Original Article

Histomorphometrical and Electron Microscopic Study of Adrenocorticocytes Following Surgically Induced Extrahepatic Biliary Obstruction in Adult Female Albino Rats

(adrenocorticocytes / bile duct / COX-II / electron microscope / P53 / suprarenal gland)

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Abstract. Cholestasis, which is a component of many liver diseases, is often associated with symptoms that resemble clinical adrenal insufficiency. This work aimed to study the histomorphometrical and electron microscopic structure of adrenocorticocytes after surgical induction of bile duct resection (BDR) in adult female albino rats. Sixty rats were randomly divided into control, BDR and sham-operated groups. Six weeks after surgery, the blood serum of the rats was examined biochemically, and the suprarenal cortexes were prepared for histological, morphometrical and statistical studies. The BDR group showed a highly significant increase in bilirubin and serum alkaline phosphatase levels, whereas aldosterone and cortisol levels were highly significantly decreased. The area percentages of positive immunoreactions for P53, cyclooxygenase II (COX-II) and inducible nitric oxide synthase (INOS) revealed highly significant increases in the BDR group. Electron microscopic examination of the BDR group showed marked cytoplasmic vacuolations, large lipid droplets, swollen mitochondria and many small dark nuclei in the adrenocorticocytes. The zona fasciculata had heterogeneously electron-dense mitochondria and dilated smooth endoplasmic reticulum. Some of the zona reticularis cells contained lipofuscin pigments. The surgical induction of BDR produced deleterious effects on the structure and function of the adrenocorticocytes. A long-term study using different animal species is recommended for further examination.

Introduction

Stricture or obstruction of the biliary system is most commonly presented by obstructive jaundice. Strictures of the biliary system could be due to ascending cholangitis, gallstones or neoplasms, whereas obstructions are caused by either extrinsic reasons due to compression by enlarged abdominal lymph nodes or intrinsic abnormality due to neoplasms within the bile duct system (Vadmal et al., 2000).

Cholangiocytes line the intrahepatic and extrahepatic biliary system of the liver and participate in several cellular processes, including modification of the bile of canalicular origin during the passage through the biliary system before it reaches the duodenum (Glaser et al., 2009; Mamta et al., 2013). Increased preoperative morbidity and mortality in patients with cholestasis has been recognized. Cholestasis is often associated with symptoms resembling the clinical features of adrenal insufficiency (Calamita et al., 2008). Cholestasis is typically associated with the accumulation of cytokines (Elkjaer et al., 2000) and endogenous opioids (Fricker et al., 1989; Ferri et al., 2003), which might contribute to the vulnerability of cholestatic patients to surgical stress (Carreras et al., 2007).

In a study done by Mc Neilly et al. (2010) the authors suggested an important and novel role for bile acids in regulating the pattern and consequences of glucocorticoid metabolism within the liver of female patients. The elevated levels of bile acids arising during cholestasis may contribute to down-regulation of the hypothalamic-pituitary-adrenal (HPA) axis and hence the apparent ad-

Received July 30, 2014. Accepted October 17, 2014.

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Abbreviations: ACTH – adrenocorticotropin hormone, BA – bile acid, BDR – bile duct resection, COX-II – cyclooxygenase II, CRH – corticotropin-releasing hormone, ELISA – enzyme-linked immunosorbent assay, INOS – inducible nitric oxide synthase, H&E - haematoxylin and eosin (stain), HPA – hypothalamic-pituitary-adrenal (axis), LPS – lipopolysaccharides, NO – nitric oxide, NOS – nitric oxide synthase, PBS – phosphate-buffered saline, PG – prostaglandin, ZF – zona fasciculata, ZG – zona glomerulosa, ZR – zona reticularis.

renal insufficiency associated with liver disease. Bile acids (BAs) enter the enterohepatic circulation as primary acids synthesized from cholesterol in hepatocytes. They are actively secreted across the canalicular membrane and carried in bile to the gallbladder, where they are concentrated during digestion. About 95 % of BAs are actively taken up from the lumen of terminal ileum, leaving only approximately 5 % (or approximately 0.5 g/d) in the colon, and a fraction of bile acids are passively reabsorbed after a series of modifications in the large intestine (Cai and Chen, 2014).

The HPA axis describes a complex set of positive- and negative-feedback loops between the hypothalamus, pituitary and adrenal glands during the body's response to stress (Jacobson, 2005; Quinn et al., 2012) that is mediated by circulation of the adrenocorticotropin hormone (ACTH) (Mitevska and Spiroski, 2012). The cells of the adrenal cortex (adrenocorticocytes) secrete more than 30 different hormones called corticosteroids; these hormones include mineralocorticoids (aldosterone) and glucocorticoids (corticosterone), which play roles in homeostasis and the stress response (Randall et al., 2002).

Nitric oxide (NO) is an important endogenous biological modulator that is produced by various cell types; NO functions as a modulator of vascular resistance, tissue perfusion, and cell proliferation. Several studies have demonstrated that NO could regulate secretion of aldosterone (Sainz et al., 2004), corticosterone (Cymeryng et al., 2002), and catecholamines (Kim et al., 2003); furthermore, NO could influence the blood flow in the adrenal cortical and medullary areas (Lai et al., 2005). Prostaglandins (PGs) and NO are important signal transducers that are involved in neurotransmitter and neurohormone secretion (Rettori et al., 2009). PGs are formed from arachidonic acid by the phospholipase A2 and cyclooxygenase (COX) enzymes, and they stimulate secretion of corticotropin-releasing hormone (CRH) and ACTH (Gadek-Michalska et al., 2005). PGs could also stimulate steroidogenesis and corticosterone release by acting directly on the adrenal gland (Mohn et al., 2005, 2011).

Taking these points into consideration and because there have been few reports relating extrahepatic cholestasis with the histological structure of the suprarenal gland cortex, this study was designed to examine the histomorphometrical distribution and localization of immunohistochemical stains and the electron microscopic structure of adrenocorticocytes after chronic surgical induction of extrahepatic biliary obstruction in adult female albino rats.

Material and Methods

Animals

Sixty adult female Wistar rats (N = 60, weight 240 ± 2.5 g, 6 months old) were obtained from the Animal House, Faculty of Veterinary Medicine, Zagazig University, Egypt. The rats were housed in stainless-steel cages with three animals per cage and maintained in a controlled environment for acclimatization for one week (room temperature 21 ± 2 °C, standard light/dark cycle of 12 h with lights turned on at 07:00 am) with standard food and water ad libitum. The handling and care of the animals were conducted in accordance with the guidelines for the Care and Use of Laboratory Animals (NIH-USA). The protocol of this study was approved by the ethics committee of the Faculty of Medicine, Zagazig University, Egypt.

