species	Usage (concentration mg/ml)	
			IB	IF
N-cadherin	BD Biosciences	Mouse	0.083	50
α-catenin	BD Biosciences	Mouse	0.5	50
β-catenin	BD Biosciences	Mouse	0.5	50
p-120 catenin	BD Biosciences	Mouse	0.25	50
occludin	Invitrogen	Mouse	1	
	BD Biosciences	Mouse		100
Claudin-11	Santa Cruz Biotech	Rabbit	0.05	
	AbCam	Rabbit		4
ZO-1-FITC	Invitrogen	Rabbit		10
ZO-1	Invitrogen	Rabbit	0.25	
Cx-43	BD Biosciences	Mouse	2.5	50
β-actin	Sigma Chemical Co	Rabbit		

IB: immunoblot; IF: immunofluorescence.

2.2. Immunofluorescence

To study the distribution and localization of cell junction proteins: adherens (*N*-cadherin and catenins), tight (claudin-11, occludin and ZO-1), and gap (Cx-43) junctions, cells cultured on chamber slides coated with laminin were fixed with methanol for 10 min at -20 °C. Cells were permeabilized with 0.1% Triton X-100 and blocked with 5% normal horse serum. Then, slides were incubated with primary antibodies (Table 1), as described previously [9,6]. Afterwards, slides were incubated with an anti-mouse or anti-rabbit IgG fluorescein isothiocyanate (FITC)-conjugated (1:25; Vector Laboratories, Burlingame, CA, USA). For negative controls, primary antibodies were replaced by PBS. Sertoli cell cultures were mounted in buffered glycerine and observed in an Axiophot fluorescent microscope with epi-illumination (Carl Zeiss Inc., Göttingen, Germany).

To detect ZO-1 expression, Sertoli cells were incubated with FITC conjugated antibody against ZO-1 (Table 1). Cells were then incubated with a biotinylated antibody anti-FITC diluted 1/100 in 5% BSA followed by incubation with Neutralite avidin-FITC conjugated (Southern Biotech, Birmingham, AL, USA) diluted 1/200 in 5% BSA. Omission of the first antibody or incubation with normal IgG isotype were used as negative controls. Finally, cells were mounted and observed in a fluorescent microscope as mentioned above.

2.3. Western blot analysis

Sertoli cells cultured in 6-multiwell plates were homogenized in 0.150 ml PBS buffer containing 1% protease inhibitor cocktail (Sigma Chemical Co., St. Louis MO, USA) and phenylmethanesulfonyl fluoride 2 mM (pH 7.4). Cells were disrupted by ultrasonic irradiation. Concentration of protein in homogenates was determined by the Bio-Rad protein assay (Bio-Rad Laboratories, Hercules, CA, USA). Extracts were dissolved with buffer sample as described previously [9], and equal amounts of proteins (~40 μg/lane) under reducing conditions were loaded and resolved on 10% and 12% acrylamide-bisacrylamide gel (SDS-PAGE). After SDS-PAGE, gels were electrotransferred at 100 V onto Polyvinylide difluoride membranes (PVDF) (Bio-Rad Laboratories, USA). Non-specific binding was blocked [9] and membranes were incubated with commercial antibodies as described in Table 1. Horse anti-mouse IgG (1:6000) and goat anti-rabbit (1:6000) biotinylated secondary antibodies (Vector Laboratories) were used. The reaction was enhanced with horseradish peroxidase-conjugated (Chemicon International Inc., Temecula, CA, USA) and proteins were visualized by chemiluminiscence. Intensity of autoradiographic bands was quantified by densitometry related to β-actin. Each value represented the mean ± SD of three densitometric readings. Prestained protein standards (Bio-Rad) with a molecular weight range of approximately 250-10 kDa were used.

2.4. Glutathione (GSH) assay

A modified method [35] was used to determine intracellular concentration of total GSH. Briefly, Sertoli cell monolayers in 24-multiwell plates were homogenized in 0.15 M Tris–HCl buffer (pH 7.4) and disrupted by sonication. Adequate aliquots for DNA determinations were saved. In microtitulation plates, two different aliquots of homogenate were added to the reaction buffer (0.1 ml) consisting of a mix of 100 mM sodium phosphate, pH 7.5–1 mM EDTA (5.75 ml), 1 mM NADPH (5 ml) and 1 mM 5.5′-Dithiobis (2-nitrobenzoic acid) (DTNB, 5 ml). Glutathione reductase from baker's yeast (20 U) was added to the mix to initiate the assay. The rate of 5-thio-2-nitrobenzoic acid formation (TNB) was followed at 2-min intervals for 10 min using a spectrophotometer at 405 nm. Absorbance was proportional to the sum of reduced and oxidized GSH present and was compared to a standard curve generated with GSH. Results were expressed as pmol GSH/μg DNA.

2.5. Activity of glutathione S-transferases

Total enzymatic activity of glutathione *S*-transferases (GSTs) was determined by the method of Habig and Jakoby [36]. Activity was measured, at room temperature, by determining the rate of conjugate formation between GSH and 1-chloro-2,4-nitrobenzene (CDNB) in a spectrophotometer at 340 nm. Results are expressed as nmol of product/μg DNA/min. Change in absorbance is a linear function of enzyme concentration.

