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Experimental Biomedical Research *Original article*

Adrenal cortex regeneration in ethane dimethanesulfonate administered rat model

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ABSTRACT

Aim: To investigate the parallel morphological and biochemical regeneration of Leydig cells in the adrenal gland after ethane dimethanesulfonate (EDS) administration.

Materials and methods: We divided 72 male rats into two groups: the control and EDS groups. Rats in the EDS group received a single dose of EDS (75 mg/kg) intraperitoneally and were sacrificed on days 7, 21, 35, and 63 respectively. Adrenal gland tissue samples obtained from the sacrificed rats were analyzed under light microscopic, immunohistochemical, biochemical, and quantitative methods.

Results: In contrast to Caspase-3 expression, 3βHSD and Ki67 expression and thickness of the zona reticularis (ZR) layer and testosterone levels significantly decreased on the 7th, 21st, and 35th days after single-dose EDS. However, 3βHSD and Ki67 expression, testosterone levels, and the ratio of ZR thickness of the EDS group were seen similar to the control group on the 63rd day following EDS administration, but experienced decreasing Caspase-3 expression. After EDS administration, we observed a significant regeneration in the ZR layer of the adrenal gland on the 63rd day.

Conclusion: EDS-injected animal models can be used to investigate the development of Leydig cells and assess the turnover of the adrenal cortex (adrenal insufficiency) per time.

Key words: Ethane dimethanesulfonate, EDS, caspase-3, Ki67, 3βHSD, adrenal gland.

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Introduction

Ethane Dimethane Sulfonate (EDS), is an alkylating sulfonic acid ester that specifically destroys adult Leydig cells, disrupts spermatogenesis by shunting androgen support and causes transient infertility [1-5)]. After an intraperitoneal injection of 75 mg/kg of EDS, all adult Leydig cells were destroyed within 4–7 days and within 7–10 weeks; new adult Leydig cells began to proliferate from Leydig stem cells. EDS inhibits the production of testosterone, resulting in a decrease in the weight of the testes, seminal vesicles, and prostate glands [6-10]. Studies have shown that Leydig cell apoptosis occurs as a result of chromatin condensation and nuclear fragmentation with the increase in glutathione levels in the cell within the first 24 h following EDS administration. The Bcl-2 family and caspases are the main mediators of the apoptotic process. However, the Bcl-2 family is not involved in Leydig cell apoptosis after EDS administration. Leydig cell apoptosis induced by EDS is mediated by caspase-3 activation [11,12].

The adrenal cortex and gonads originate from a common structure(the adreno-genital

primordium) that arises from the intermediate mesoderm [13]. The adrenal cortex produces cortisol, corticosterone, aldosterone, and adrenal androgens (precursors of sex hormones in adrenal tissues in the testis). The major androgens secreted by the zona reticularis layer of the adrenal cortex are dehydroepiandrosterone (DHEA) and its sulfate and androstenedione, whereas approximately 5% of the circulating testosterone is directly synthesized by the adrenal cortex of males [14,15]. In the literature, there is a conflict of hypotheses on the compensation between the adrenal gland and testis. Some articles depicted that there is no compensatory effect of the adrenal gland on testosterone production following castration [16] whereas some demonstrated this compensatory effect [17]. An EDS dose has a strong deleterious effect on steroidogenic cells of the adult male rat adrenal cortex similar to Leydig cells from the same origin. The decrease in adrenal cortex weight and atrophy, especially in the zona fasciculata and ZR layers, is one of the toxic effects of EDS on the adrenal gland [18]. Moreover, 11β HSD (a corticosteroid hormone activator) expression decreased on the 7th day after EDS administration, followed by progressive increase in immunoreactivity by days 14 and 21 [19]. However, there is no evidence of long-term regeneration of the adrenal cortex, as in Leydig cells.