Bile duct ligation and resection model

The bile duct ligation procedure was conducted as described previously (Scott-Conner and Grogan, 1994; Leke et al., 2012). The rats were anaesthetized with ketamine (90 mg/kg) and xylazine (12 mg/kg) by intraperitoneal injection and placed in the supine position on an operating table. A thermal controlled mattress (37 °C) was used to maintain a constant body temperature throughout the surgical procedure. A middle abdominal incision was performed after shaving the fur, and the hepatic ligament was exposed. The common bile duct was ligated with two 4-0 non-absorbent surgical sutures. The first suture was placed below the junction of the biliary-hepatic duct, and the second suture was placed above the entrance to the pancreatic duct. The common bile duct was resected between the two ligatures. The abdominal incision was closed with 4-0 sutures in two layers. The rats were then returned to their home cages. Surgical intervention for bile duct ligation and resection was done at the Animal House, Faculty of Medicine, Zagazig University.

Study design

The animals were randomly distributed into three groups (N = 20): control, bile duct-resected (BDR) and sham-operated rats. After the operation, the animals were housed in their cages and allowed free access to food and water for six weeks. The surgical procedure was performed using a strict sterile technique. The sham-operated rats, considered as the control group (Koko et al., 2004), underwent middle abdominal incision; the hepatic ligament was exposed, and the common bile duct was manipulated without ligation or resection.

At the end of the experiment, six weeks after the surgery, the animals were decapitated using a guillotine (Harward-Apparatus, Holliston, MA). At the time of sacrifice, blood samples were obtained from the tail veins for biochemical study. The right adrenal glands were quickly excised, weighed (Koko et al., 2004) and examined by light microscopy, whereas the left suprarenal glands were processed for examination by electron microscopy.

I. Biochemical study

For each animal, 3-ml blood samples were collected in plain tubes under completely aseptic conditions; the samples were allowed to sit for 30-60 min to allow spon-

taneous clotting at room temperature. The tubes were then centrifuged at 3000 rpm (LMC-3000 Laboratory centrifuge, Cat. No BS-010208-AAA, Biosan inc., Riva, Latvia) for 10 min. The serum samples were separated into another set of tubes and kept frozen at -80 °C.

As an indication of biliary obstruction, the serum was used for the determination of bilirubin and alkaline phosphatase levels by the colorimetric method using an available commercial kit (Boehringer, Mannheim, Germany) (Moss et al., 1987). The serum cortisol was measured by an immunoassay using automated equipment (IMMULITE 2000; Siemens Healthcare Diagnosis, Inc, Tarrytown, NY).

The serum aldosterone concentration was determined using an available commercial kit (Diagnostic Products Corp., Los Angeles, CA). The enzyme-linked immunosorbent assay (ELISA) method was used according to the manufacturer's protocol. Biochemical study was done at Biochemistry Department, Faculty of Medicine, Zagazig University.

II. Histological study

Specimens from the right suprarenal glands were fixed in 10% buffered formalin overnight at 4 °C. The tissue samples were then dehydrated in alcohol, cleared in xylol, and embedded in paraffin. The tissue sections (5 μm thickness) were stained with haematoxylin and eosin (H&E) stain (Drury and Wallington, 1980; Bancroft and Gamble, 2008).

III. Immunohistochemical study

The immunohistochemistry was done using the peroxidase-labelled streptavidin biotin technique. The sections were cut to a thickness of 3–5 μm. The endogenous peroxidase activity was inhibited using 0.3% H_2O_2 in methanol. The sections were blocked in 5% normal horse serum (Vectastain, Vector Laboratories, Burlingame, CA) and incubated overnight. Monoclonal anti-mouse antibody for p53 (M7001) was diluted at 1 : 50, and polyclonal rabbit anti-COX-II and anti-INOS antibodies were diluted at 1 : 300 and 1 : 250, respectively. The anti-P53 and anti-COX-II antibodies were obtained from Dako, Glostrup, Denmark, and the anti-INOS antibody was obtained from Lab Vision, Neo Markers, Fremont, CA. The samples were incubated with the primary antibodies for 1 h in a humidified chamber, washed in phosphate-buffered saline (PBS), incubated for 30 min with the secondary biotinylated antibody, and then incubated with the avidin peroxidase complex for another 30 min, according to the manufacturer's instructions (Universal Detection Kit, Dako). A brown colour was developed with 3,3-diaminobenzidine tetrahydrochloride (DAB), (Dako k0411 kit) for 5 min; the slides were then washed in distilled water and counterstained with Mayer's haematoxylin for 1 min. All steps of the procedure were performed at room temperature. A negative control for all of the markers, in which the primary antibody was replaced with PBS, was used (Ram et al., 2013). The brown colour observed in the nucleus was an indication of positive P53 staining, whereas the brown colour in the cytoplasm was used for evaluation of the presence or absence of INOS and COX-II (Hassan et al., 2013).

IV. Electron microscopic study

Small specimens of the left suprarenal glands (1 mm^3) were immediately fixed in 2.5% phosphate-buffered glutaraldehyde (pH 7.4). The specimens were then post-fixed in 1% osmium tetroxide in the same buffer at 4 °C, dehydrated, and embedded in epoxy resin. Semi-thin sections were stained with toluidine blue. Ultrathin sections were stained with uranyl acetate and lead citrate (Glauert and Lewis, 1998) and then examined and photographed using a JEOL JEM 1010 electron microscope (Jeol Ltd, Tokyo, Japan) in the Electron Microscope Research Laboratory of the Histology and Cell Biology Department, Faculty of Medicine, Zagazig University, Egypt.

V. Morphometrical study

The Leica Qwin 500 (Leica Ltd, Cambridge, UK) image analyser computer system at the Image Analysis Unit in the Histology Department, Faculty of Medicine, Zagazig University, Egypt, was used to evaluate the height of the three zones of the suprarenal cortex using the H&E slides and the area percentage of positive immune reactions for P53, COX-II and INOS using the immunostained slides. The measurements were performed using the interactive measurements menu. A measurement frame of a standard area equal to 118,476.6 um^2 was selected to allow the brown positive immune reactions to be visualized and masked by the blue binary colour. Five readings from five non-overlapping sections from each rat were examined. These measurements were obtained at a total magnification of × 200. For measuring the height of each zone, digital images of each adrenal section that contained full-height cross-section of the cortex were prepared. Image-editing software (Adobe Photoshop Elements 10.0) was then used to adjust this captured micrograph. A straight line was drawn from the subcapsular area of the adrenal gland to the edge of zona glomerulosa (ZG), then inward in the direction of cord-like zona fasciculata (ZF), followed by drawing another line representative of the height of zona reticularis (ZR) (Xiao-Gang et al., 2009).

