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Lindane induces testicular apoptosis in adult Wistar rats through the involvement of Fas–FasL and mitochondria-dependent pathways

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ABSTRACT

Lindane, an organochlorine pesticide, is known to impair testicular functions and fertility. To elucidate the mechanism(s) underpinning the gonadal effects of lindane, we sought to investigate the levels of apoptosis-related proteins, namely cytochrome *c*, caspase-3 and-9, Fas and FasL in the testis of adult rats. Furthermore, the study aims to delineate whether nuclear factor kappa B (NF-κB) is involved in meditating the testicular effects of lindane. Animals were administered with a single dose of lindane (5 mg/kg body weight) and sacrificed at specific post-treatment intervals (0, 3, 6, 12, 24 and 72 h). Significant elevations in the levels of cytosolic cytochrome *c* with a parallel increase in pro-caspase-9 were observed as early as 6 h following exposure. Time-dependent elevations in the levels of Fas, FasL and caspase-3 were observed. Immunofluorescence studies revealed increased colocalization of Fas and caspase-3 in peritubular germ cells. FasL levels were increased in Sertoli and peritubular germ cells. The cytoplasmic levels of NF-κB p65 decreased from 3 h following exposure with a maximal decline at 12 and 24 h. Changes in the localization of NF-κB were observed with maximal nuclear translocation in germ cells at 12 and 24 h. Terminal deoxynucleotidyl transferase-mediated dUTP nickend-labeling (TUNEL) assay revealed a time-dependent increase in the number of apoptotic cells. Taken together, the data illustrate induction of testicular apoptosis in adult rats following exposure to a single dose of lindane. Early activation of NF-κB in contrast to late increase in Fas expression suggests a pro-apoptotic role of NF-κB in testicular response to lindane.

1. Introduction

The rapid expansion of chemical industry has resulted in the release of a plethora of chemicals into the environment. These chemicals have become part and parcel of our lives. Of these, potentially hazardous chemicals and chemicals endowed with hormone-like activity have been implicated in the burgeoning high incidence of male infertility (Irvine, 2000; Aitken et al., 2004; Saradha and Mathur, 2006a). Lindane, the gamma isomer of hexachlorocyclohexane (HCH), has driven much attention due to its estrogenic (Raizada et al., 1980; El-Mubarak and Huisingh, 2001) as well as anti-estrogenic properties (Chadwick et al., 1988; Oropeza-Hernandez et al., 2001). Lindane belongs to the family of organochlorine pesticides and has been widely used to eradicate insects in agriculture and to treat scabies. The extensive use, chemical stability and bioaccumulation potential of lindane have resulted in its ubiquitous distribution in the ecosystem and thereby considered to be a global pollutant.

A vast array of literature exists that demonstrate detrimental effects of lindane on reproduction (Dikshith et al., 1978; Dalsenter et al., 1997; Suwalsky et al., 2000; Pages et al., 2002) and in fact, testes have been found to be highly sensitive target organs for lindane. It has been shown to accumulate in rat testis thereby causing degenerative changes in germinal epithelium and Sertoli cell fragmentation (Dalsenter et al., 1996). Impaired androgen synthesis in Leydig cells (Ronco et al., 2001), decreased sperm counts (Saradha and Mathur, 2006b) and increased sperm abnormalities (Prasad et al., 1995) have also been reported. Recently, we have demonstrated alteration in the levels of heat shock protein (HSP) and clusterin accompanied by an induction of oxidative stress in rat testis as early as 12 h following exposure to a single dose (5 mg/kg body weight) of lindane (Saradha et al., 2008a). We have also demonstrated transient inhibitory effect of lindane on testicular steroidogenesis and the possible role of hydrogen peroxide (H_2O_2) in mediating these

Abbreviations: DAB, diaminobenzidine; H_2O_2, hydrogen peroxide; HSP, heat shock protein; HCH, hexachlorocyclohexane; IκB, inhibitory kappa B; NF-κB, nuclear factor kappa B; NOAEL, no observed adverse effect level; PBS, phosphate-buffered saline; ROS, reactive oxygen species; rTDT, terminal deoxynucleotidyl transferase, recombinant; TMB/H_2O_2, tetramethylbenzidine/hydrogen peroxide; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling.

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effects (Saradha et al., 2008b). Studies from our laboratory and of others clearly demonstrate the detrimental effects of lindane on testicular functions and its strong association with oxidative stress and reactive oxygen species (ROS) (Samanta and Chainy, 1997; Sujatha et al., 2001). The stress response and programmed cell death are cellular reactions to stressful stimuli. Stress-induced apoptotic alterations have been implicated as a cause or consequence of various pathological states including infertility (Sikka et al., 1995; Wang et al., 2003). In the present study, we sought to investigate whether low dose of lindane would alter the levels of apoptosis-related proteins in adult rat testis within a short time following exposure. The objective of this study was to evaluate the protein levels of cytochrome *c*, caspase-3 and -9, Fas and FasL in the testis of lindane-treated rat, and also to delineate the role of nuclear factor kappa B (NF-κB) in meditating the testicular effects of lindane.

2. Materials and methods

2.1. Chemicals

Lindane (99% pure) and rabbit monoclonal antibody against β-actin and Lamin-B were procured from Sigma Chemical Company (St. Louis, MO, USA). Rabbit polyclonal antibodies against caspase-9 (sc-7885), Fas (sc-7886), Fas-L (sc-834) and NF-κB p65 (sc-7151), and goat polyclonal antibodies against caspase-3 (sc-1225) and cytochrome *c* (sc-8385) were procured from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Goat anti-rabbit IgG and rabbit anti-goat IgG conjugated to FITC, goat anti-rabbit IgG conjugated to Cy3™, horseradish peroxidase-conjugated goat anti-rabbit IgG and rabbit anti-goat IgG were obtained from Bangalore Genei (Bangalore, India). All other chemicals used were of analytical grade and were obtained from local commercial sources.

2.2. Experimental design

Adult male rats of Wistar strain (80–90 days old) were used in this study. The animals were procured from an authorized vendor (M/S Raghavendra Enterprises, Banglore, India) and were housed in polypropylene cages and maintained at 22–25 °C under a well-regulated light and dark (12 h:12 h) schedule at the Animal Facility of the Pondicherry University. The animals were provided standard rat chow (Sai Durga Feeds and Foods, Bangalore, India) and water *ad libitum*. The weights of the rats were recorded on alternate days and the animals showing poor growth rate were eliminated from the study. For 7 days prior to the start of experiments, rats were handled daily for 5 min to acclimatize them to human contact and minimize their physiological response to handling.

Animals used for experimentation were divided into six groups and four animals were maintained in each group. Lindane was dissolved in olive oil (5 mg/kg body weight) and administered by oral gavage to all treatment groups. After treatment with a single dose of lindane, animals were sacrificed at 0, 3, 6, 12, 24 and 72 h of post-exposure using overdose of anesthetic ether. Testes were collected, cleared of the adhering tissues, weighed and stored at −80 °C until analysis. The experiments were carried out in accordance with the guidelines of the Committee for the Purpose of Control and Supervision on Experiments on Animals (CPCSEA), Government of India (CPCSEA, 2003).