2.6. Lipid peroxidation (LP) quantification assay

LP was quantified in chloroform: methanol lipid extracts by the FOX2 method [37]. Briefly, Sertoli cells cultured in 25 cm^2 flasks were washed and recovered in PBS (1.5 ml). After centrifuging, the tubes with resultant cell pellets were kept frozen (−80 °C) until analysis. Adequate aliquots for protein determinations were saved. For lipid extraction, 1.5 ml of chloroform: methanol (2:1, v:v) were added to each tube, sonicated and centrifuged. The lipid extract was transferred to clean glass tubes and dried out under a N_2 stream to a final volume of ~0.3 ml. For LP measurement 0.050 ml of extract was added to 0.450 ml of the FOX-2 reaction mixture (100 μM xylenol orange, 250 μM ammonium ferrous sulfate, 25 mM H_2SO_4 and 4 mM BHT in 90% methanol) and incubated for 30 min at RT. Hydrogen peroxide (100, 50, 25, 12.5, 6.2, 3.1 and 1.6 μM) was used as standard. Prior to measurement an aliquot of 0.300 ml of each tube was transferred to a micro plate and read at 570 nm with a reference filter of 690 nm. Data were corrected for extract volume and are expressed as nM/mg of protein.

2.7. Statistical analysis

For Western blot densitometry data, one-way analysis of variance (ANOVA) and the Student-Newman-Keuls multiple comparison test were applied using an Instat program. For GSH and GST assays, data were presented as mean ± SEM. ANOVA was used to compare more than two experimental conditions, followed by Tukey's test for comparison of all conditions against each other. A One-sample *t*-test was used to compare results of an experimental condition to a 100% expected theoretical value. Statistical analyses were done with GraphPad version 4.00 (GraphPad Software, San Diego, CA, USA). Results were considered significantly different if $p < 0.05$.

3. Results

3.1. Morphology of Sertoli cell cultures

In a preliminary experiment, cell viability test (MTS) was performed in Sertoli cells exposed to 10, 100 and 200 μM of MEHP at 6, 24 and 48 h. Since >95% of cell viability was achieved at all times and doses (Table 2 Supplementary), experiments were finally performed at 24 h only and at 200 μM of MEHP. A confluent monolayer of wide columnar or irregular-shaped cells with thick cytoplasmic processes was observed in 5-day Sertoli cell cultures by phase-contrast microscopy. After 24 h MEHP treatment a discontinuity in cell monolayer confluence was observed (Fig. 1A and B).

3.2. MEHP effect on intercellular Sertoli cell junctional protein localization, distribution and expression

Distribution and localization of cell junction proteins in Sertoli cell cultures was evaluated by immunofluorescence (IF). *N*-Cadherin and α and β-catenins were localized at cell membrane level in the contact zone between adjacent Sertoli cells and visualized as a continuous IF line in basal and MEHP-treated cells. A higher IF intensity was detected in MEHP-treated cells (Fig. 2 and Fig. 2 Supplementary). Occludin and claudin-11 were detected at intercellular Sertoli cell junctions in basal and MEHP-treated cells. However, in cells treated with the toxicant, the immunoreactive signal was delocalized and redistributed at cell cytoplasm with a higher intensity (Fig. 3). ZO-1 protein in Sertoli cells showed regular and continuous IF staining around the cell periphery in basal cell cultures, whereas in MEHP-treated cells a discontinuous IF pattern with higher fluorescent intensity was detected (Fig. 3). Cx-43 expressed at gap junction plaques between adjacent cells showed a

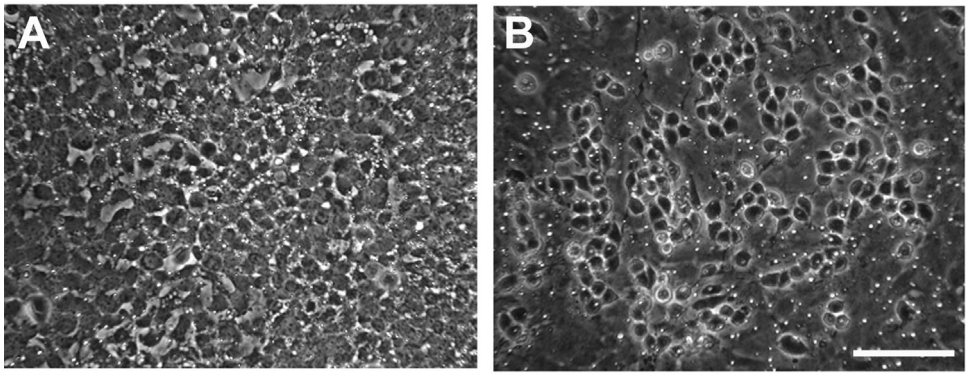

Fig. 1. Sertoli cell monolayer morphology (phase contrast microscopy) after 5 day culture. Confluent cell monolayer under basal conditions (A) and a discontinuous cell monolayer in MEHP-treated cells is observed (B). Scale bar: 100 μm.