VI. Statistical analysis

All statistical data were obtained and significance tests were performed using the Statistical Program for Social Science, version 19 (SPSS Inc. Chicago, Illinois, IL). Statistical significance was determined by a one-way analysis of variance for differences between the means of the different groups. Further analysis was performed using a post-hoc test to compare the parameters in the different groups. Probabilities of $P \leq 0.05$, $P \leq 0.01$ and $P > 0.05$ were considered statistically significant, highly significant and non-significant, respectively. All data were expressed as the mean ± SD.

Table 1. Mean ± SD of suprarenal gland weight (mg) and height (mm) of each zone for all groups at the end of the experiment

	Control	BDR	Sham	P value and significance
Suprarenal weight/mg	0.22 ± 0.02	0.18 ± 0.03	0.2 3± 0.02	*P = 0.000 **HS** **P = 0.000 **HS** #P = 0.023 **S**
ZG height/mm	1.7 ± 0.36	0.97 ± 0.20	1.6 ± 0.33	*P = 0.000 **HS** ** P= 0.000 **HS** #P = 0.590 **NS**
ZF height/mm	6.1 ± 0.7	5.3 ± 1.7	6.3 ± 0.7	*P = 0.005 **S** **P = 0.006 **S** #P = 0.866 **NS**
ZR height/mm	0.77 ± 0.02	0.66 ± 0.02	0.76 ± 0.05	*P = 0.000 **HS** **P = 0.000 **HS** #P = 0.326 **NS**

*P: BDR vs control; **P: BDR vs sham; #P: control vs sham; HS: highly significant; S: significant; NS: non-significant

Results

1. Survival rate of animals

One case of death was reported in the control group at the end of the experiment. Two rats in the BDR group died; one died during the operation, and the other rat died two days after the ligation. In the sham group, one rat died one week after middle abdominal incision.

2. Observations and measurements

The rats in the bile duct resection group showed clinical evidence of cholestasis, including jaundice and dark urine. The mean suprarenal gland weight (gm) of the BDR group was highly significantly decreased compared with those of the control and sham groups (P = 0.000); there were significant differences (P = 0.023) in weight between the control and sham groups (Table 1). In comparison of both the control and sham groups, the BDR group revealed a highly significant decrease in the mean height of ZG and ZR (P = 0.000), whereas there was only a significant decrease in the mean height of ZF (P = 0.005 and P = 0.006 for the control and sham groups, respectively). There was no significant difference (P = 0.590, P = 0.866 and P = 0.326) in the height of the entire zones between the control and sham groups (Table 1).

3. Biochemical results

The mean serum bilirubin (mg/dl) and serum alkaline phosphatase (IU/l) levels of the BDR group showed a highly significant increase (P = 0.000) compared with those of the control and sham groups; there were no significant differences in the serum bilirubin and alkaline phosphatase levels (P = 0.952 and P = 0.311, respectively) between the control and sham groups (Table 2).

The mean aldosterone (pg/ml) and cortisol (µg/dl) levels in the serum of the BDR group showed a highly significant decrease (P = 0.000) compared with those of the control and sham groups; there were no significant differences in the aldosterone and cortisol levels (P = 0.832 and P = 0.898, respectively) between the control and sham groups (Table 2).

4. Histological results

Examination of the H&E-stained sections of the control suprarenal cortex revealed arches of ZG and parallel cords of ZF separated by blood sinusoids. The cells of ZF were polyhedral and had vacuolated pale cytoplasm and rounded nuclei with prominent nucleoli. The cells

Table 2. Mean ± SD of serum bilirubin (mg/dl), alkaline phosphatase (IU/L), aldosterone (pg/ml) and cortisol (µg/dl) for all groups at the end of the experiment

	Control	BDR	Sham	P value and significance
Bilirubin (mg/dl)	0.21 ± 0.03	9.2 ± 0.84	0.22 ± 0.02	*P = 0.000 **HS** **P = 0.000 **HS** #P = 0.952 **NS**
Alkaline phosphatase (IU/l)	126.6 ± 4.45	412.03 ± 0.41	127.1 ± 5.4	*P = 0.000 **HS** **P = 0.000 **HS** #P = 0.311 **NS**
Aldosterone (pg/ml)	589.3 ± 7.2	561 ± 28.4	588 ± 8.8	*P = 0.000 **HS** **P = 0.000 **HS** #P = 0.832 **NS**
Cortisol (µg/dl)	2.64 ± 0.07	1.48 ± 0.06	2.69 ± 0.08	*P = 0.000 **HS** **P = 0.000 **HS** #P = 0.898 **NS**

*P: BDR vs control; **P: BDR vs sham; #P: control vs sham; HS: highly significant; NS: non-significant

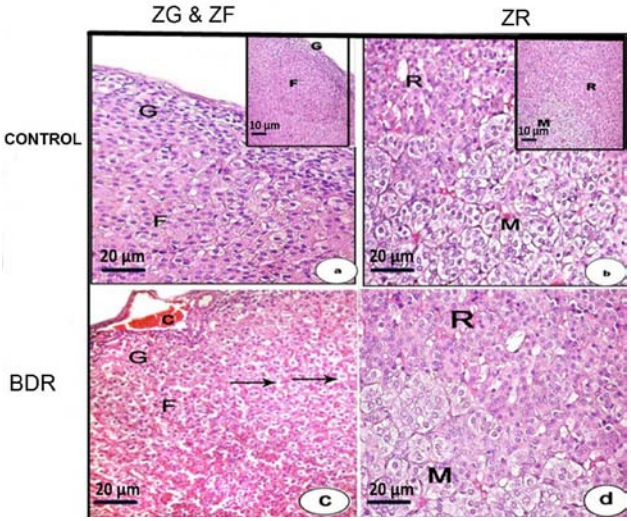


Fig. 1. Photomicrographs of sections from the suprarenal cortex of adult female rats. (**a, b**) The control group is formed of cells arranged into arches in zona glomerulosa (G) and parallel cords in zona fasciculata (F) separated by blood sinusoids. The cells of the ZF are polyhedral with vacuolated pale cytoplasm and rounded nuclei with prominent nucleoli. The cells of the zona reticularis (R) are arranged in anastomosing cords separated by blood sinusoids. The cells have few vacuoles in their cytoplasm. Note the pale medulla (M). (**c**, **d**) The BDR group has marked cytoplasmic vacuolations and many dark, small nuclei (arrows) in the cells of the ZG (G) and ZF (F). Congested blood vessels (C) are also present. The ZR cells (R) have marked focal cytoplasmic vacuolation. Notice the pale medulla (M).
(H&E; a–d ×400, inset ×100)