2.3. Immunoblot analyses of caspase-3, -9, cytochrome c, Fas, and FasL

Testes lysates were prepared in lysis buffer (50 mM Tris, pH 7.4 containing 0.15 M NaCl, 10% glycerol (v/v), 1% NP-40 (v/v), 1 mM sodium fluoride, 1 mM sodium orthovanadate, 1 mM PMSF, 1 mM EDTA, 150 μM bestatin, 1 μM leupeptin and 1 μM aprotinin) using a tissue: buffer ratio of 1:5. After homogenization, samples were centrifuged at 1000 × *g* for 2 min to remove large tissue fragments and then centrifuged again at 10,000 × *g* for 30 min. The supernatants were collected and stored at −70 °C until analysis. The protein concentration was determined (Lowry et al., 1951) and equal quantities of protein were loaded per lane and subjected to 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis [SDS-PAGE] (Mini Protean II System, Bio-Rad) as described by Laemmli (1970). Electrophoresis was performed at 75 V and the resolved proteins were electrophoretically transferred onto a nitrocellulose membrane (NYTRAN, Keene, NH, USA) in Transfer buffer (0.2 mol/L glycine, 25 mM Tris, and 20% methanol). The membranes were incubated in a blocking buffer (phosphate-buffered saline [PBS] containing 0.1% (v/v) Tween 20 and 5% (w/v) non-fat dry milk powder) for 5 h at room temperature, followed by incubation in respective primary antibodies. Anti-caspase-3 was diluted at 1/100, and anticaspase-9, anti-Fas and anti-FasL were diluted at 1/200. Incubations with the primary antibodies were carried out overnight at 4 °C. The following day, blots were washed in PBS and incubated for 1 h at room temperature with either horseradish peroxidase conjugated anti-rabbit IgG or anti-goat IgG (1:1000 dilution). Immunodetection of proteins were visualized by using tetramethylbenzidine/hydrogen peroxide (TMB/H_2O_2) (Bangalore Genei, Bangalore, India) as substrate and resulting immunospecfic bands were quantified by densitometry.

For measurement of cytochrome *c* release, cytosolic fractions, free of mitochondria, were isolated by homogenizing the testes in a buffer containing 0.25 M sucrose, 50 mM HEPES, 0.1 mM EDTA, pH 7.4, 10 mM NaCl and 2 mM DTT using a glass–teflon homogenizer (Remi RQ-127A, Remi Motors, Mumbai, India). The homogenate was centrifuged at 1000 × *g* for 10 min at 4 °C to remove debris and the resulting supernatant was further centrifuged at 12,800 × *g* for 30 min. The supernatants (cytosolic fractions) were collected and protein content was determined (Lowry et al., 1951). Equal quantities of protein were subjected to one-dimensional SDS-PAGE and subsequently electroblotted onto nitrocellulose membrane as describe above. Blots were incubated with primary antibody for cytochrome *c* (1:200) overnight at 4 °C. The following day, blots were washed in PBS and incubated for 1 h at room temperature with horseradish peroxidase conjugated anti-goat IgG (1:1000 dilution). Immunodetection of proteins were visualized by using TMB/H_2O_2 (Bangalore Genei, Bangalore, India) as substrate and resulting immnunospecfic bands were quantified by densitometry. Densitometric scanning was performed using Gene tool (version 3.05, Synoptics Ltd., Cambridge, UK).

2.4. Immunoblot analysis of NF-κB p65

Testis were gently homogenized into a hypotonic buffer containing 50 mM HEPES, pH 7.4, 10 mM KCl, 1 mM EDTA, 1 mM DTT, 0.2 mM PMSF, 1 μg/ml pepstatin, 1 μg/ml leupeptin, 0.5% Nonidet P-40 using a glass–teflon homogenizer (Remi RQ-127A, Remi Motors, Mumbai, India). Cytoplasmic and nuclear extracts were prepared as previously described (Han and Brasier, 1997) and protein concentrations were determined (Lowry et al., 1951). Equal quantities of protein were subjected to one-dimensional SDS-PAGE and subsequently electroblotted onto nitrocellulose membrane as described above. Blots were incubated with primary antibody for NF-κB p65 (1:200) overnight at 4 °C. The following day, blots were washed in PBS and incubated for 1 h at room temperature with horseradish peroxidase conjugated anti-rabbit IgG (1:1000 dilution). Immunodetection of proteins were visualized by using TMB/H_2O_2 (Bangalore Genei, Bangalore, India) as substrate and resulting immnunospecfic bands were quantified by densitometry. Densitometric scanning was performed using Gene tool (version 3.05, Synoptics Ltd., Cambridge, UK).

2.5. Immunoflurosecent staining of Fas, FasL, caspase 3 and NF-κB p65

Bouin's-fixed testes were embedded in paraffin and sectioned at a thickness of 5 μm on poly-L-lysine-coated slides. After deparaffinization and rehydration, sections were washed in PBS (10 mM NaH_2PO_4, pH 7.4 at 22 °C containing 0.15 M NaCl) and permeabilized in 0.2% Triton X-100 (v/v). Sections were then blocked with 10% non-immune goat serum for 1 h. Sections were incubated with anti-FasL and anti NF-κB (1:100 dilution each containing 1% non-immune goat serum) antibodies overnight at 4 °C. After repeated rinsing with PBS, the sections were saturated with goat anti-rabbit IgG conjugated to FITC (1:100 dilution containing 10% non-immune goat serum) for 2 h in dark. After being washed in PBS three to four times (5 min each), the slides were mounted and examined under the microscope (Olympus System, model CX41RF, Tokyo, Japan). Negative control consisted of sections incubated with non-immune rabbit IgG (1:100 dilution) instead of primary antibody and PBS instead of secondary antibody.

To determine whether Fas and caspase-3 were colocalized, the sections were incubated with primary antibody mixture which consisted of rabbit anti-Fas and goat anti-caspase-3 (1:100 dilution each containing 1% non-immune goat serum) overnight at 4 °C. After repeated rinsing with PBS, the sections were incubated with secondary antibody mixture which consisted of goat anti-rabbit IgG conjugated to Cy3™ and rabbit anti-goat IgG conjugated to FITC (1:100 dilution each containing 10% non-immune goat serum). After being washed in PBS three to four times (5 min each), the slides were mounted and examined under the microscope (Olympus System, model CX41RF, Tokyo, Japan). Negative controls consisted of sections incubated with non-immune rabbit and goat IgG in place of primary antibodies and PBS instead of secondary antibodies.

2.6. Detection of apoptosis

Apoptotic cells were detected using the DeadEnd Colorimetric Apoptosis Detection System (Promega Corp., Madison, WI, USA). This system end-labels the fragmented DNA of apoptotic cells using a modified terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) assay. It consists of incorporation of biotinylated nucleotides at the 3′-OH DNA ends using the terminal deoxynucleotidyl transferase, recombinant (rTDT) enzyme. Horseradish-peroxidase-labeled streptavidin is then bound to these biotinylated nucleotides, which are detected using the peroxidase substrate, hydrogen peroxide and the stable chromogen diaminobenzidine (DAB). Briefly, the tissue sections were deparaffinized, rehydrated and fixed in 4% paraformaldehyde solution in PBS. Proteinase K (20 μg/ml) treatment for 15 min was followed by a second fixation in 4%

paraformaldehyde solution in PBS. The sections were incubated with equilibration buffer (200 mM potassium cacodylate, pH 6.6, 25 mM Tris–HCl, pH 6.6, 0.2 mM DTT, 0.25 mg/ml BSA, 2.5 mM cobalt chloride) for 10 min. Subsequently, the sections were allowed to react with rTDT reaction mixture (98 μl equilibration buffer, 1 μl biotinylated nucleotide mix and 1 μl rTDT enzyme) for 1 h at 37 °C in a humidified chamber. The slides were immersed in 2× SCC for 15 min to stop the reaction and the endogenous peroxidase were blocked by incubating the sections with 0.3% H_2O_2 in PBS for 5 min. After incubation with streptavidin–HRP solution for 30 min at room temperature, slides were stained with DAB-H_2O_2 in the dark. Positive nuclei stained dark-brown and were visualized under a light microscope. Negative controls with TUNEL assay were performed according to the instructions provided by the manufacturer.

2.7. Statistical analyses

The data were expressed as mean ± S.D. for four animals per group. Statistical analyses were performed by one-way analysis of variance (ANOVA) followed by Dunnett's post-test using SPSS (student version 7.5, SPSS Inc., Surrey, UK). We used the significant level alpha = 0.05.