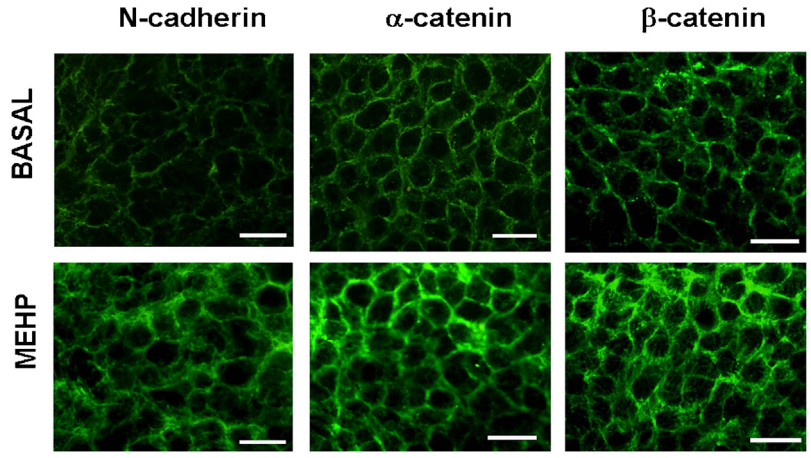

Fig. 2. Immunofluorescence to detect expression and distribution of N-cadherin, αand β catenins in basal and MEHP-treated Sertoli cell culture. Note that immunosignals were detected in basal and MEHP-treated cells as continuous lines specifically localized at cell membrane level or in cell to cell contacts. A higher IF intensity is observed after MEHP treatment. Scale bar: 20 μm.

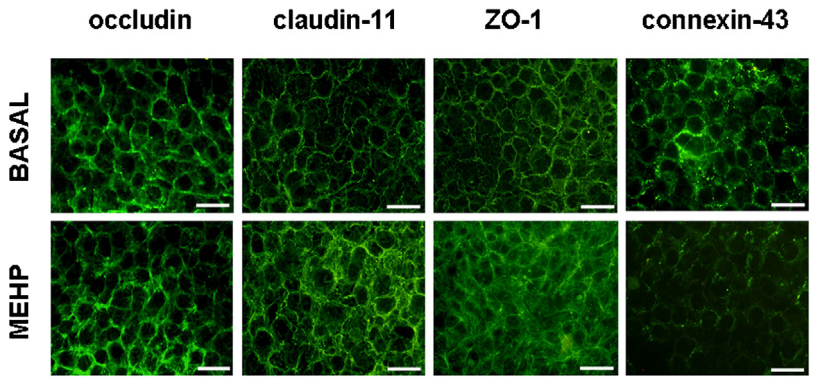

Fig. 3. Immunofluorescent (IF) localization and distribution of occludin, claudin-11, ZO-1 and Cx-43 in basal and MEHP-treated Sertoli cell culture. A linear and continuous IF was localized at the level of cellular contacts, except for Cx-43 in which IF has a punctuate pattern. In the presence of MEHP, the IF pattern shows a redistribution of ZO-1 and a decrease in the immunosignal for Cx-43. Scale bar: 20 μm.

reduced IF signal and punctuate pattern in MEHP-treated cells suggesting that MEHP induces disruption of cell–cell contacts (Fig. 3).

Western blot analysis was performed on Sertoli cell lysates in basal and 24 h MEHP-treated cells. A significant increase of *N*-cadherin and α and β catenin expression was observed in MEHP-treated Sertoli cells (Fig. 4). No changes in p120 catenin, occludin and claudin-11 expression were detected in basal and MEHP-treated cells (Fig. 4). However, we detected a significant increase in ZO-1 protein and a significant decrease in Cx-43 gap junction protein expression in MEHP-treated Sertoli cells (Figs. 5 and 6).

3.3. Oxidative stress evaluation and lipid damage after MEHP-treated Sertoli cell cultures

Glutathione levels and lipoperoxide index were determined in basal and 24 h MEHP-treated Sertoli cells. Intracellular glutathione