EDS is used to the eliminate and re-differentiate of Leydig cells without affecting other testicular cell types in animal models. Although literature strongly describes the effect of EDS on Leydig cells, only one study reveals the short-term effect of EDS on adrenal glands through its structure and function. Thus, the systemic endocrine effects of EDS on the adrenal gland should be explored in experimental studies where an EDS model could be created. In this study, parallel with the regeneration of adult Leydig cells, we aimed at demonstrating the long-term effects of EDS on adrenocortical cell renewal via Ki67, 3βHSD, and Caspase-3 immunohistochemical expression and morphometric analysis for the first time in the literature. Therefore, we assessed the relationship between two steroidogenic organs: the testis and adrenal glands.

Materials and metods

Animals: We obtained rats from the Medical Sciences and Experimental Research and Application Center of Cukurova University and housed in well-ventilated polypropylene cages with food and tap water *ad libitum*. They were kept under controlled laboratory conditions of a normal light/dark cycle and normal temperature (25 °C ± 2 °C), and were allowed to acclimatize for one week.

Ethical statement: The research protocol was approved by the Scientific Ethics Committee of Cukurova University (Dated 16.02.2017), and all experimental procedures were carried out according to the Universal Declaration of International Animal Rights.

Chemicals: EDS was synthesized by the Chemistry Department of the Faculty of Sciences in Cukurova University as specified by Jackson and Jackson [20]. Moreover, EDS was adminsitered at the dose specified from another study. First, 75 mg of EDS was dissolved in 0.5 mL of dimethylsulfoxide (DMSO) (Sigma Aldrich, St. Louis, MO, USA), where 1.5 mL of distilled water was added to prepare 2 mL of EDS solution that will be injected intraperitoneally into the rats (2 mL per kg).

Injection procedure: Rats in the control group were given a single dose i.p. injection of normal saline. We preferred normal saline over EDS vehicle dimethyl sulfoxide in the control group because in a previous study, no differences were

found between the vehicle-treated and untreated rats [21]. They received a single dose (75 mg/kg body weight) i.p. injection of EDS [20]

Surgical procedure: Seventy-two adult Sprague-Dawley type, 12–13-week-old male rats were divided into two groups. In addition, we formed four subgroups within each group in accordance with the adult Leydig cell development stages [8].

Group 1: Control group (n = 32).

Group 1A: Sacrificed on the 7th day (n = 8)

Group 1B: Sacrificed on the 21st day (n = 8)

Group 1C: Sacrificed on the 35th day (n = 8)

Group 1D: Sacrificed on the 63rd day (n = 8)

Group 2: EDS group (n = 40).

Group 2A: Sacrificed on the 7th day after EDS application (n = 8)

Group 2B: Sacrificed on the 21st day after EDS application (n = 8)

Group 2C: Sacrificed on the 35th day after EDS application (n = 8)

Group 2D: Sacrificed on the 63rd day after EDS application (n = 8)

No animal was lost to follow-up due to side effects of EDS.

Light microscope methods: To explore samples under light microscopic examinations, one adrenal gland from each sacrificed animal were fixed in Bouin's solution for 8 hours and dehydrated for 6 hours using 50% ethyl alcohol and then 70% ethyl alcohol. Each animal was fixed with Bouin's solution, dehydrated and embedded in paraffin, tissue blocks were sectioned at 5 μm thickness, and stained with haematoxylin and eosin (H&E). Tissue sections were examined under a light microscope (Olympus BX53, Tokyo, Japan) [22].

Quantitative measurement of the adrenal cortex: Micrographs containing a full-thickness cross section of the adrenal cortex were prepared at 20 x magnification. First, a straight line was drawn from the subcapsular area of the adrenal cortex to the edge of the medulla. Then, another parallel line was drwan through the thickness of the Zona reticularis (ZR). Finally, the ratio of ZR thickness was calculated according to the total thickness of the adrenal cortex. Five values were measured for each section of each rat, and the average value was determined for the analysis [23].