3. Results

3.1. Effect of lindane on the protein levels of cytochrome c, caspase-9 and caspase-3 in the testis of adult rats

To assess the effect of lindane on mitochondria-dependent cell death pathway, the protein levels of cytochrome *c*, caspase-9 and caspase-3 were evaluated in the testis of adult rat. Western blot analysis revealed a significant elevation in the levels of cytosolic cytochrome *c* as early as 6 h following exposure to a single dose of lindane (Fig. 1). A parallel increase in the level of procapase-9 was also observed. Maximum change in the levels of cytochrome *c* and procaspse-9 were observed at 12 and 24 h post-treatment. The levels declined at 72 h, yet remained significantly higher than control. A time-dependent elevation in the levels of procaspase-3 accompanied by an increase in 17 kDa protein band (p17), which represents larger subunit of activated caspase-3, was observed following exposure to a single dose of lindane (Fig. 1). A faint band corresponding to the active fragment (p17) of caspase-3 was also detected at 0 h, which could be attributed to the spontaneous germ cell apoptosis that takes place in the testis under physiological conditions. The level of p17 was observed to be maximum at 72 h following lindane treatment.

3.2. Effect of lindane on the protein levels of Fas and FasL in the testis of adult rats

To assess the effect of lindane on Fas–FasL pathway, the levels of Fas and FasL were evaluated in the testis of adult rats. Administration of lindane resulted in significant increase in the levels of Fas and FasL in a time-dependent manner from 6 h onwards reaching a peak at 72 h (Fig. 2).

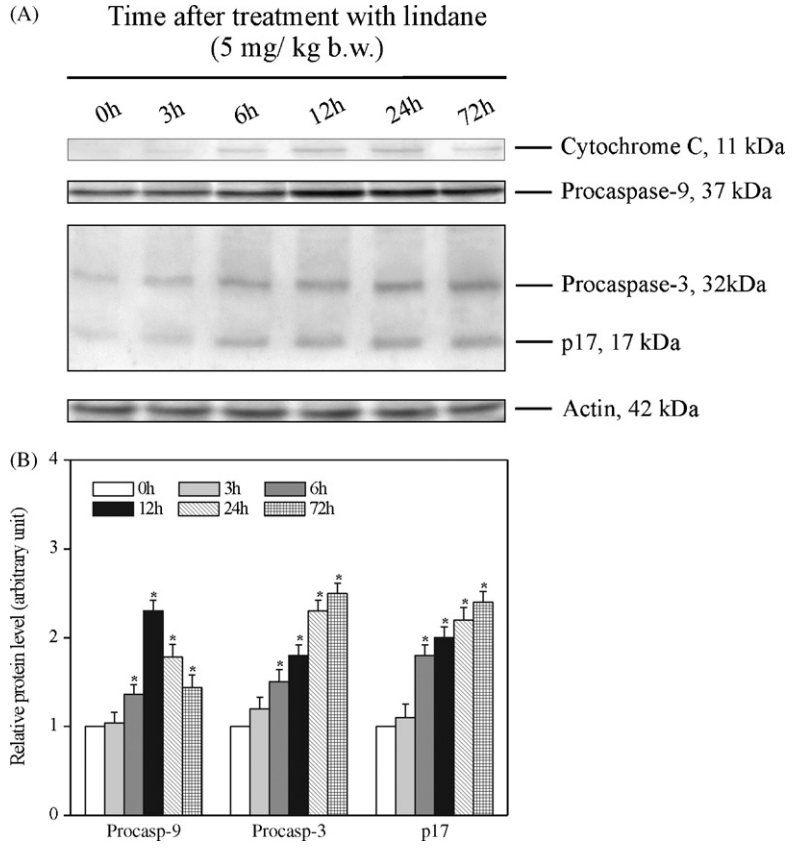

Fig. 1. Changes in the levels of cytochrome *c*, caspase-9 and -3 in the testis of lindane-treated rats over different time points. Lindane (5 mg/kg/day) was administered to adult male rats ($n = 4$) which were sacrificed at specific time points thereafter. (A) Immunoblots showing sequential increase in the cytosolic levels of cytochrome *c* from 6 h post-treatment. Immunoblot of procaspase-9 shows a parallel increase from 6 h onwards. Maximum changes in the levels of cytochrome *c* and procaspse-9 are observed at 12 and 24 h post-treatment. The levels of cytochrome *c* and procapase-9 declines at 72 h, yet remains significantly higher than control. A sequential increase in the levels both procaspase-3 and the cleaved fragment (p17) is observed from 6 to 72 h post-exposure. Actin blot shows equal protein loading. Each of these depicted blots is representative of four experiments. (B) Graph representing densitometrically scanned results corresponding to the blots depicted in A. Each data point represents the average value from four independent experiments normalized against the control (0 h). The control was arbitrarily set at 1. Asterisk indicates values significantly different (*alpha = 0.05) as compared with control.

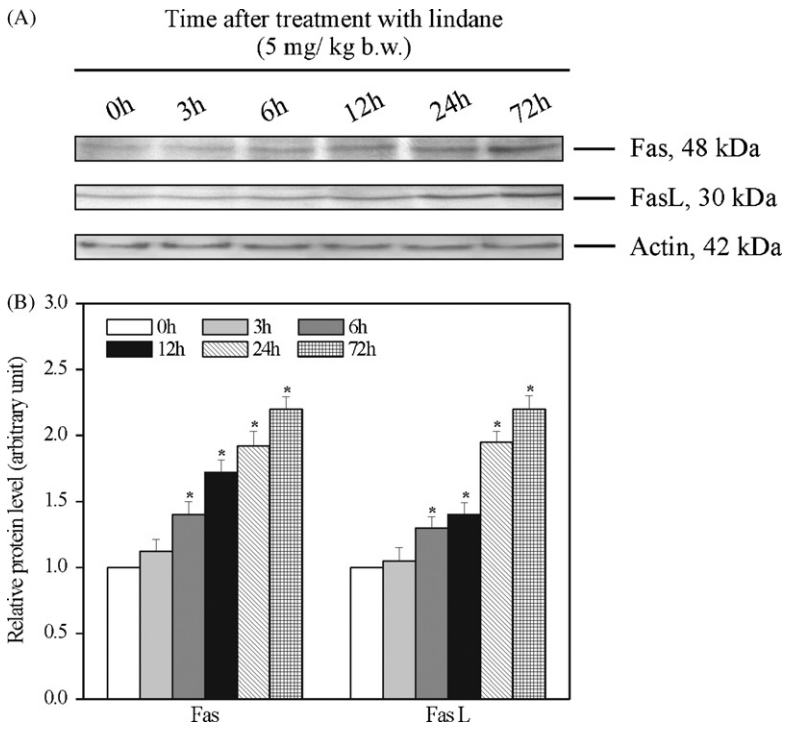

Fig. 2. Changes in the levels of Fas and FasL in the testis of lindane-treated rats over different time points. Lindane (5 mg/kg/day) was administered to adult male rats ($n = 4$) which were sacrificed at specific time points thereafter. (A) Immunoblot showing sequential increase in the levels of Fas and FasL from 6 to 72 h post-treatment. Actin blot shows equal protein loading. Each of these depicted blots is representative of four experiments. (B) Graph representing densitometrically scanned results corresponding to the blots depicted in A. Each data point represents the average value from four independent experiments normalized against the control (0 h). The control was arbitrarily set at 1. Asterisk indicates values significantly different (*alpha = 0.05) as compared with control.

3.3. Colocalized expression of caspase-3 and Fas in testis of lindane-treated rats

The localization of caspase-3 and Fas in the testis was examined by immunofluorescence microscopy following lindane treatment. A time-dependent increase in colocalized expression of caspase-3 and Fas was observed in the testes of lindane-treated rats (Fig. 3). Fas and caspase-3 were localized in peritubular germ cells (spermatogonia and spermatocytes) and their coexistence were visualized when green (caspase-3) and red fluorescence (Fas) were superimposed (yellow to orange in Fig. 3). The experiment when repeated by replacing the primary antibodies with rabbit and goat IgG, yielded no detectable staining (data not shown).