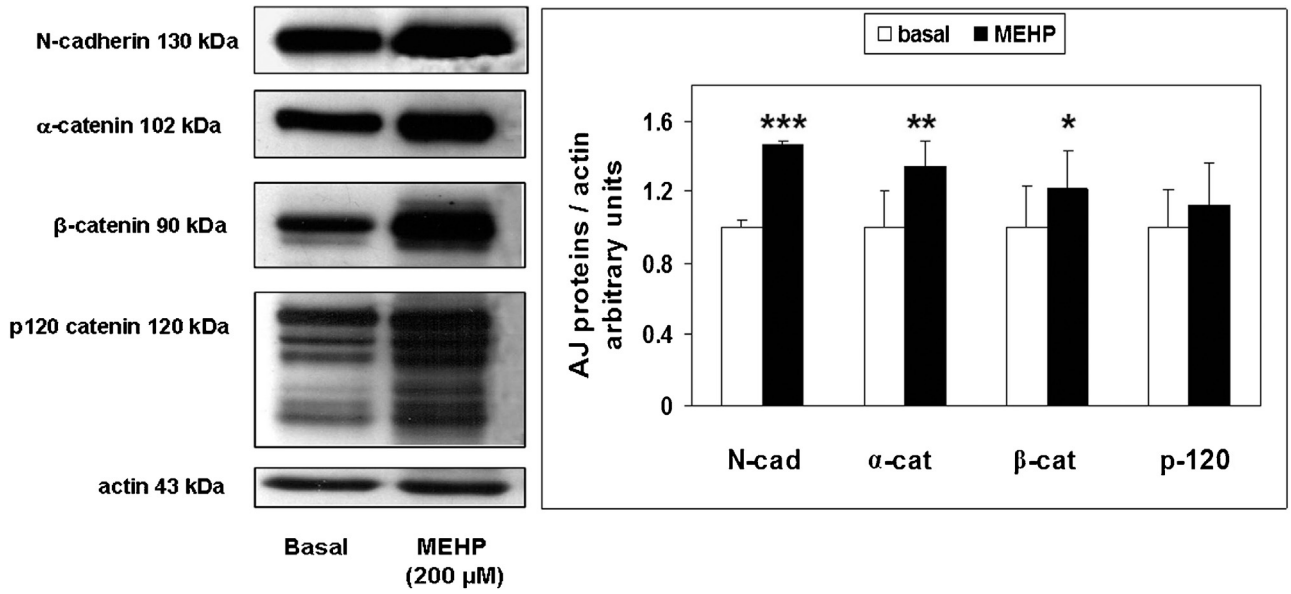

Fig. 4. Representative Western blot (Wb) and densitometric analysis of adherens junction (AJ) proteins (N-cadherin and catenins) expression in basal and MEHP treated Sertoli cell culture (***$p < 0.001$; **$p < 0.01$; *$p < 0.05$ *vs.* basal). Each bar represents the mean ± SD pooled data from three independent experiments.

levels significantly decreased in 200 μM MEHP-treated Sertoli cells compared to basal cultured cells (Fig. 7A). When the effect of exposure to MEHP was analyzed in Sertoli cell lipoperoxides as an indicator of oxidative stress, a significant increase in lipoperoxide levels was observed (Fig. 7B). The effect of the antioxidant *N*-acetylcysteine (NAC) on MEHP-exposed Sertoli cells was tested. Fig. 8 shows NAC-effect on glutathione levels in Sertoli cells under basal and MEPH-conditions. NAC alone or in combination with MEHP increased glutathione levels compared to basal conditions. NAC treatment helped to prevent glutathione decrease in MEHP-treated cells. A concomitant increase in GST activity was observed in 24 h MEHP-treated cells, indicating that glutathione could conjugate to the toxicant to detoxify MEHP-treated Sertoli cells. Although this enzymatic activity was not affected by NAC alone, this antioxidant prevented increase of GST activity in MEHP-treated cells. We were unable to detect lipoperoxide levels in MEHP-treated Sertoli cells plus NAC.

3.4. Effects of NAC on intercellular junction expression in MEHP-treated Sertoli cells

Effects of incubation of Sertoli cells with NAC were analyzed only for junctional proteins that underwent changes in their expression, evaluated by Western blot (*N*-cadherin, α, β-catenins, ZO-1 and Cx-43) during the cell culture period. We previously showed the increase in adherens junction proteins, *N*-cadherin, αand β-catenin expression in MEHP-treated Sertoli cells. We now evaluated whether NAC could down-regulate this expression. Results

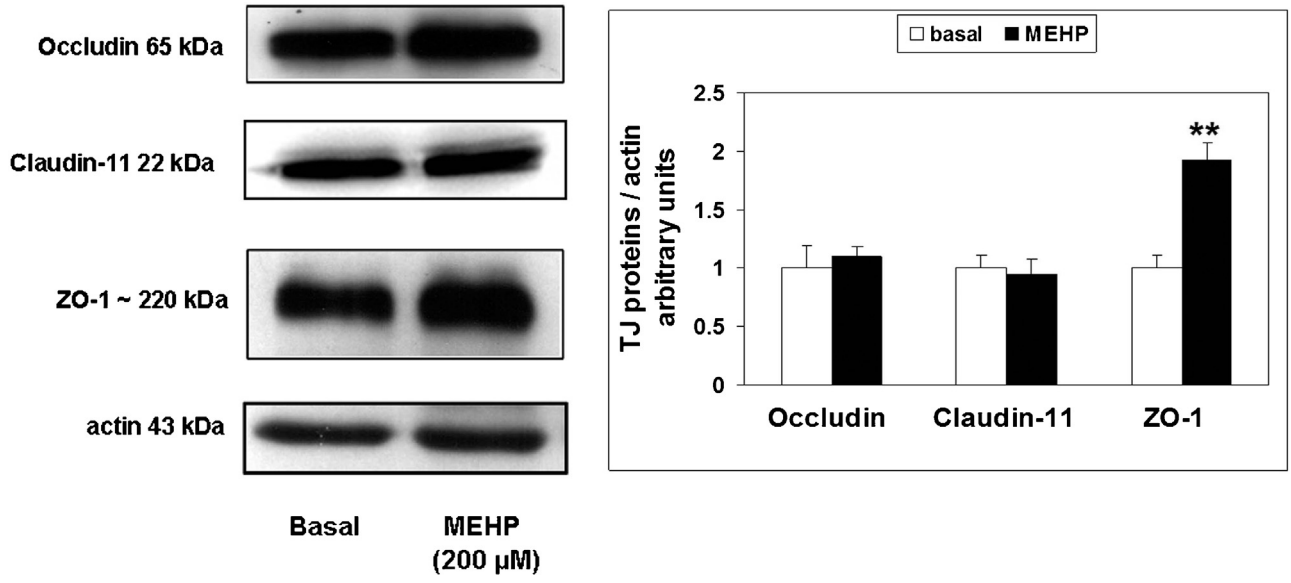

Fig. 5. Representative Wb and densitometric analysis of tight junction (TJ) proteins (occludin, Claudin-11 and ZO-1) expression in basal and MEHP-treated Sertoli cell culture. (**$p < 0.01$ *vs.* basal). Each bar represents the mean ± SD pooled data from three independent experiments.