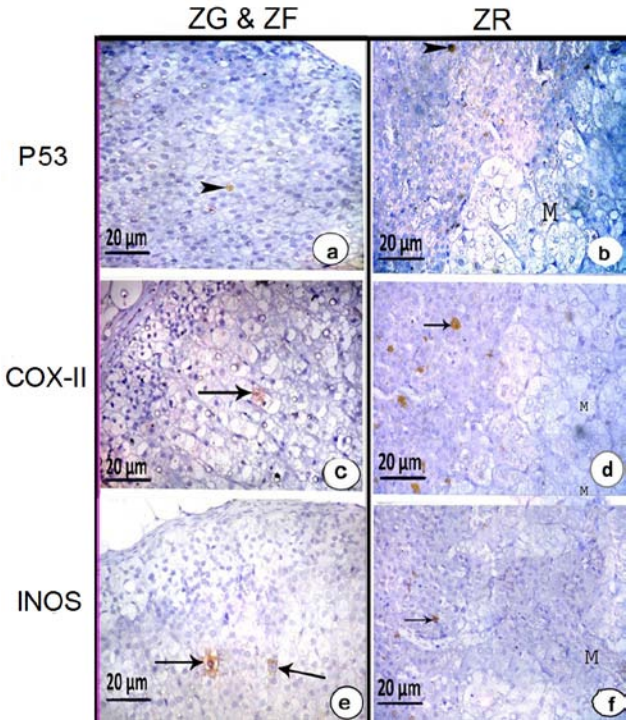


Fig. 2. Photomicrographs of immunostained sections from the suprarenal cortex of adult female rats from the control group. (**a**, **b**) Negative reaction for P53 in the nuclei of most adrenocorticocytes of all zones. Weak positive nuclear P53 immune reaction (arrowheads) in some cells of the ZF. (**c**, **d**) Weak positive reaction for COX-II (arrows) in the cytoplasm of some adrenocorticocytes in the ZF. (**e**, **f**) Weak positive reaction for INOS (arrows) in the cytoplasm of some adrenocorticocytes of the ZG, ZF and ZR. Note: cells of medulla (M) showed negative immune reaction for P53, COX-II and INOS in the control group.
(Immunoperoxidase reactions for P53, COX-II and INOS; a–f ×400)

of the ZR were arranged in anastomosing cords separated by blood sinusoids. The cells had few vacuoles in the cytoplasm. A pale medulla was observed (Fig. 1a, b). The BDR group had marked cytoplasmic vacuolations and many dark small nuclei in the cells of ZG and ZF; these regions also showed congested blood vessels. The cells of ZR had marked focal cytoplasmic vacuolation (Fig. 1c, d).

Examination of the immunostained sections from the suprarenal cortex of adult female rats from the control group showed negative reactions for P53 in the nuclei of most adrenocorticocytes of all the zones. A few cells that were positive for P53 could be observed in ZF (Fig. 2a, b). The cytoplasm of a few adrenocorticocytes of ZF revealed weakly positive COX-II immune reactions (Fig. 2c, d). Weak positive reactions for INOS were observed in the cytoplasm of some of the adrenocorticocytes of ZG, ZF and ZR. Cells of medulla showed negative immune reaction for P53, COX-II and INOS in the control group (Fig. 2e, f).

Examination of the immunostained sections from the suprarenal cortex of adult female rats from the BDR group showed strong positive reactions for P53 in the nuclei of most adrenocorticocytes of ZG, ZF and ZR (Fig. 3a, b). Strong positive immune reactions for COX-II were present in the cytoplasm of most adrenocorticocytes of ZG and ZF and in a few cells of ZR (Fig. 3c, d). Strong positive immune reactions for INOS were observed in the cytoplasm of most adrenocorticocytes of all zones. Cells of medulla showed negative immune reaction for P53, COX-II and INOS in the control group (Fig. 3e, f).

Ultrastructural examination of the adrenocorticocytes from adult female rats from the control group showed that the cells of ZG had regular euchromatic nuclei, numerous mitochondria and some lipid droplets (Fig. 4a). The cells of ZF had rounded euchromatic nuclei, many lipid droplets, and many ovoid mitochondria of variable sizes with closely packed vesicular and tubular cristae in a moderate electron-dense matrix (Fig. 4b, c). ZR had cells with euchromatic nuclei, mitochondria, and some lipid droplets. Blood capillaries were observed between the ZR cells (Fig. 4d).

Ultrastructural examination of the adrenocorticocytes from adult female rats from the BDR group showed columnar cells in ZG with some irregular and euchromatic

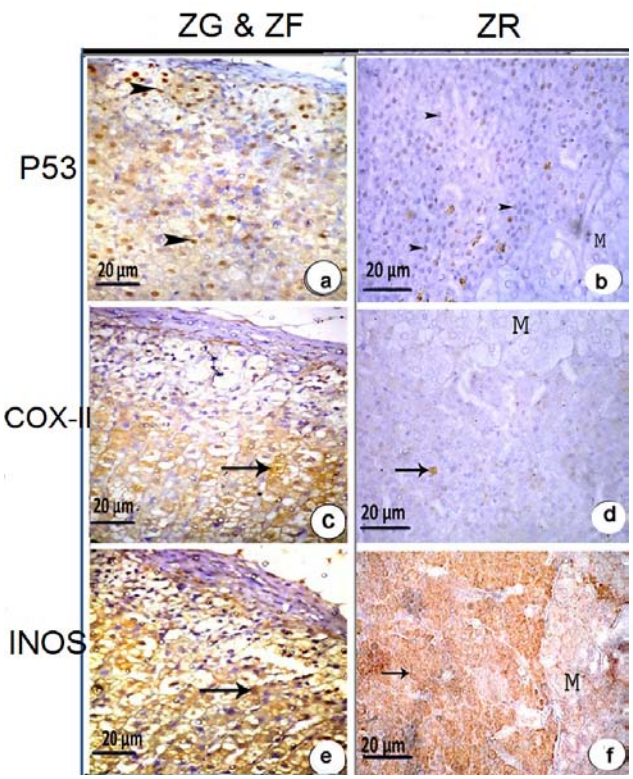


Fig. 3. Photomicrographs of immunostained sections from the suprarenal cortex of adult female rats from the BDR group. (**a**, **b**) Strong positive reaction for P53 in the nuclei of many adrenocorticocytes (arrowheads) of the ZG, ZF and ZR. (**c**, **d**) Strong positive immune reaction for COX-II in the cytoplasm of most adrenocorticocytes (arrows) of the ZG and ZF and a few positive cells in the ZR. (**e**, **f**) Strong positive immune reaction for INOS in the cytoplasm of most adrenocorticocytes (arrows) of the ZG, ZF and ZR. Note: cells of medulla (M) showed negative immune reaction for P53, COX-II and INOS.
(Immunoperoxidase reactions for P53, COX II and INOS; a–f ×400)