3.4. Immunolocalization of FasL in testis of lindane-treated rats

The localization of FasL in the testis was examined by immunofluorescent microscopy following lindane treatment. FasL was visualized in the cytoplasmic extensions of Sertoli cells and the germ cells in peritubular regions involving spermatogonia and spermatocytes (Fig. 4). Immunoreactivity of FasL increased in these cells in a time-dependent manner from 6 to 72 h following exposure to lindane (Fig. 4). Replacing the primary antibody with non-immune rabbit IgG, yielded no detectable staining (data not shown).

3.5. Effect of lindane on the protein levels of NF-κB p65 in the cytoplasmic and nuclear extracts of the testis

To delineate the role of NF-κB in testicular effects of lindane, the levels of NF-κB p65 were evaluated in the cytoplasmic and nuclear extracts of the testis. The cytoplasmic levels of NF-κB p65 decreased in a time-dependent manner, with a maximum decline at 12 and 24 h after treatment with lindane. However, the levels started recouping at 72 h post-exposure (Fig. 5). On the contrary, the levels of NF-κB p65 in the nuclear extracts of the testis increased in a time-dependent manner from 3 h onwards. The intensity of the bands was maximum at 12 and 24 h post-treatment (Fig. 5). By 72 h, the levels of NF-κB p65 in the nuclear extracts started declining, yet remained significantly higher than control.

3.6. Immunolocalization of NF-κB p65 in testis of lindane-treated rats

The localization of NF-κB p65 in the testis was examined by immunofluorescent microscopy following lindane treatment. Localization of NF-κB p65 changed from cytoplasm to nucleus of germ cells in lindane-treated rats. This change in the localization of NF-κB p65 was observed from 3 h with maximum nuclear staining at 12 h post-exposure (Fig. 5). However, at 72 h the nuclear localization of NF-κB p65 was less pronounced. Replacing the primary antibody with non-immune rabbit IgG, yielded no detectable staining (data not shown).

3.7. Lindane-induced germ cell death in the testis of rats

TUNEL analysis was performed to detect programmed cell death *in situ*. The number of apoptotic cells increased in a time-dependent manner following exposure to a single dose of lindane (Fig. 6). The TUNEL-positive cells were found in the peripheral region near the basement membrane of the seminiferous tubules.

4. Discussion

Substantial evidence has piled up portending the adverse effects of environmental toxicants on male reproduction (Aitken et al., 2004; Mathur et al., 2008). Most of these toxicants, including

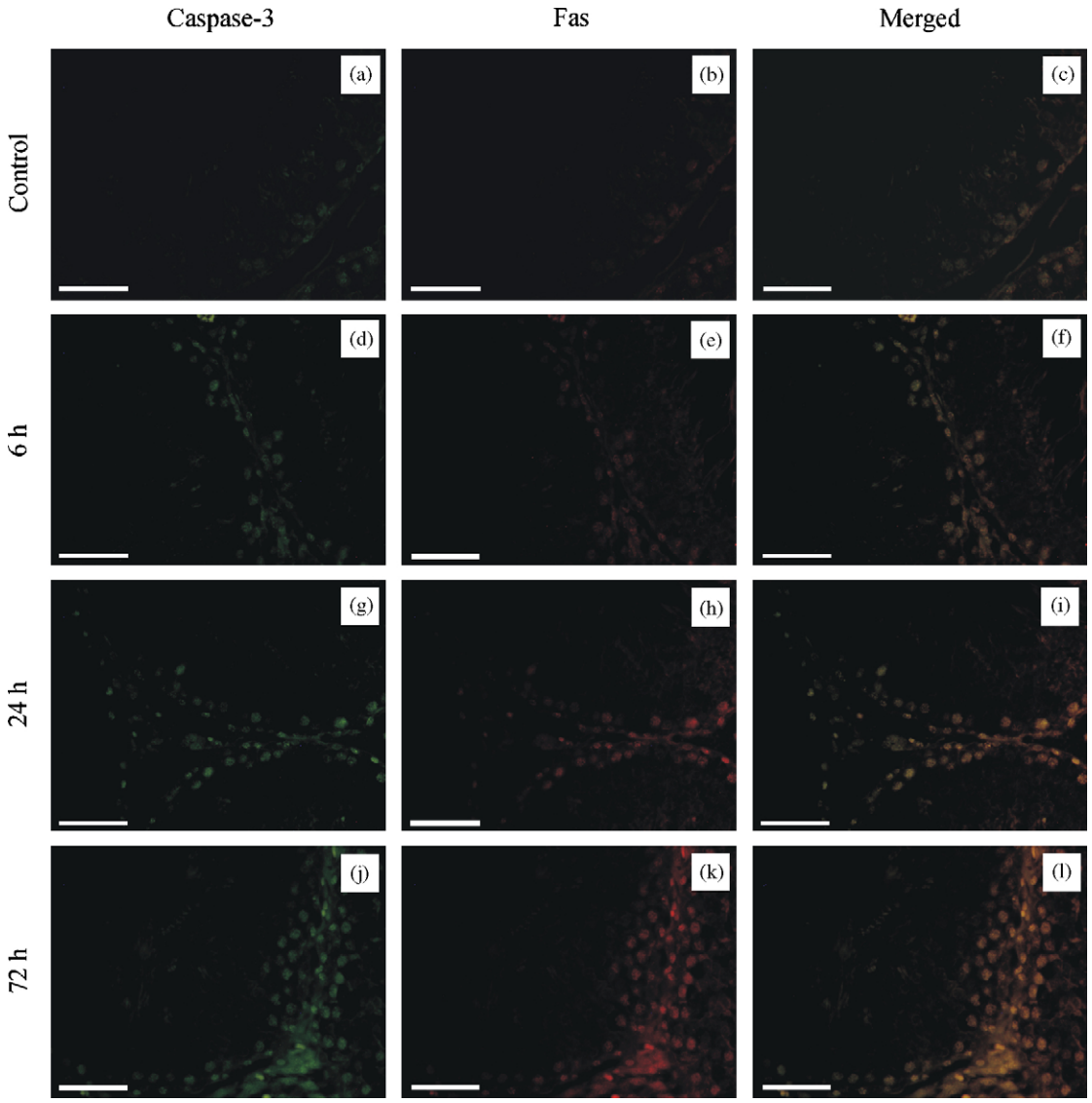

Fig. 3. Colocalized expression of caspase-3 and Fas in the testis of lindane-treated rats; (a, d, g, and j) visualization of caspase-3 staining; (b, e, h, and k) visualization of Fas staining; (c, f, i, and l) visualization of overlap of Fas and caspase-3 staining. Fas and caspase-3 is localized to peritubular germ cells (spermatogonia and spermatocytes) and their coexistence is visualized (c, f, i, and l) when green (caspase-3) and red fluorescence (Fas) are superimposed (yellow to orange). A time-dependent increase in the expression of caspase-3 and Fas in the testis of lindane-treated rats may be noted. The bar represents 50 μm (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article).

lindane, have been shown to compromise the sperm quality and impair fertility in rodent models (Chitra et al., 2003; Saradha and Mathur, 2006b). Exposure of adult rats to lindane (6 mg/kg body weight) for 5 days has been shown to bring about numeric reduction of spermatids and fragmentation of Sertoli cells thereby leading to impaired spermatogenesis (Dalsenter et al., 1996). Studies from our laboratory have demonstrated induction of oxidative stress and inhibition of steroidogenesis in adult rat testis within a few hours following exposure to a single dose of lindane (5 mg/kg body weight) (Saradha et al., 2008a,b). Thereafter, we were interested to investigate the possible role of apoptosis in mediating the gonadal effects of lindane. In the present study we sought to explore the sequential changes in the levels of cytochrome *c*, caspase-9 and caspase-3, Fas and FasL proteins in adult rat testis following exposure to a single dose of lindane. Furthermore, we were interested to uncover if there is any correlation between the expression of these proteins with changes in the levels of NF-κB, a transcription factor and a major regulator of stress responses. A dose of 5 mg/kg body weight, which is approximately three times the reported no observed adverse effect level (NOAEL) for reproductive toxicity of lindane (1.7 mg/kg body weight) in rats (FAO/WHO, 2002), was selected for the present study.