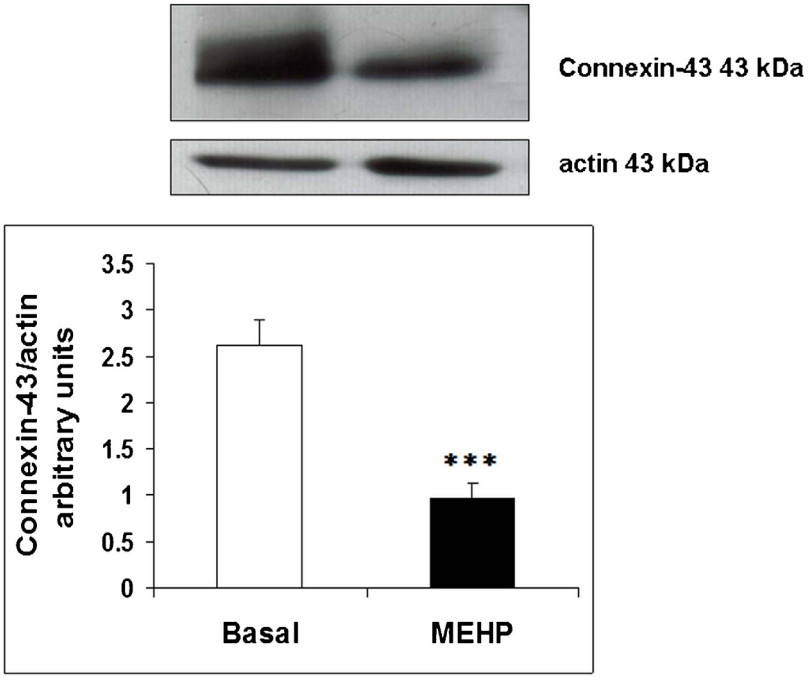

Fig. 6. Representative Wb and densitometric analysis of Cx43 expression in basal and MEHP-treated Sertoli cell cultures (***$p < 0.001$ *vs.* basal). Each bar represents the mean ± SD pooled data from three independent experiments.

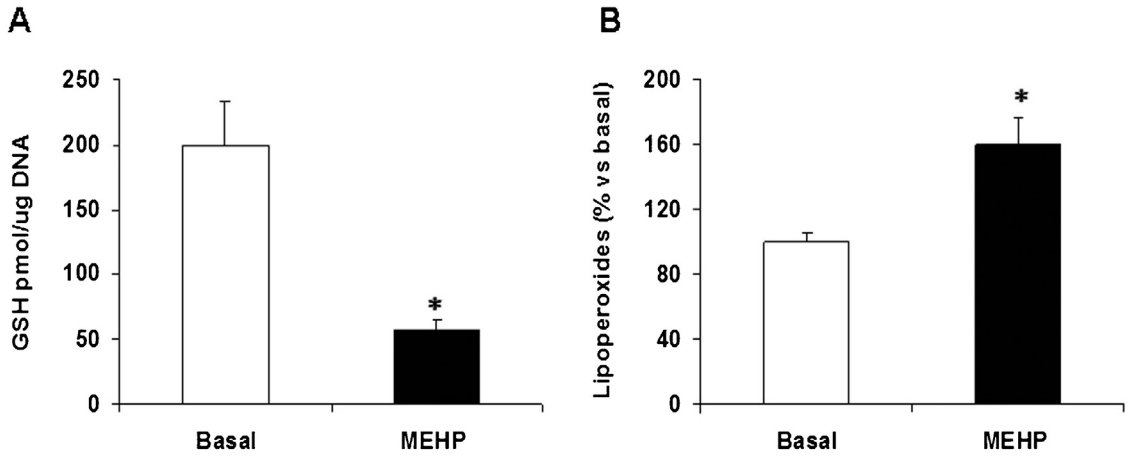

Fig. 7. Levels of Glutathione (A) and lipoperoxidases (B) in MEHP exposed Sertoli cells *vs.* basal condition (*$p < 0.05$ *vs.* basal) ($n = 4$). Glutathione is expressed as pmol GSH/μg. DNA and lipoperoxides are expressed as percentage of basal cells in each experiment.

showed that in the presence of NAC, MEHP-treated Sertoli cells exhibited, *N*-cadherin and α-catenin IF patterns similar to those of Sertoli cell cultures at basal conditions (Fig. 9), thereby demonstrating the ability of this antioxidant to prevent up-regulation of adherent cell junction protein expression induced by MEHP. MEHP-treated Sertoli cells incubated with NAC showed by Western blot analysis that expression of *N*-cadherin and α-catenin was down-regulated to levels similar to those of basal cells (Fig. 10 A and B). In contrast, the IF increased intensity or immunosignal redistribution observed for β-catenin and ZO-1 in MEHP plus NAC treated cells was similar to that of cells incubated with MEHP only (Fig. 9; arrows). A similar reduction of IF was observed for Cx-43 protein in MEHP plus NAC compared to MEHP-treated cells (Fig. 9; arrow). Results of Wb are in accordance with immunofluorescent data (Figs. 10 and 11).

4. Discussion

Present results demonstrate that cultured rat Sertoli cells exposed to MEHP undergo up-regulation of adherent junction proteins (*N*-cadherin and α-catenin) concomitant with down-regulation of Cx-43 expression. Data suggest that oxidative stress generated by cell exposure to this toxicant is involved in modulation of Sertoli cell junction protein expression. In fact, the reduced GSH level observed in MEHP treated Sertoli cells was prevented by NAC. Lipoperoxidation, an indicator of oxidative stress, was increased in Sertoli cells exposed to the toxicant.