Immunohistochemical methods: For 3βHSD, caspase-3, and Ki67 immunoreactivity analysis, adrenal gland specimens obtained from rats sacrificed on days 7, 21, 35, and 63 were fixed in 4% paraformaldehyde for 24 h at room temperature. After dewaxing and rehydrating the specimens in graded alcohol, 4 μm thick paraffin sections were treated with a heat-induced epitope retrieval solution and put into microwave irradiation for 10 min. Then, the sections were cooled off, washed in PBS, and incubated with 3% H_2O_2 for 15 min at room temperature in order to inhibit endogenous peroxidase. After washing three times in PBS, the sections were blocked with blocking agent (IHC Kit, ab93705, Abcam, MA, USA) for 15 min. The primary antibodies including anti-HSD3B1 (1:2000; anti-rat rabbit monoclonal antibody, ab150384, Abcam, MA, USA), anti-Caspase-3 (1:500; anti-rat rabbit polyclonal antibody, ab4051, Abcam, MA, USA), and anti-Ki67 (1:200; anti-rabbit polyclonal antibody, ab16667, Abcam, MA, USA) were added before the samples were incubated overnight at 4°C. After washing them on the next day, a secondary antibody (IHC Kit, ab93705, Abcam, MA, USA) was added, followed by staining with 3-amino-9-ethylcarbazole (AEC, IHC Kit, ab93705, Abcam, MA, USA) for 10 min and haematoxylin reagent for 3 min before dehydration. Images of the stained sections were viewed under a light microscope (Olympus BX53, Olympus, Tokyo, Japan). The immunostainings were scored as 0–3 for weak to strong staining. The number of cells

in each field and those stained at each intensity were counted. Staining intensity was evaluated with a grading score of 0, 1, 2, or 3, corresponding to a negative, weak, moderate, or strong staining intensity, respectively. The average percentage positive was calculated and the following formula was applied: IHC H-score = (% of cells stained at intensity category 1x1) + (% of cells stained at intensity category 2x2) + (% of cells stained at intensity category 3x3). An H-score < 2 was rated as low (weak staining), whereas an H-score ≥ 2 was rated as high (strong staining) [24].

Serum assays: Intracardiac blood samples were centrifuged at 3600 rpm for 5 min to obtain serum. The resulting serum was stored in Eppendorf tubes at −20°C. Serum testosterone levels were measured using testosterone (CSB-E05100r, Cusabio) Elisa kits in the Elisa Reader (Epoch) at the Biochemistry Department of Cukurova University Medicine Faculty.

Statistical analysis: The GraphPad Prism 5 (GraphPad Software Inc. USA) program was used for quantitative analysis, immunohistochemical scoring, and analyses of biochemical data. Using the D'Agostino & Pearson omnibus normality test, the normality of data distribution was checked. One-Way analysis of variance (ANOVA) and the Tukey test were used to compare the differences in immunoreactivity scores and biochemical data between experimental and control groups. Moreover, *p* values less than 0.05 were considered significant, and *p*:0.33 were characterized non-significant.

Results

Light Microscope Results: The adrenal cortex and medulla were normal in structure when samples from the control group were exqmined under the light microscope (Figures 1A, 1B, 1C, and 1D). In the adrenal gland tissue sections of the EDS group, most cells exhibited nuclear pycnotic changes, especially in the ZR layer of the adrenal cortex in rats sacrificed on the 7th, 21st and 35th days after EDS (Figures 1E, 1F, and 1G). However, the ZR layer of the adrenal cortex in rats sacrificed on the 63rd day after adminstering EDS were similar in appearance to those from the control group ($p = 0.07$) (Fig. 1H). Moreover, the ratio of the ZR thickness in the adrenal cortex significantly decreased in rats sacrificed on the 7^{th,} 21st, and 35th days after administering EDS ($p < 0.0001$, $p < 0.0001$, $p < 0.001$ respectively) (Fig. 1I). When the EDS group was evaluated according to sacrificed days, the ratio of ZR thickness significantly increased daily (Figure 1J). The values of ZR thickness are summarized in table 1. We equally monitored the testicular expression of 3βHSD in rats sacrificed 7 days after EDS to confirm if adult Leydig cells were eliminated as a result of single dose EDS administration, and none was observed. The absence of any expression in the interstitium was interpreted as the effective elimination of Leydig cells by EDS (Figure 2).