Most of the studies on lindane illustrate the undoubted role of ROS in executing its detrimental effects (Samanta and Chainy, 1997; Sujatha et al., 2001). Oxidative changes and ROS have been associated with apoptosis in many cell types including spermatogenic cells (Kasahara et al., 2002). Mitochondria have been described as the sensor of oxidative stress and thus, we have focused on the possible role of mitochondria-dependent pathway in the manifestation of the effects of lindane in gonads. Translocation of cytochrome *c* into cytosol is the primary event in mitochondrial pathway that leads to the formation of apoptosomes and activation of caspase cascade (Gao et al., 2003). In the present study, we observed a significant elevation in the levels of cytosolic cytochrome *c* as early as 6 h following exposure to a single dose of lindane. A parallel increase in the level of procapase-9 was also observed. As the time points (12

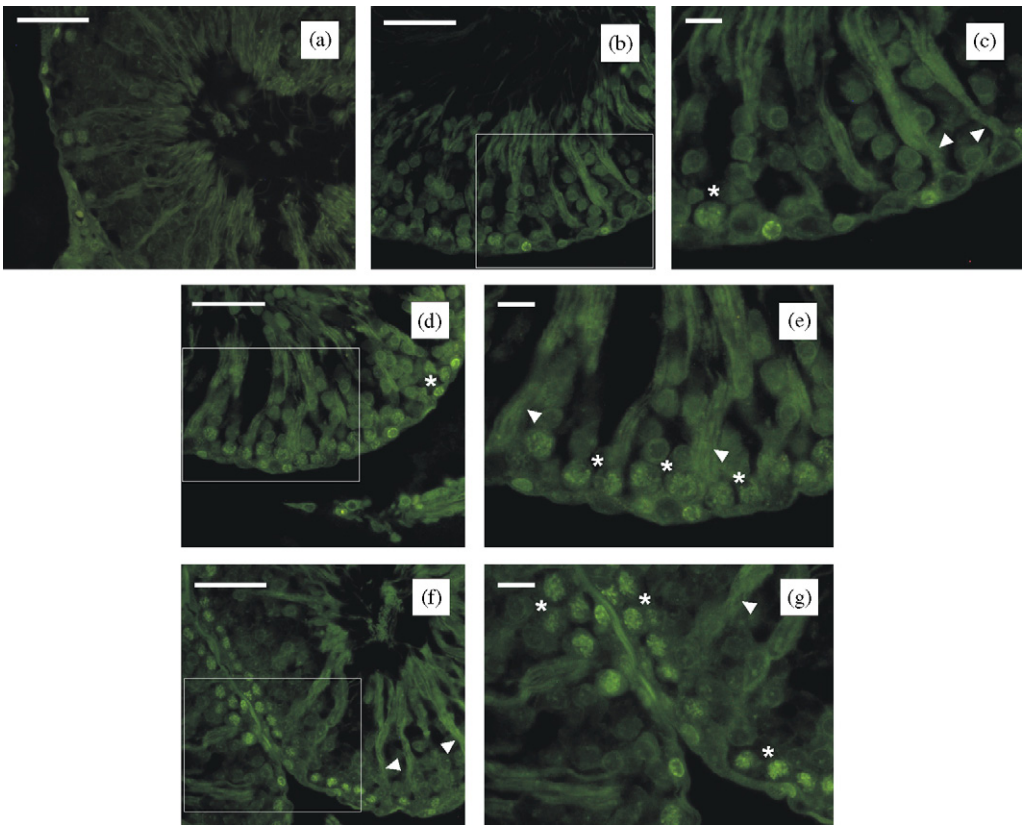

Fig. 4. Immunofluorescent staining of FasL in the testis of lindane-treated rats. Representative images for FasL immunoreactivity in control (a), 6 h (b), 12 h (d) and 72 h (f) post-treated groups. c, e, and g, magnified image of b, d, and f, respectively. FasL is localized to the cytoplasmic extensions of Sertoli cells (arrows heads) and peritubular germ cells (asterisks). A time-dependent increase in the expression of FasL in the testis of rats exposed to a single dose of lindane may be noted. The bar represents 50 μm.

and 24 h) displaying maximal changes in the levels of cytochrome *c* and caspase-9 coincide with the time points exhibiting transient increase in the levels of H_2O_2 (Saradha et al., 2008b), the possible role of ROS in mediating these changes cannot be ruled out. Elevated levels of ROS can cause oxidation of the mitochondrial pore thereby disrupting the mitochondrial membrane potential and releasing cytochrome *c* (Kasahara et al., 2002). Once free of the mitochondrial membrane, cytochrome *c* rapidly assembles a multi-protein complex involving Apaf-1 and propcaspase-9 leading to the activation of the caspase-9, which subsequently triggers the effector caspases-3 (Gao et al., 2003). Increased generation of ROS could not only be a cause but also the consequence of mitochondrial dysfunction (Orrenius et al., 2007). Lindane has also been shown to influence the mitochondrial function by uncoupling oxidative phosphorylation and inhibiting ATPase activity (Gopalaswamy and Aiyar, 1984). A marked inhibition of Na^+, K^+-ATPase has been demonstrated in the testicular plasma membrane of rats administrated with HCH (Srivastava et al., 1995). Inhibition of Na^+, K^+-ATPase in turn could lead to apoptosis by lowering the intracellular K^+ level which can affect the mitochondrial function by altering membrane potential (Nobel et al., 2000) and by increasing the intracellular Ca^{2+} level (Xiao et al., 2002; Srivastava and Shivanandappa, 2006). Furthermore, oxidative stress associated with increased levels of ROS could also lead to inhibition of Na^+, K^+-ATPase (Rodrigo et al., 2002).

In the present study, we observed a significant increase in the levels of pro-caspase-3 accompanied with an increase in its active form (p17) from 6 to 72 h post-exposure. Translocation of cytochrome *c* associated with activation of caspase-9 and -3 support the notion that lindane induces testicular apoptosis via mitochondrial pathway. However, at this point, the activation of caspase-3, could also be due to direct activation by Fas–FasL system via caspase-8 (Nair and Shaha, 2003).

Apoptotic degeneration of early spermatogenic cells is proposed to be a mechanism regulating the quantity and quality of sperm produced in mammalian testis (Blanco-Rodriguez and Martinez-Garcia, 1996; Sinha Hikim and Swerdloff, 1999). The Fas–FasL system has been recognized as a key regulator of germ cell apoptosis. Germ cells expressing Fas have been shown to transmit an apoptotic signal within the cells when bound to its cognate ligand, FasL, expressed in Sertoli cells (Lee et al., 1997). This form of cell death has been shown to be embellished following various testicular insults such as toxicant treatment, radiation and heat exposure (Lee et al., 1997, 1999; Vaithinathan et al., 2008). Recently, it has been demonstrated that FasL is expressed in spermatogenic cells (D'Alessio et al., 2001). Exposure to synthetic estrogen, diethylstilbestrol, has been shown to induce Fas–FasL upregulation and apoptosis in spermatogenic cells of adult male rats (Nair and Shaha, 2003). Administration of cadmium chloride, a heavy metal, has been demonstrated to enhance FasL immunoreactivity in Sertoli cells and peritubular germ cells, including spermatogonia and spermatocytes of adult rats (Ozawa et al., 2002). In the present study, we have observed a significant elevation in the levels of Fas within 6 h of exposure, with a further increase from 12 to 72 h, reflecting the incidence of germ cell apoptosis. A similar pattern of increment in the levels of FasL was observed in the testis of rat following a single exposure to lindane. Immunofluorescent staining of FasL was visualized in the cytoplasmic extensions of Sertoli cells and the germ cells in peritubular regions involving spermatogonia and spermatocytes. Immunoreactivity of FasL increased in these cells in a time-dependent manner from 6 to 72 h. Upregulation of FasL, following exposure to toxicants, has been considered as a novel