Sertoli cells and their intercellular junctions (adherens, gap, and tight junctions) are known to play a pivotal role in the process of spermatogenesis. Consequently, perturbations of Sertoli cell junc-

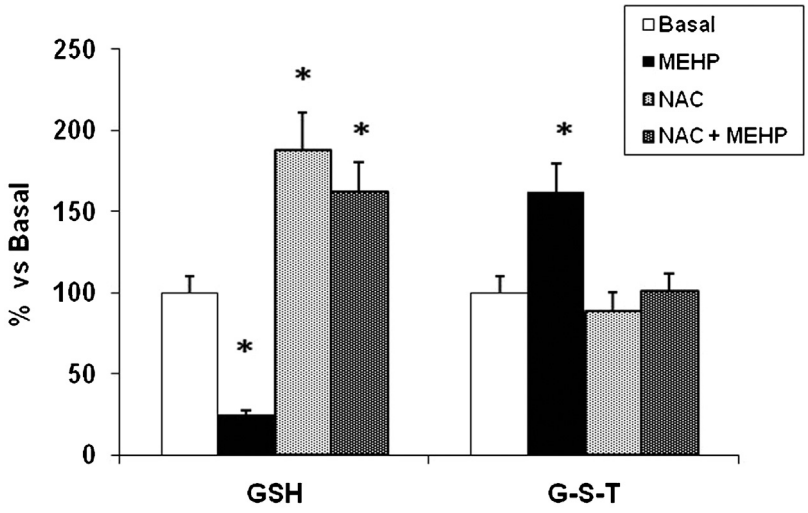

Fig. 8. Antioxidant NAC effect on glutathione levels of MEHP-exposed Sertoli cells and GST activity in presence of NAC and NAC plus MEHP (*$p < 0.01$ *vs.* basal) ($n = 4$). Results are expressed as percentage of basal cells in each experiment.

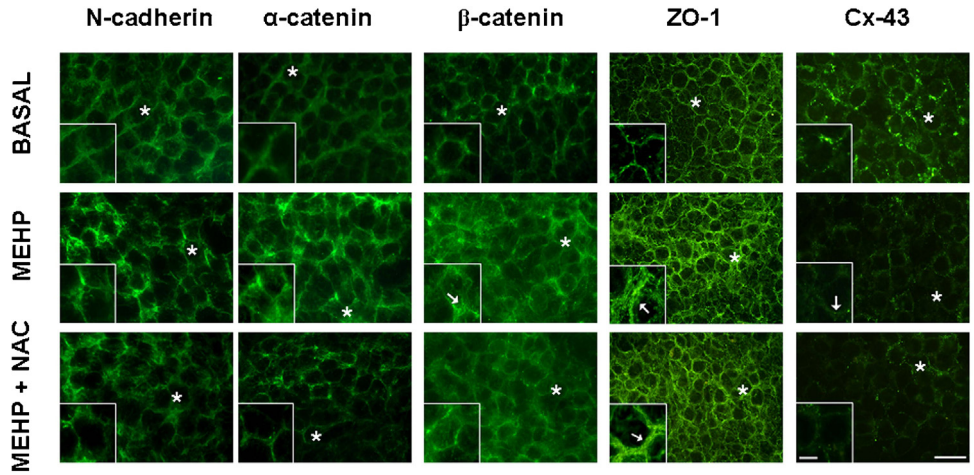

Fig. 9. Effect of MEHP and NAC co-treatment on cell junction protein expression in Sertoli cell cultures. *N*-cadherin and α-catenin IF pattern were similar to basal conditions. Instead IF pattern for β-catenin, ZO-1 and Cx43 proteins did not changed compared to MEHP only (arrows). Inserts show magnification of areas indicated by asterisks. Scale bar for pictures: 20 μm; scale bars for inserts: 5 μm.

tional proteins have been widely associated with impairment of spermatogenesis [8,9,38].

In vitro, Sertoli cells are able to form junctional interactions and express major and specific proteins involved in cell adhesion (*N*-cadherin), in the formation of tight (occludin, claudin-11 and ZO-1) and gap (Cx-43) junctions.

Present results confirm previous data from our group in rats exposed to di-(ethylhexyl) phthalate (DEHP) [6] and from others in Sertoli cell cultures [39] showing down-regulation of Cx43 expression suggesting impairment of gap junction structure and function. We observed delocalization of occludin and claudin-11 expression in MEHP-treated Sertoli cells by IF, whereas we detected no changes in the expression of these proteins by Western blot. With a different methodology and MEHP concentration Zhang et al. [40], and Chiba et al. [41], used RT-PCR to demonstrate down-regulation of occludin and claudin-11 expression in Sertoli cell cultures exposed to MEHP.

No previous data describe effects of MEHP on *in vitro* Sertoli cell adherens junction proteins. However, Lahousse et al. [42] reported in HeLa cells exposed to MEHP the phosphorylation of cell adhesion Celsr2 protein (Flamingo 1). Our results demonstrated that rat Sertoli cells *in vitro* exposed to MEHP undergo up-regulation of adherent junction proteins (*N*-cadherin and α-catenin) suggesting a mechanism to compensate for disruption of Sertoli cell junctions. These observations are in accordance with our previous data obtained in an *in vivo* model in which DEHP was administered to immature rats [9]. In another experimental model, we previously reported that deregulation of β-catenin/*N*-cadherin complex is associated with an increase in β-catenin phosphorylation leading to loss of cell adhesion function [43].