nuclei, whereas other nuclei were irregular, small electron-dense, and heterochromatic, marked increase in lipid droplets and the presence of destroyed mitochondria with heterogeneous electron density. A congested capillary was also observed (Fig. 5a, b). The cells of ZF had some nuclei that were regular and euchromatic, whereas other nuclei were small, and irregular heterochromatic. The cells had a marked increase in lipid droplets, compared with the control group, and some lipid droplets coalesced together. The mitochondria were either swollen with swollen cristae and had no outer mitochondrial membrane, or destroyed and had a heterogeneous electron-dense matrix, and dilated smooth endoplasmic reticulum (Fig. 5c, d, e, f). The cells of the ZR had nuclei that were either regularly euchromatic or irregularly heterochromatic, many lipid droplets, large cytoplasmic vacuoles, lipofuscin pigments and some mitochondria were swollen, whereas other mitochondria had intact tubular cristae (Fig. 5g, h, i).

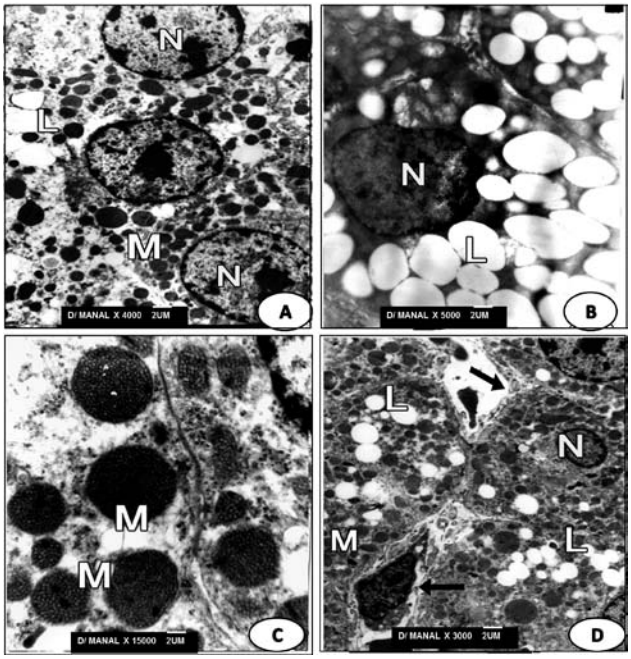


Fig. 4. Electron micrographs of adrenocorticocytes from adult female rats from the control group. (**a**) The cells of ZG have regular euchromatic nuclei (N), numerous mitochondria (M) and some lipid droplets (L). (**b**, **c**) The cells of ZF have rounded euchromatic nuclei (N), many lipid droplets (L), many ovoid mitochondria with variable size (M), and closely packed vesicular and tubular cristae in a moderate electron-dense matrix. (**d**) The cells of ZR have euchromatic nuclei (N), mitochondria (M), and some lipid droplets (L). Blood capillaries (arrows) can also be observed.
(TEM; a ×4000, b ×5000, c ×15000, d ×3000)

5. Morphometrical results

Immunohistochemical distribution of the P53-positive area percentage in the adrenocorticocytes of all zones revealed highly significant increase (P = 0.000) in the BDR group compared with the control and sham groups. There were no significant changes in the immunohistochemical distribution of P53 between the control and sham groups with regard to ZG, ZF, and ZR (P = 1.00, P = 0.09 and P = 0.90, respectively) (Fig. 6).

Immunohistochemical distribution of the COX-II-positive area percentage in the adrenocorticocytes of all zones revealed highly significant increase (P = 0.000) in the BDR group compared with the control and sham groups. There were no significant changes in the immunohistochemical distribution of COX-II between the control and sham groups with regard to ZG, ZF, and ZR (P = 0.10, P = 0.60 and P = 0.71, respectively) (Fig. 7).

Immunohistochemical distribution of the INOS-positive area percentage in the adrenocorticocytes of all zones revealed highly significant increase (P = 0.000) in the BDR group compared with the control and sham groups. There were no significant changes in the immunohistochemical distribution of INOS between the con-