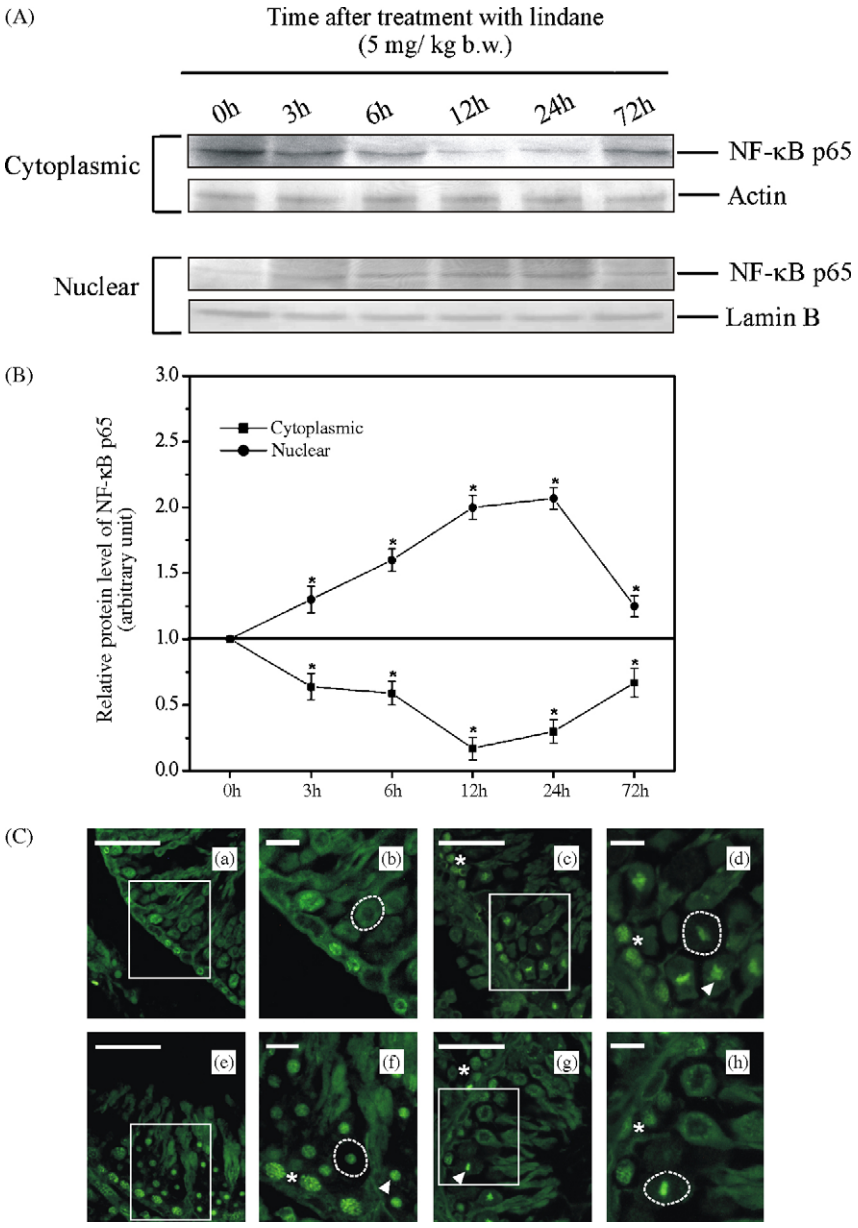

Fig. 5. (A) Changes in the levels of NF-κB p65 in the cytoplasmic and nuclear extracts of testis following lindane treatment. Immunoblot of NF-κB shows time-dependent decrease in the cytosolic levels of NF-κB p65 with a maximum decline at 12 h post-exposure. The levels of NF-κB p65 in the nuclear extracts of the testis show a time-dependent increase from 3 h onwards. By 72 h, the levels of NF-κB p65 in the nuclear extract declines, yet remains significantly higher than control. Actin and lamin blots shows equal protein loading of cytoplasmic and nuclear extracts, respectively. (B) Graph representing densitometrically scanned results corresponding to the blots depicted in A. Each data point represents the average value from four independent experiments normalized against the control (0 h). The control was arbitrarily set at 1. Asterisk indicates values significantly different (*alpha = 0.05) as compared with control. (C) Immunofluorescent staining of NF-κB p65 in the testis of lindane-treated rats. Representative images for NF-κB p65 immunoreactivity in control (a), 6 h (c), 12 h (e) and 72 h (g) post-treated groups. b, d, f and h, magnified image of a, c, e, and g, respectively. A time-dependent increase in nuclear localization of NF-κB p65 in spermatogonia (asterisks) and spermatocytes (arrow heads) with a maximal nuclear staining at 12 h post-exposure (e, f) may be noted. At 72 h, the nuclear staining is less pronounced (g, h). The bar represents 50 μm.

marker to identify a Sertoli cell toxicant (Lee et al., 1999). Recently, we have demonstrated colocalized expression of caspase-3 and Fas in the peritubular germ cells undergoing apoptosis following exposure to piperine, a major alkaloid present in black pepper (D'Cruz et al., accepted). In the present study, a time-dependent increase in colocalized expression of caspase-3 and Fas was observed in the peritubular germ cells of lindane-treated rats. These observations further confirm the involvement of Fas-mediated pathway in lindane-induced testicular apoptosis.

Recent studies have shown that ligation of Fas by FasL could stimulate NF-κB activation (Kreuz et al., 2004), while active NF-κB can in turn induce Fas transcription (Malewicz et al., 2003). The NF-κB transcriptional factors are composed of homodimers or heterodimers of Rel protein, of which, p65/p50 heterodimer is the predominant complex in testicular germ cells (Delfino and Walker, 1998). In unstimulated cells, NF-κB dimers are sequestered in the cytoplasm by inhibitory kappa B (IκB) protein. Upon exposure to various extracellular signals that leads to phosphorylation and degradation of IκB, free NF-κB dimers rapidly translocates to nucleus, wherein they activate transcription of target genes (Karin, 1999). A few studies have demonstrated either pro-apoptotic or antiapoptotic role of NF-κB in testicular response to toxicant injury