Yao et al. [44] showed that β-catenin mRNA level and vinculin are up-regulated by MEHP in NT2/D1 cells, a human testicular embryonal carcinoma cell line. Also, Kopera et al. [44] described up-regulation of *N*-cadherin and α and β-catenins in adjudin-treated pre-pubertal rats. This phenomenon was also described after exposing animals during puberty and adulthood to other toxicants such as bisphenol-A [25] or adjudin [45]. An early report by Gray and Beamand [46] demonstrated germ cell loss induced by MEHP in co-cultures of Sertoli-germ cells. Deregulation of *N*-cadherin and β-catenin expression positively correlates with severity of testicular pathology in these models characterized by increased sloughing of germ cells and frequency of apoptotic cell death. Therefore, alterations in expression of Sertoli AJ complexes may be one of the possible mechanisms leading to impaired

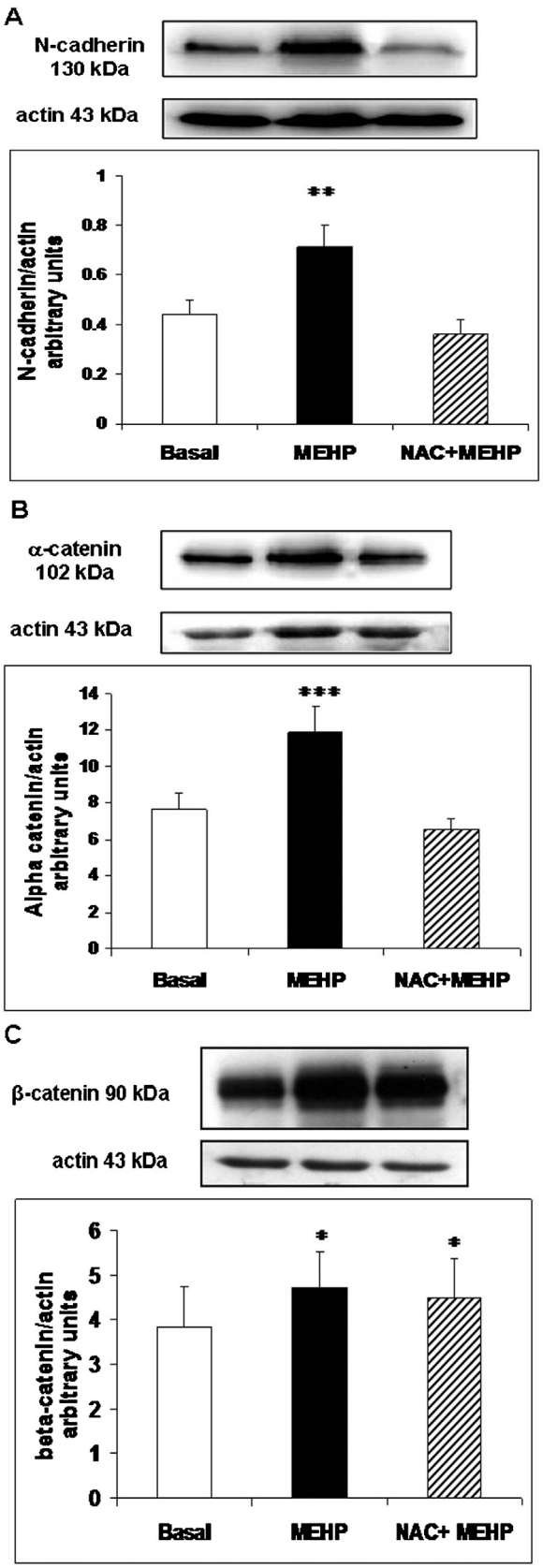

Fig. 10. Effect of MEHP and NAC co-treatment on adherens junction protein expression in Sertoli cell cultures analyzed by Wb (***$p < 0.001$; **$p < 0.01$; *$p < 0.05$ *vs.* basal). Each bar represents the mean ± SD pooled data from three independent experiments.