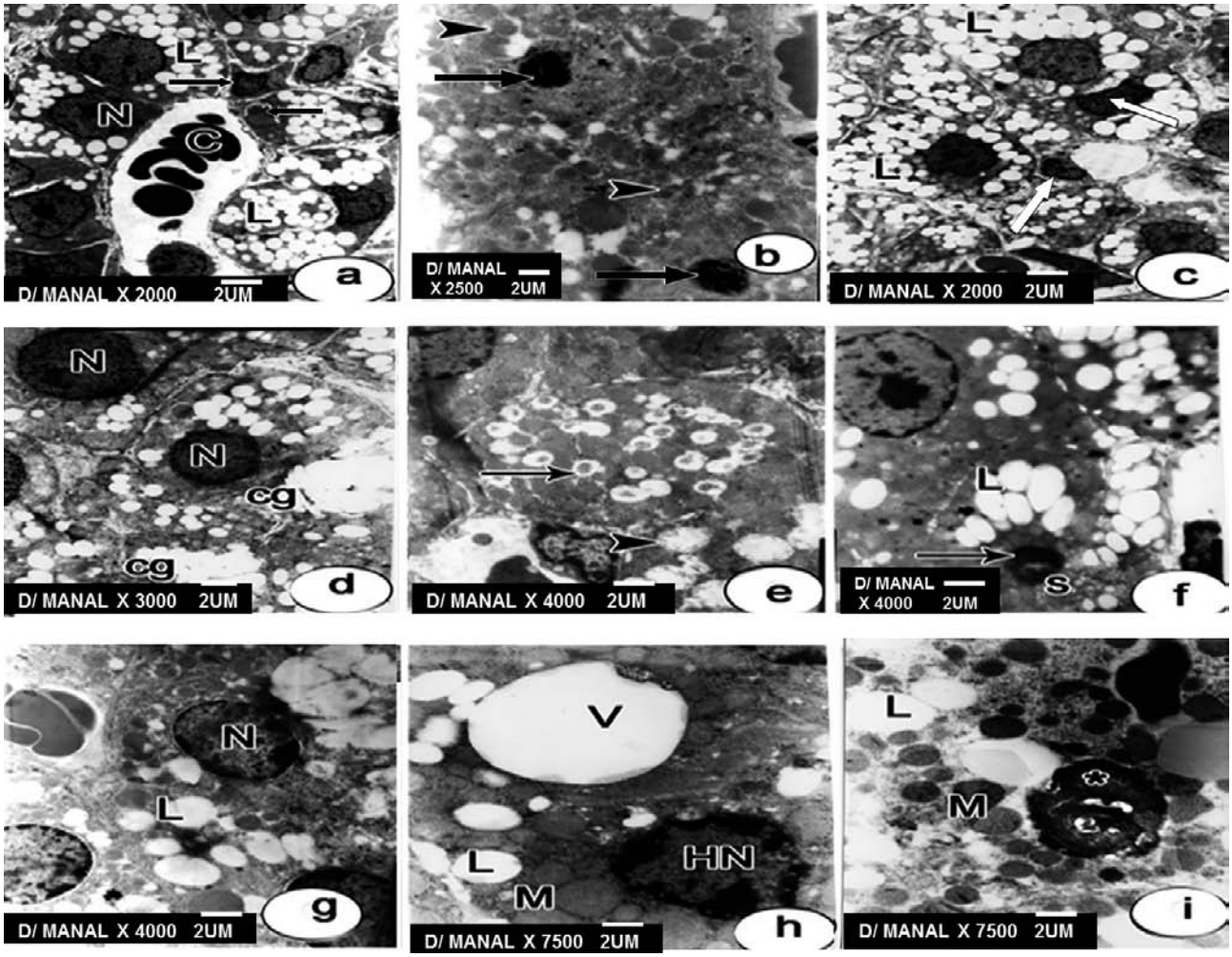


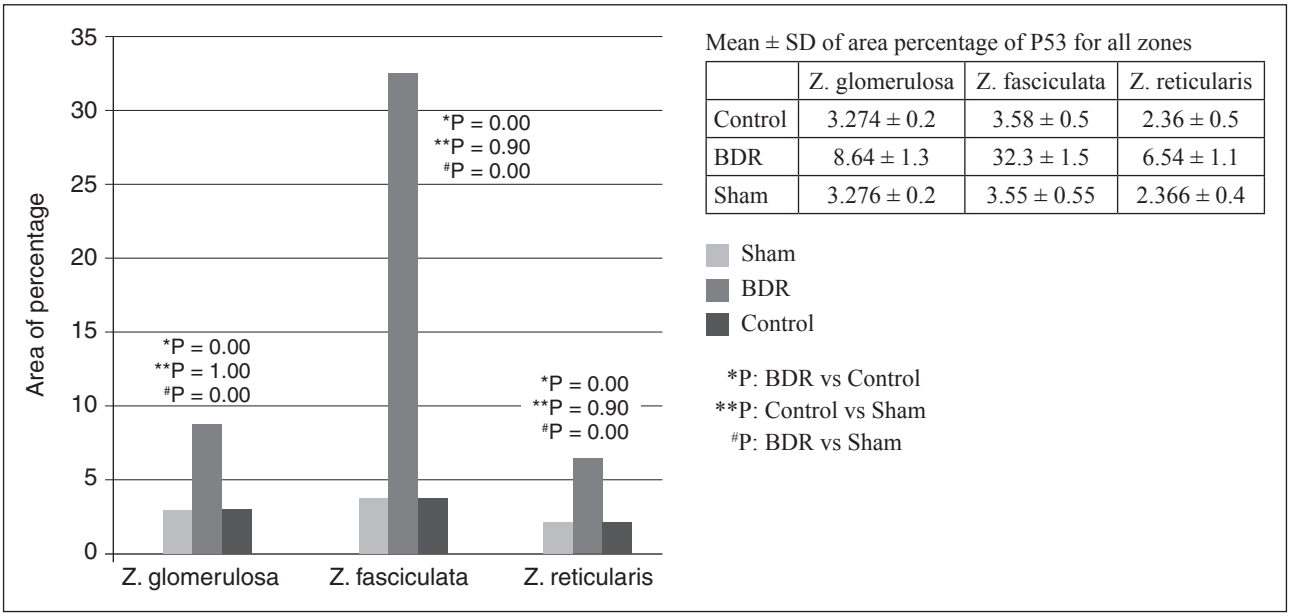
Fig. 5. Electron micrographs of adrenocorticocytes from adult female rats from the BDR group. (**a**, **b**): The cells of the ZG are columnar-shaped and have some nuclei that are irregular and euchromatic (N), whereas other nuclei are irregular with small electron-dense heterochromatic nuclei (arrows), a marked increase in lipid droplets (L), and destroyed mitochondria with heterogeneous electron density (arrowheads). Congested capillaries (C) can also be observed. (**c**, **d**, **e**, **f**) The cells of the ZF have some nuclei that are regular and euchromatic, whereas other nuclei are small, irregular and heterochromatic (arrows) with a marked increase in lipid droplets (L) compared with the control group. Some lipid droplets have coalesced (cg) together, and the mitochondria are either swollen with swollen cristae and have no outer mitochondrial membrane (arrowheads) or destroyed with a heterogeneous electron-dense matrix (arrows) and dilated smooth endoplasmic reticulum (s). (**g**, **h**, **i**) The ZR cells have nuclei that are either regularly euchromatic (N) or irregularly heterochromatic (HN), many lipid droplets (L), large cytoplasmic vacuoles (V), and lipofuscin pigments (*). Some mitochondria are swollen, whereas other mitochondria have intact tubular cristae (M).
(TEM; a ×2000, b ×2500, c ×2000, d ×3000, e, f-g ×4000, h–i ×7500)

trol and sham groups with regard to ZG, ZF, and ZR ($P = 0.31$, $P = 0.26$ and $P = 0.65$, respectively) (Fig. 8).