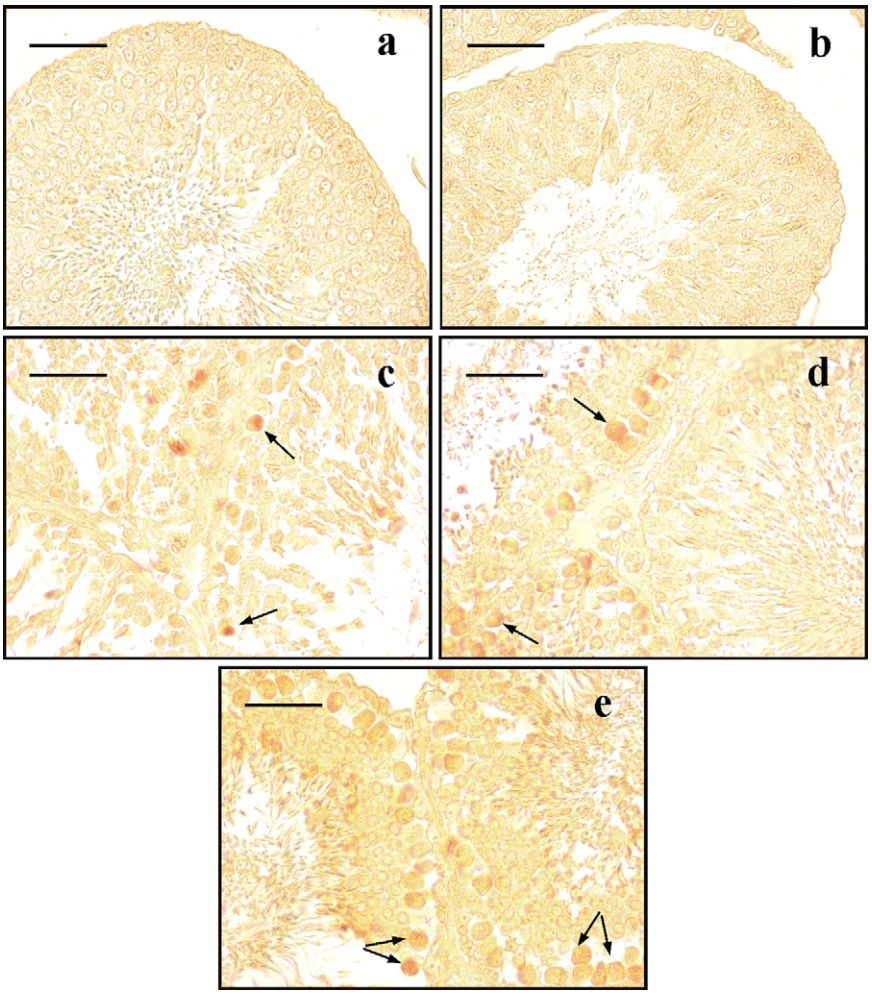

Fig. 6. Lindane-induced germ cell death in the testis of rats as revealed by TUNEL assay. Representative images for TUNEL staining in negative control (a), control (b), 6 h (c), 12 h (d) and 72 h (e) post-treatment groups. The TUNEL-positive cells (arrows) are found in the peripheral region near the basement membrane of the seminiferous tubules. A time-dependent increase in the number of apoptotic cells in the testis of lindane-treated rats may be noted. The bar represents 50 μm.

(Rasoulpour and Boekelheide, 2005; Kaur et al., 2006). In the present study, we observed a significant decline in the levels of p65 subunit of NF-κB in cytoplasmic extract of testis as early as 3 h following exposure to lindane with a maximal decrement at 12 and 24 h. On the contrary, the levels of NF-κB p65 in the nuclear extracts increased in a time-dependent manner from 3 h onwards reaching a peak at 12 and 24 h post-exposure. Immunofluorescence studies showed nuclear translocation of NF-κB p65 in germs cells (spermatogonia and spermatocytes) of lindane-treated rats from 3 h post-exposure with a maximal nuclear staining at 12 h. However, by 72 h the nuclear localization of NF-κB p65 became less pronounced. Early activation of NF-κB (3 h) in contrast to late increase in Fas expression (6 h) suggests a pro-apoptotic role of NF-κB in testicular response to lindane.

The two divergent pathways of apoptosis converge on the common down stream effector, caspase 3, which is known to activate endonuclease and induce DNA fragmentation. In the present study, TUNEL assay was performed to detect apoptotic cells in the testis of lindane-treated rats. In the testicular sections of control animals, only a very few tubules displayed TUNEL-positive cells, which could be due to spontaneous germ cells death that takes place in the testis as a normal homeostatic process. A time-dependent increase in the number of apoptotic germ cells were observed in the testicular section of rats following exposure to a single dose of lindane. The TUNEL-positive cells were found in the peripheral region near the basement membrane of the seminiferous tubules.

To sum up, the data illustrate induction of apoptosis in testis of adult rats involving both mitochondria-dependent and Fas–FasL pathways following exposure to a single dose of lindane. Early activation of NF-κB, in contrast to late increase in Fas expression, suggests a pro-apoptotic role of NF-κB in testicular response to lindane.

Conflict of interest

None.

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References

Aitken, R.J., Koopman, P., Lewis, S.E., 2004. Seeds of concern. Nature 432, 48–52.

Blanco-Rodriguez, J., Martinez-Garcia, C., 1996. Spontaneous germ cell death in the testis of the adult rat takes the form of apoptosis: re-evaluation of cell types that exhibit the ability to die during spermatogenesis. Cell Prolif. 29, 13–31.

Chadwick, R.W., Cooper, R.L., Chang, J., Rehnberg, G.L., McElroy, W.K., 1988. Possible antiestrogenic activity of lindane in female rats. J. Biochem. Toxicol. 3, 147–158.

Chitra, K.C., Latchoumycandane, C., Mathur, P.P., 2003. Induction of oxidative stress by bisphenol A in the epididymal sperm of rats. Toxicology 185, 119–127.

CPCSEA, 2003. CPCSEA guidelines for laboratory animal facility. Indian J. Pharmacol. 35, 257–274.

D'Alessio, A., Riccioli, A., Lauretti, P., Padula, F., Muciaccia, B., De Cesaris, P., Filippini, A., Nagata, S., Ziparo, E., 2001. Testicular FasL is expressed by sperm cells. Proc. Natl. Acad. Sci. U.S.A. 98, 3316–3321.

D'Cruz, S.C., Vaithinathan, S., Saradha, B., Mathur P.P., accepted. Piperine activates testicular apoptosis in adult rats. J. Biochem. Mol. Toxicol.

Dalsenter, P.R., Faqi, A.S., Webb, J., Merker, H.J., Chahoud, I., 1996. Reproductive toxicity and tissue concentrations of lindane in adult male rats. Hum. Exp. Toxicol. 15, 406–410.

Dalsenter, P.R., Faqi, A.S., Webb, J., Merker, H.J., Chahoud, I., 1997. Reproductive toxicity and toxicokinetics of lindane in the male offspring of rats exposed during lactation. Hum. Exp. Toxicol. 16, 146–153.

Delfino, F., Walker, W.H., 1998. Stage-specific nuclear expression of NF-kappaB in mammalian testis. Mol. Endocrinol. 12, 1696–1707.

Dikshith, T.S., Tandon, S.K., Datta, K.K., Gupta, P.K., Behari, J.R., 1978. Comparative response of male rats to parathion and lindane: histopathological and biochemical studies. Environ. Res. 17, 1–9.

El-Mubarak, A., Huisingh, D., 2001. Environmental estrogens: short-term exposure of low doses of lindane, dieldrin, dibutyl phthalate, and diethylhexyl phthalate increases uterine weight in young female mice. Anal. Sci. 17, i261–264.

FAO/WHO, 2002. Pesticide residues in food-2002. Joint Meeting of the FAO Panel of Experts on Pesticide Residues in Food and the Environment and the WHO Core Assessment Group, Rome.

Gao, H.B., Tong, M.H., Hu, Y.Q., You, H.Y., Guo, Q.S., Ge, R.S., Hardy, M.P., 2003. Mechanisms of glucocorticoid-induced Leydig cell apoptosis. Mol. Cell Endocrinol. 199, 153–163.

Gopalaswamy, U.V., Aiyar, A.S., 1984. Effects of lindane on liver mitochondrial function in the rat. Bull. Environ. Contam. Toxicol. 33, 106–113.

Han, Y., Brasier, A.R., 1997. Mechanism for biphasic RelA NF-κB1 nuclear translocation in tumor necrosis factor α-stimulated hepatocytes. J. Biol. Chem. 272, 9825–9832.

Irvine, D.S., 2000. Male reproductive health: cause for concern? Andrologia 32, 195–208.

Karin, M., 1999. How NF-kappaB is activated: the role of the IkappaB kinase (IKK) complex. Oncogene 18, 6867–6874.