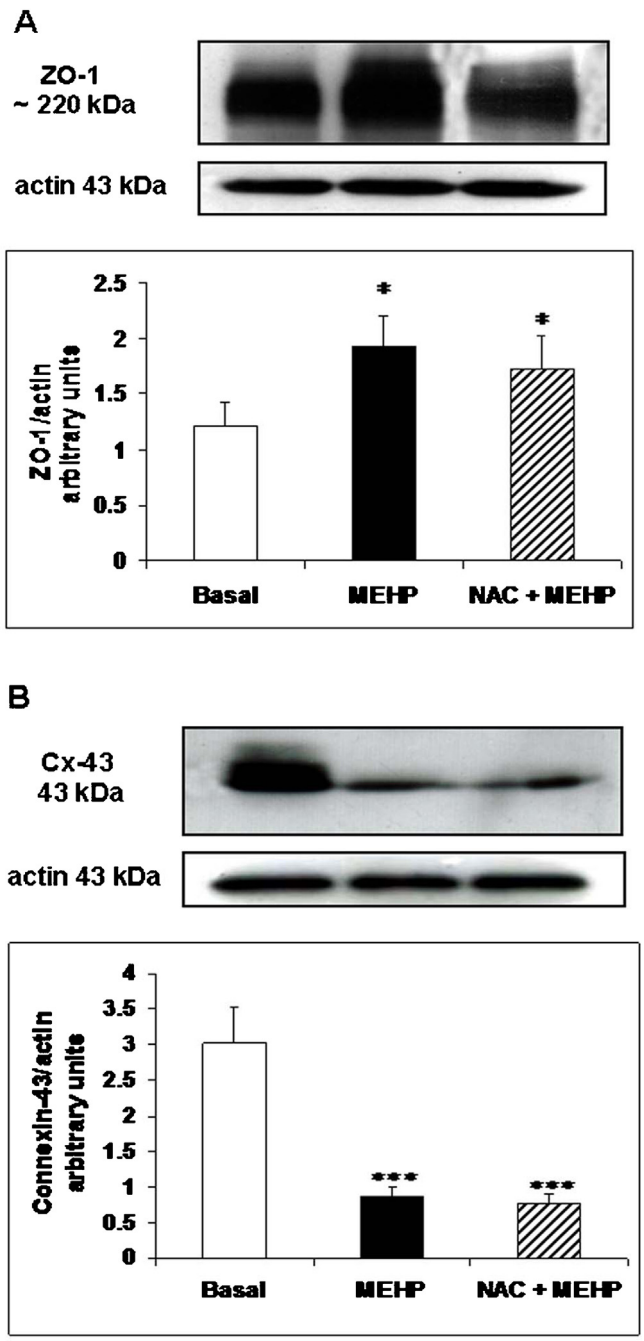

Fig. 11. Effect of MEHP and NAC co-treatment on ZO-1 (A) and Cx43 (B) protein expression in Sertoli cell cultures analyzed by Wb. (*$p < 0.05$; ***$p < 0.001$ *vs.* basal). Each bar represents the mean ± SD pooled data from three independent experiments.

cell–cell adhesion within the tubules, resulting in impairment of spermatogenesis and fertility.

As suggested by Yao et al. [8] and Chiba et al. [41], Sertoli cell junctional protein expression seems to be particularly susceptible to toxicants and therefore a suitable specific biomarker for cytotoxicity studies. Present results in MEHP-treated Sertoli cells showed a decrease of total GSH content and increased lipoperoxide content, demonstrating oxidative stress cell status. As a novel finding, we demonstrated that up-regulation of AJ proteins induced in Sertoli cells exposed to MEHP can be prevented by co-treatment with the anti-oxidant, *N*-acetyl-cysteine.

Wang et al. [47] demonstrated that MEHP induces oxidative stress, inhibiting growth of mouse ovarian antral follicles. NAC blocked MEHP toxic effect, restoring reactive oxygen species levels. Also, an increment in intracellular ROS was observed in MA-10 Leydig cell line exposed to MEHP [48].

It has been reported that oxidative stress might be a mechanism responsible for delocalization of testicular tight junction proteins and focal adhesion complexes in testis of adjudin-treated rats [22,49]. Bailey et al. [50] demonstrated that oxidative stress induces delocalization of *N*-cadherin and an increase in cytosolic β-catenin expression, disrupting retinal pigment epithelial cell junctions and barrier integrity. Also Usatyuk et al. [51] reported that 4-hydroxy-2-nonenal, an active aldehyde formed during inflammation and oxidative stress, affects endothelial cell permeability, modulating adherens and tight junction proteins.

Intracellular GSH is a key redox regulator crucial for multiple biological functions. The decrease of GSH observed in phthalate treated cells supported occurrence of oxidative stress and disturbance of intracellular redox equilibrium, since removal of H_2O_2 by glutathione peroxidase-1 requires GSH as a cofactor. Loss of GSH also occurs through conjugation to endogenous and exogenous electrophilic centers in reactions catalyzed by GSTs, enzymes capable of detoxifying genotoxic electrophilic compounds by catalyzing their conjugation to GSH, thereby inactivating several environmental chemicals [52].

The increase in GST activity detected in MEHP-treated Sertoli cells might be involved in detoxification of phthalates in the *in vitro* assay. NAC supplementation prevented the GST-increment induced by MEHP alone, indicating that mechanisms other than oxidative stress might also be involved in GST increment. Incubation of MEHP-treated Sertoli cell with NAC showed differential behaviour in intercellular junction protein expression. Up-regulation of *N*-cadherin and α-catenin induced by MEHP was prevented by NAC but not β-catenin, ZO-1 and Cx-43 expression, suggesting that the effect of the toxicant on adherens junction proteins occurs through oxidative stress.

Tight and gap junction proteins seem to be more susceptible to oxidative stress than adherens junction since in Sertoli cells treated with NAC, the effects of MEHP remained unchanged. We can speculate that tight and gap junction proteins might possess ROS-dependent as well as ROS-independent pathways to modulate junction protein expression, as has been suggested by Ko et al. [53] in diabetic cardiomyocytes.

Our study demonstrated that in Sertoli cell culture, expression of adherens junction proteins (*N*-cadherin and α-catenin), as well as tight junction associated protein (ZO-1) and gap junction protein (Cx-43), were affected by MEHP. Overall results also suggest that oxidative stress is a mechanism relevant to MEHP-induced damage of rat Sertoli cells, affecting particularly the structure and function of intercellular cell junctions.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.reprotox.2015.10.010.

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