Immunohistochemical results: The proliferation marker (Ki67 expression) was high in all layers of adrenal glands in the control group (Figs. 3A, 3B, 3C, and 3D). However, the Ki67 expression significantly decreased in rats sacrificed on days 7, 21, and 35 after EDS administration (Figs. 3E and 3F). Moreover, in those sacrificed on day 63 following EDS administrations, we observed a significant increase in Ki67 immunoreactivity, especially in the ZR layer when compared to that observed in those sacrificed on days 7, 21 and 35 after EDS (Figs. 3G and 3H). Ki67 immunoreactivity scores were significantly lower in adrenal gland tissue sections of rats sacrificed on the 7th, 21st and 35th days after EDS administration ($p < 0.0001$ respectively), when compared to those of the control group. However, when the EDS group was evaluated

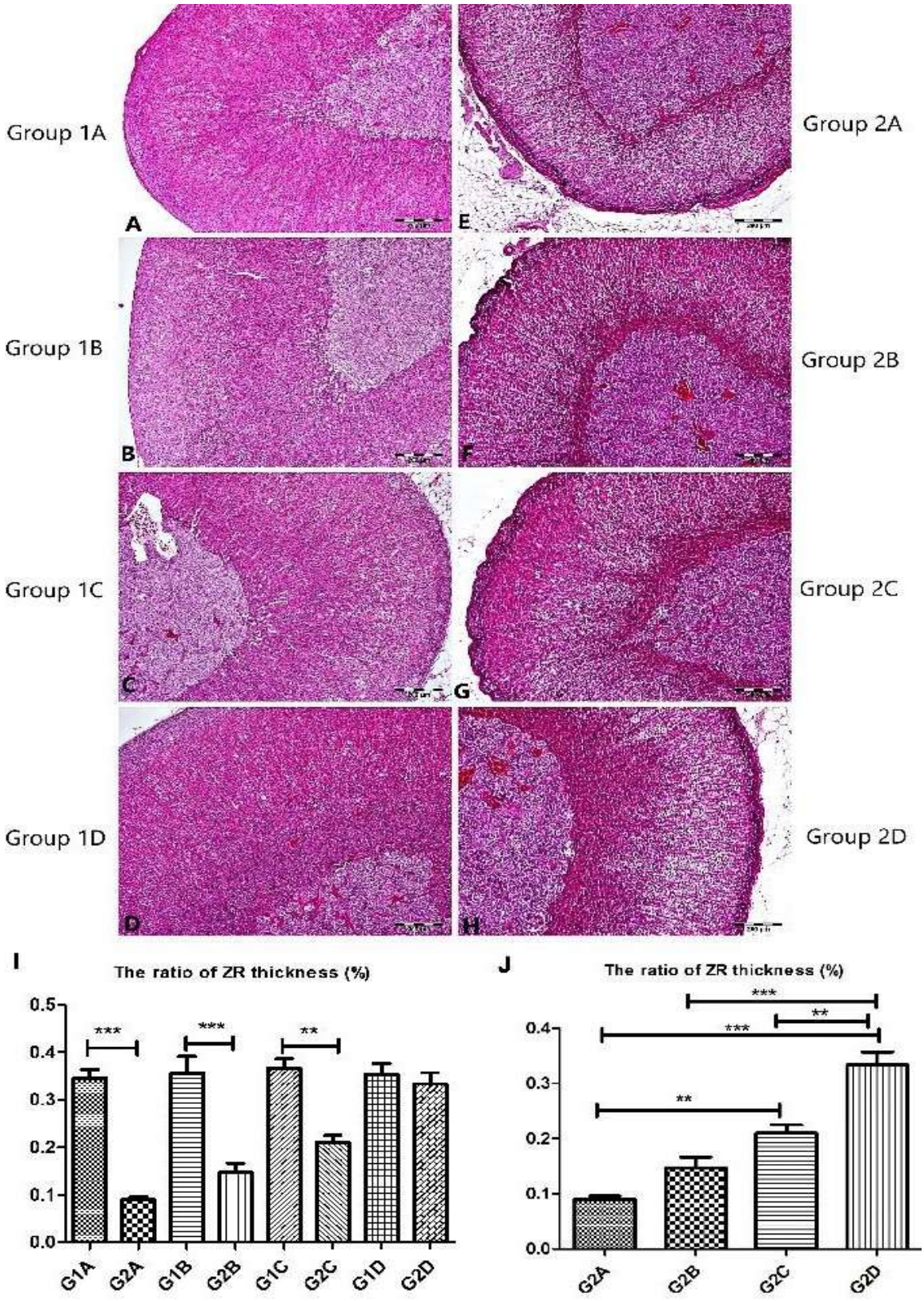

Figure 1. Light microscopic view of adrenal gland tissue sections (H&E). **(A–D)** In the control group, the adrenal cortex and adrenal medulla were intact in groups 1A–1D, sacrificed on days 7, 21, 35, and 63 