Kasahara, E., Sato, E.F., Miyoshi, M., Konaka, R., Hiramoto, K., Sasaki, J., Tokuda, M., Nakano, Y., Inoue, M., 2002. Role of oxidative stress in germ cell apoptosis induced by di(2-ethylhexyl)phthalate. Biochem. J. 365, 849–856.

Kaur, P., Kaur, G., Bansal, M.P., 2006. Tertiary-butyl hydroperoxide induced oxidative stress and male reproductive activity in mice: role of transcription factor NF-kappaB and testicular antioxidant enzymes. Reprod. Toxicol. 22, 479–484.

Kreuz, S., Siegmund, D., Rumpf, J.J., Samel, D., Leverkus, M., Janssen, O., Hacker, G., Dittrich-Breiholz, O., Kracht, M., Scheurich, P., Wajant, H., 2004. NFkappaB activation by Fas is mediated through FADD, caspase-8, and RIP and is inhibited by FLIP. J. Cell. Biol. 166, 369–380.

Laemmli, U.K., 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227, 680–685.

Lee, J., Richburg, J.H., Younkin, S.C., Boekelheide, K., 1997. The Fas system is a key regulator of germ cell apoptosis in the testis. Endocrinology 138, 2081–2088.

Lee, J., Richburg, J.H., Shipp, E.B., Meistrich, M.L., Boekelheide, K., 1999. The Fas system, a regulator of testicular germ cell apoptosis, is differentially up-regulated in Sertoli cell versus germ cell injury of the testis. Endocrinology 140, 852–858.

Lowry, O.H., Rosebrough, N.J., Farr, A.L., Randall, R.J., 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193, 265–275.

Malewicz, M., Zeller, N., Yilmaz, Z.B., Weih, F., 2003. NF kappa B controls the balance between Fas and tumor necrosis factor cell death pathways during T cell receptor-induced apoptosis via the expression of its target gene A20. J. Biol. Chem. 278, 32825–32833.

Mathur, P.P., Saradha, B., Vaithinathan, S., 2008. Impact of environmental toxicants on testicular function. Immun. Endoc. Metab. Agents Med. Chem. 8, 79–90.

Nair, R., Shaha, C., 2003. Diethylstilbestrol induces rat spermatogenic cell apoptosis in vivo through increased expression of spermatogenic cell Fas/FasL system. J. Biol. Chem. 278, 6470–6481.

Nobel, C.S., Aronson, J.K., van den Dobbelsteen, D.J., Slater, A.F., 2000. Inhibition of Na+/K+-ATPase may be one mechanism contributing to potassium efflux and cell shrinkage in CD95-induced apoptosis. Apoptosis 5, 153–163.

Oropeza-Hernandez, L.F., Sierra-Santoyo, A., Cebrian, M.E., Manno, M., Albores, A., 2001. Ovariectomy modulates the response of some cytochrome P450 isozymes to lindane in the rat. Toxicol. Lett. 124, 91–99.

Orrenius, S., Gogvadze, V., Zhivotovsky, B., 2007. Mitochondrial oxidative stress: implications for cell death. Annu. Rev. Pharmacol. Toxicol. 47, 143–183.

Ozawa, N., Goda, N., Makino, N., Yamaguchi, T., Yoshimura, Y., Suematsu, M., 2002. Leydig cell-derived heme oxygenase-1 regulates apoptosis of premeiotic germ cells in response to stress. J. Clin. Invest. 109, 457–467.

Pages, N., Sauviat, M.P., Bouvet, S., Goudey-Perriere, F., 2002. Reproductive toxicity of lindane. J. Soc. Biol. 196, 325–338.

Prasad, A.K., Pant, N., Srivastava, S.C., Kumar, R., Srivastava, S.P., 1995. Effect of dermal application of hexachlorocyclohexane (HCH) on male reproductive system of rat. Hum. Exp. Toxicol. 14, 484–488.

Raizada, R.B., Misra, P., Saxena, P., Datta, K.K., Dikshith, T.S., 1980. Weak estrogenic activity of lindane in rats. J. Toxicol. Environ. Health 6, 483–492.

Rasoulpour, R.J., Boekelheide, K., 2005. NF-kappaB is activated in the rat testis following exposure to mono-(2-ethylhexyl) phthalate. Biol. Reprod. 72, 479–486.

Rodrigo, R., Trujillo, S., Bosco, C., Orellana, M., Thielemann, L., Araya, J., 2002. Changes in (Na+K)-adenosine triphosphatase activity and ultrastructure of lung and kidney associated with oxidative stress induced by acute ethanol intoxication. Chest 121, 589–596.

Ronco, A.M., Valdes, K., Marcus, D., Llanos, M., 2001. The mechanism for lindane-induced inhibition of steroidogenesis in cultured rat Leydig cells. Toxicology 159, 99–106.

Samanta, L., Chainy, G.B., 1997. Comparison of hexachlorocyclohexane-induced oxidative stress in the testis of immature and adult rats. Comp. Biochem. Physiol. C: Pharmacol. Toxicol. Endocrinol. 118, 319–327.

Saradha, B., Mathur, P.P., 2006a. Effect of environmental contaminants on male reproduction. Environ. Toxicol. Pharmacol. 21, 34–41.

Saradha, B., Mathur, P.P., 2006b. Induction of oxidative stress by lindane in epididymis of adult male rats. Environ. Toxicol. Pharmacol. 22, 90–96.

Saradha, B., Vaithinathan, S., Mathur, P.P., 2008a. Lindane alters the levels of HSP70 and clusterin in adult rat testis. Toxicology 243, 116–123.

Saradha, B., Vaithinathan, S., Mathur, P.P., 2008b. Single exposure to low dose of lindane causes transient decrease in testicular steroidogenesis in adult male Wistar rats. Toxicology 244, 190–197.

Sikka, S.C., Rajasekaran, M., Hellstrom, W.J., 1995. Role of oxidative stress and antioxidants in male infertility. J. Androl. 16, 464–468.

Sinha Hikim, A.P., Swerdloff, R.S., 1999. Hormonal and genetic control of germ cell apoptosis in the testis. Rev. Reprod. 4, 38–47.

Srivastava, S.C., Kumar, R., Prasad, A.K., Srivastava, S.P., 1995. Effect of hexachlorocyclohexane (HCH) on testicular plasma membrane of rat. Toxicol. Lett. 75, 153–157.

Srivastava, A., Shivanandappa, T., 2006. Causal relationship between hexachlorocyclohexane cytotoxicity, oxidative stress and Na+, K +-ATPase in Ehrlich Ascites tumor cells. Mol. Cell Biochem. 286, 87–93.

Sujatha, R., Chitra, K.C., Latchoumycandane, C., Mathur, P.P., 2001. Effect of lindane on testicular antioxidant system and steroidogenic enzymes in adult rats. Asian J. Androl. 3, 135–138.

Suwalsky, M., Villena, F., Marcus, D., Ronco, A.M., 2000. Plasma absorption and ultrastructural changes of rat testicular cells induced by lindane. Hum. Exp. Toxicol. 19, 529–533.

Vaithinathan, S., Saradha, B., D'Cruz, S.C., Mathur, P.P., 2008. Apoptosis in testis: the hostile role of environmental toxicants. In: Sharma, R.S., Rajanna, A., Rajalakshmi, M. (Eds.), Recent Advances and Challenges in Reproductive Health Research. Indian Council of Medical Research, New Delhi, pp. 379–391.

Wang, X., Sharma, R.K., Sikka, S.C., Thomas Jr., A.J., Falcone, T., Agarwal, A., 2003. Oxidative stress is associated with increased apoptosis leading to spermatozoa DNA damage in patients with male factor infertility. Fertil. Steril. 80, 531.

Xiao, A.Y., Wei, L., Xia, S., Rothman, S., Yu, S.P., 2002. Ionic mechanism of ouabain-induced concurrent apoptosis and necrosis in individual cultured cortical neurons. J. Neurosci. 22, 1350–1362.