Discussion

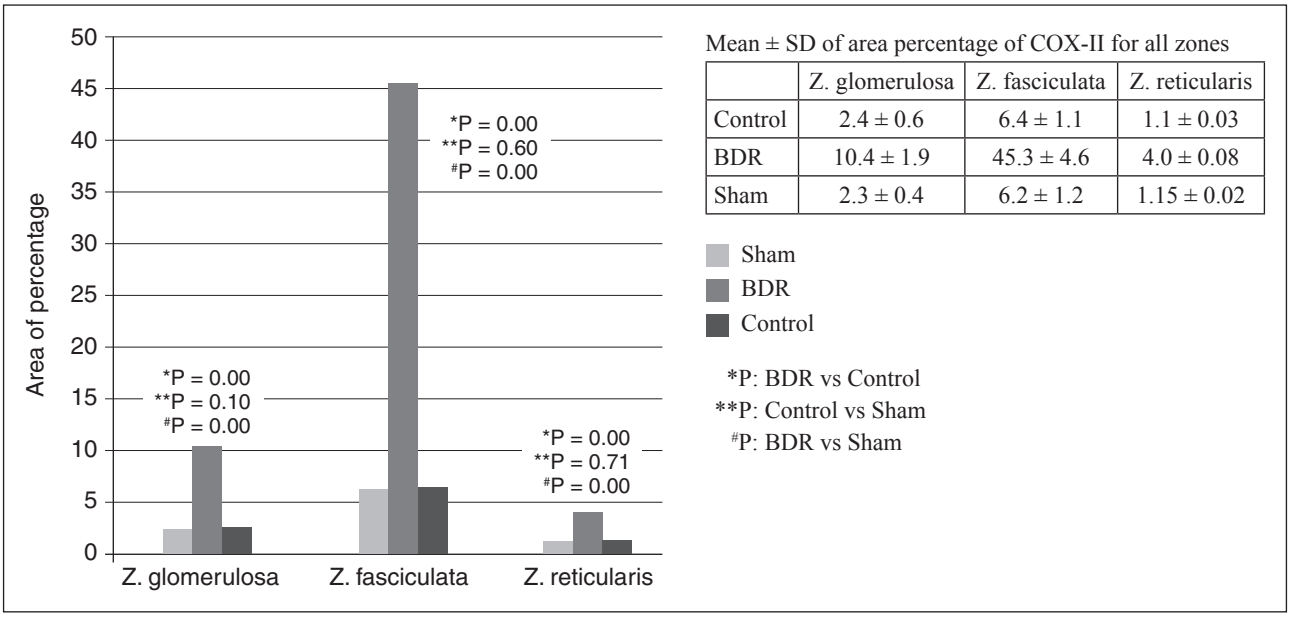
The adrenal gland is a complex, polyfunctional organ whose secretions are required for the maintenance of life. These glands are commonly susceptible to toxins and stress factors. The relationship between cholestasis and the HPA axis has long been unclear. Clinically, cholestatic patients often exhibit features suggestive of adrenal insufficiency, such as hypovolemia, hypotension, and renal failure (Harvey et al., 2007; Quinn et al., 2012). Furthermore, patients with glucocorticoid deficiency often exhibit symptoms of cholestatic hepatitis (Gonc et al., 2006).Therefore, in this study, the relationship between cholestasis and the suprarenal cortex was studied histologically, electron microscopically, morphometrically and biochemically.

Throughout this work, the BDR group showed a highly significant reduction in the mean cortisol and aldosterone levels accompanied by a highly significant increase in the mean serum bilirubin and alkaline phosphatase levels. These changes were associated with histological cortical degenerative manifestations in ZF and ZR in the cases of cholestasis. Similar changes were detected by El-Shenawany et al. (2007). Circulating glucocorticoid levels are reduced as an early event after



	Z. glomerulosa	Z. fasciculata	Z. reticularis
Control	3.274 ± 0.2	3.58 ± 0.5	2.36 ± 0.5
BDR	8.64 ± 1.3	32.3 ± 1.5	6.54 ± 1.1
Sham	3.276 ± 0.2	3.55 ± 0.55	2.366 ± 0.4

Fig. 6. Mean ± SD of area percentage of P53 in all zones for all groups



	Z. glomerulosa	Z. fasciculata	Z. reticularis
Control	2.4 ± 0.6	6.4 ± 1.1	1.1 ± 0.03
BDR	10.4 ± 1.9	45.3 ± 4.6	4.0 ± 0.08
Sham	2.3 ± 0.4	6.2 ± 1.2	1.15 ± 0.02

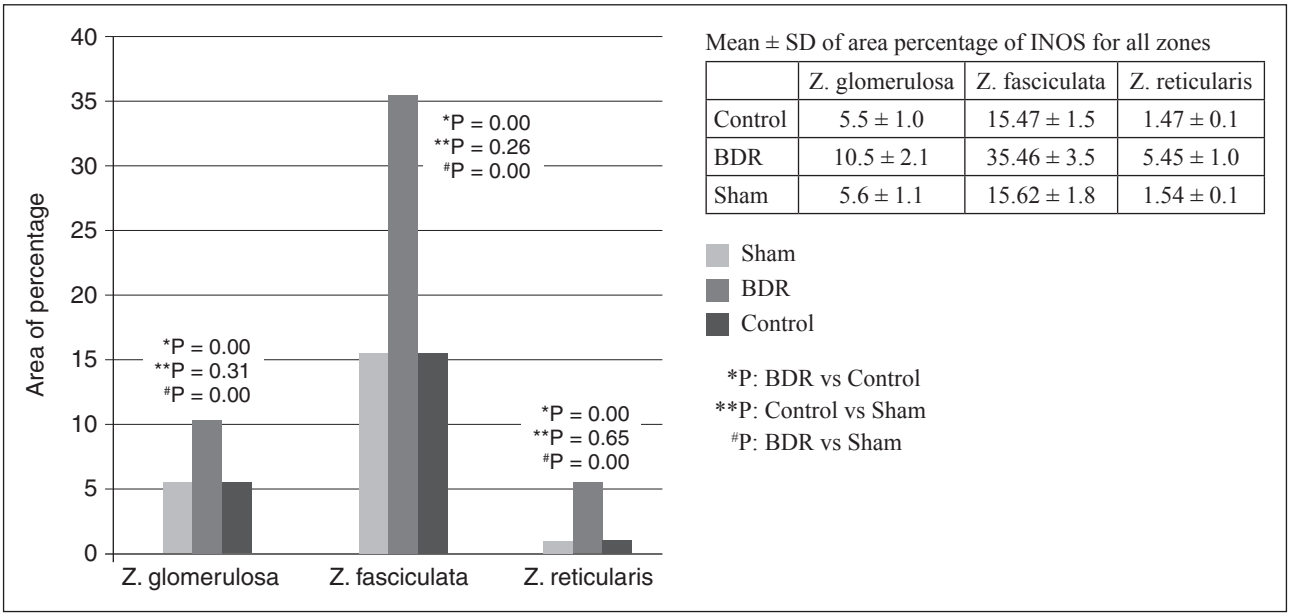
Fig. 7. Mean ± SD of area percentage of COX-II in all zones for all groups

bile duct ligation, and expression and secretion of the key regulatory molecules that drive HPA activity and regulate glucocorticoid levels are rapidly suppressed in experimental rodent models of cholestatic liver diseases (Quinn et al., 2012).

Regarding the highly significant reduction in the aldosterone level observed in our study compared to the opposite result obtained from previous studies, it could be duration, diurnal or species dependent. However, the damage seen in ZG denoted by electron microscope and the reduction in its height can support our result.