respectively. **(E–G)** Most cells show nuclear pyknotic changes, especially in the ZR layer of the adrenal cortex in rats of groups 2A, 2B, and 2C, sacrificed on days 7, 21, and 35 respectively. **(H)** Adrenal cortex and adrenal medulla were seen normal in rats in group 2D, sacrificed on day 63. **(I)** Differences between the ratio of ZR thickness of subgroups in groups 1 and 2. **(J)** Differences between the ratio of ZR thickness of subgroups in group 2 according to sacrificed days. ∗∗∗$p < 0.0001$; ∗∗$p < 0.001$; ∗$p < 0.01$.

Table 1. Values of ZR thickness compared among the control and EDS groups.

	Control group				EDS group			
ZR thickness (μm)	7th day	21st day	35th day	63rd day	7th day	21st day	35th day	63rd day
	3.1 ± 0.3	3.4 ± 0.1	3.32 ± 0.2	3.12 ± 0.2	0,7 ± 0.1[a]	1.4 ± 0.2[a]	1,9 ± 0.2[a,b]	2,91 ± 0.3[b,c,d]

a: $p < 0.05$, when compared with control and EDS groups.

b: $p < 0.05$, when compared with group 2A and other EDS subgroups.

c: $p < 0.05$, when compared with group 2B and other EDS subgroups.

d: $p < 0.05$, when compared with group 2C and other EDS subgroups.