Quinn et al. (2012) hypothesized that bile acids might enter the brain and suppress the HPA axis directly. Additionally, other researchers have suggested that the blood-brain barrier becomes leaky in rodent models of cholestasis (Wright et al., 2007; Faropoulos et al., 2010), allowing access of aberrant signalling molecules to the brain. The increased amount of bile acids in the brain after BDR was considered to have a dampening effect on the HPA axis, regardless of whether these bile acids worked through direct activation of the glucocorticoid receptor via a negative-feedback loop to turn off the HPA axis or via direct transcriptional suppression of CRH (Miura et al., 2001; Quinn et al., 2012).

Morphometrically, the BDR group revealed highly significant reductions in the zonal height and weight of the suprarenal gland, which supports the biochemical results that were obtained. These degenerative cortical changes affect the integrity of the cell membranes, which are essential for steroidogenesis. Accordingly, the series



Mean ± SD of area percentage of INOS for all zones

	Z. glomerulosa	Z. fasciculata	Z. reticularis
Control	5.5 ± 1.0	15.47 ± 1.5	1.47 ± 0.1
BDR	10.5 ± 2.1	35.46 ± 3.5	5.45 ± 1.0
Sham	5.6 ± 1.1	15.62 ± 1.8	1.54 ± 0.1

Fig. 8. Mean ± SD of area percentage of INOS in all zones for all groups

of reactions necessary for steroid hormone synthesis could be affected (Rosol et al., 2001; Gayathri et al., 2004). Dakine et al. (2000) attributed the reduction in cortical height to the damage observed in the mitochondria and smooth endoplasmic reticulum that was caused by the decrease in circulating levels of CRF and ACTH.

The immunohistochemical distribution of COX-II revealed a highly significant increase in its expression in the BDR group. COX-II is an inducible enzyme (also called prostaglandin synthase) that is responsible for the conversion of arachidonic acid to prostaglandins and other inflammatory mediators. COX-II is not detectable in most normal tissues; however, it is induced by cytokines, growth factors and tumour promoters at sites of inflammation (Shirahama, 2000).

The immunohistochemical studies in this work also revealed a dramatic increase in the area positive for INOS immune expression in the cytoplasm of adrenocorticocytes of the rats in the BDR group. This result could be explained by the response to increased lipopolysaccharide formation after exposure to stress that has been observed in male rats (Mohn et al., 2011). Nitric oxide is formed from L-arginine through the action of nitric oxide synthase (NOS) (Kleinert et al., 2003). Nitric oxide, similarly to PGs, could modulate release of the stress hormones such as CRH, vasopressin, ACTH, and corticosterone (Bugajski et al., 2004; Rettori et al., 2009). The NOS activity is increased during stress and infection (Gadek-Michalska et al., 2005), and NO could regulate secretion of aldosterone and corticosterone (Sainz et al., 2004).

In this study, the significant increase in INOS and COX-II immunoexpression in the BDR group confirms the direct action of bile on the adrenal gland. Some authors have confirmed the presence of these receptors in human and mouse adrenal glands and their activation by lipopolysaccharides (LPS). These receptors have also been shown to be able to interact directly with the steroid biosynthetic pathway to induce cytokine production (Sanchez-Lemus et al., 2008).

The current study revealed a highly significant increase in the positive area for the immunoexpression of P53, in addition to the ultrastructural appearance of polymorphic and hyperchromatic nuclei in the adrenocorticocytes of the BDR group. Some authors consider p53 to act as the "guardian of the genome" because it limits tissue damage by allowing time for repair or by eliminating the damaged cells through apoptosis (Everds et al., 2013). P53 either eliminates damaged cells or proceeds to induce necrosis that is commonly associated with the functional failure of tissues. In the BDR group, some cells with apoptotic morphology were observed by electron microscopic examination, and the endpoint of the damage appears to be cell death (Canman et al., 1994; Hidenori et al., 2006). Apoptosis has been described to occur in the rat adrenal cortex when the tissue is deprived of its trophic hormone, ACTH. Additionally, in the rat, apoptosis observed after ACTH withdrawal reaches a maximal level several days after surgery (McCullough et al., 2000; Elferink 2003).

Ultrastructurally, the changes observed in the mitochondria in the form of swelling and disruption of the cristae could be explained by accumulation of lipid granules caused by impaired steroidogenesis. It has been reported that the enzymes 11-hydroxylase (CYP11) in mitochondria and 17- and 21-hydroxylases (CYP 17 and 21) in the smooth endoplasmic reticulum are commonly affected by toxic chemicals such as bile acids (Rosol et al., 2001).

The mitochondrial hypertrophy observed in some sections of the BDR group might be attributed to accumulation of molecules involved in the steroid synthesis. Accordingly, inhibition of the conversion of cholesterol to pregnenolone might lead to accumulation of choles-

terol within the mitochondria. This conversion occurs by side chain cleavage of cholesterol to yield pregnenolone (Quinn et al., 2012).

The BDR group showed large heterogenic lipid droplets, which could be explained by denaturation of lipids in the cells. Lipid droplets in adrenocorticocytes usually exhibit a relatively uniform appearance. The staining of lipid droplets depends on the degree of free fatty acid saturation, which is a reflection of the impairment of steroidogenesis. Enlargement of these droplets is caused by accumulation of cholesterol needed for steroidogenesis (Cole et al, 2000; Gadek-Michalska and Bugajski, 2004).

The lipofuscin pigments and lysosomes observed in sections of the BDR group in this study might be explained by inhibition of ACTH release (Bairagi et al., 2008). In addition, lipofuscin is known to be the end product of lipid peroxidation. Lipofuscin has been considered a "wear and tear" pigment that is not easily degraded and is classified as a sign of free radical injury. Free radicals serve to induce autophagocytosis of the excess lipid droplets and oxidize the contents of these droplets, forming more lipofuscin lysosomal structures (Kumar et al., 2005).

Conclusion

Surgically induced extrahepatic biliary obstruction was characterized by a significant elevation of serum bilirubin and alkaline phosphatase levels in addition to a significant reduction of aldosterone and cortisol levels. It also caused deleterious effects on the structure and function of adrenocorticocytes in the form of highly significant decrease in the weight of the gland and height of all zones. Moreover, the percentage of positive areas for P53, COX–II and INOS were significantly elevated. Additional signs were heterochromatic nuclei, changes in the size and shape of mitochondria and lipid droplets, and appearance of lipofuscin pigments in adrenocorticocytes.

Recommendation

A long-term study using a different animal model should be performed to validate the findings of the present study.

Conflicts of interest

The authors declare that they have no conflicts of interest related to this study.

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