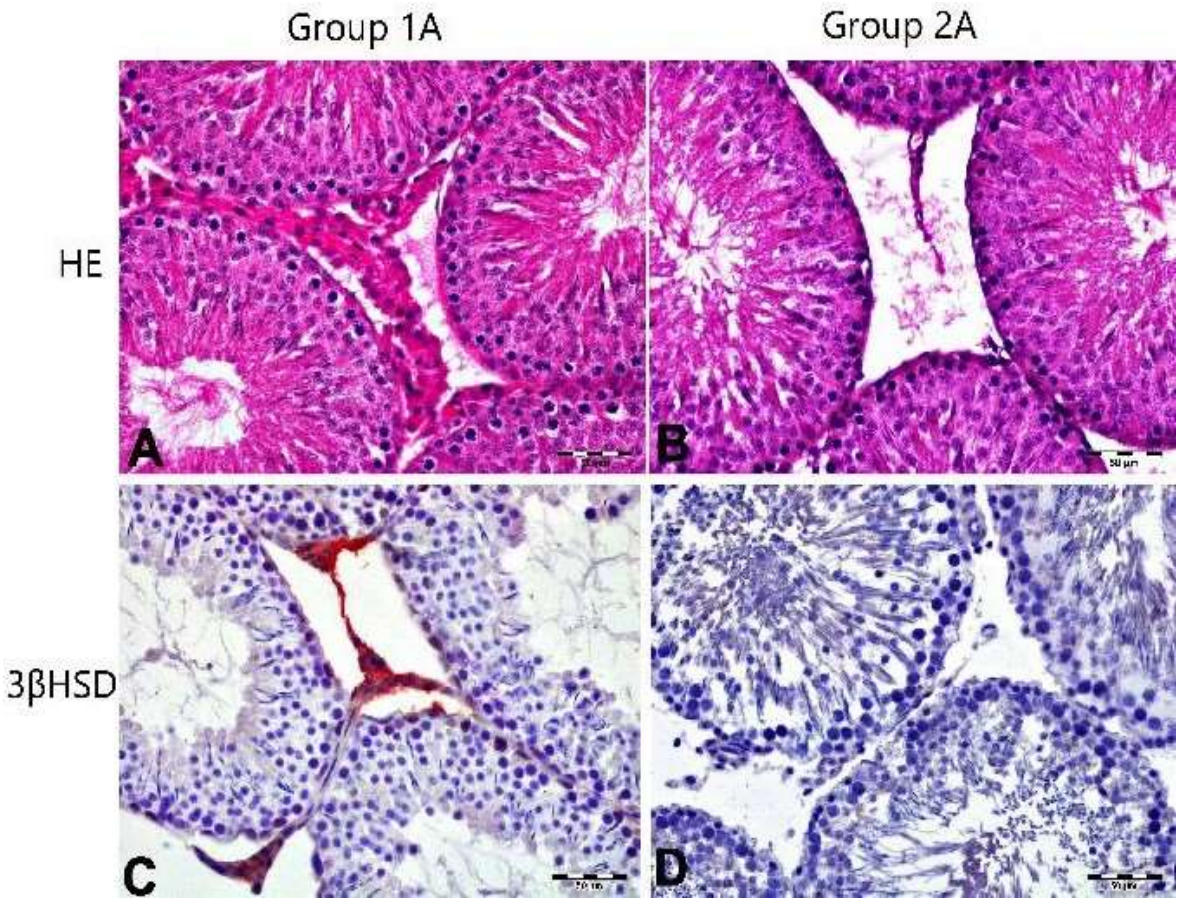

Figure 2. (A) Testicular tissue section of group 1A, sacrificed on day 7 is seen at the light microscopic level. Seminiferous tubule epithelium and interstitium are observed (H&E). **(B)** Testicular tissue section of group 2A, sacrificed on day 7 shows the elimination of Leydig cells in the interstitium (H&E). **(C)** High 3βHSD immunoreactivity was observed in Leydig cells in the testicular tissue sections of group 1A, sacrificed on day 7. **(D)** No 3βHSD immunoreactivity was observed in Leydig cells in testicular tissue sections of group 2A, sacrificed on day 7.

according to sacrificed days, Ki67 immunoreactivity scores significantly increase daily (Figs. 3I and 3J). The Caspase-3 expression (an apoptotic marker) was minimum in all layers of the adrenal glands in the control group (Figs. 4A, 4B, 4C, and 4D). The Caspase-3 expression significantly increased in the ZR layer of adrenal glands of rats sacrificed on the 7th, 21st and 35th days after EDS administration (Figs. 4E and 4F). However, in those sacrificed on day 63 after EDS administration, we observed a significant decrease in Caspase-3 immunoreactivity in the ZR layer when compared to that observed in those sacrificed on days 7, 21, and 35 after EDS

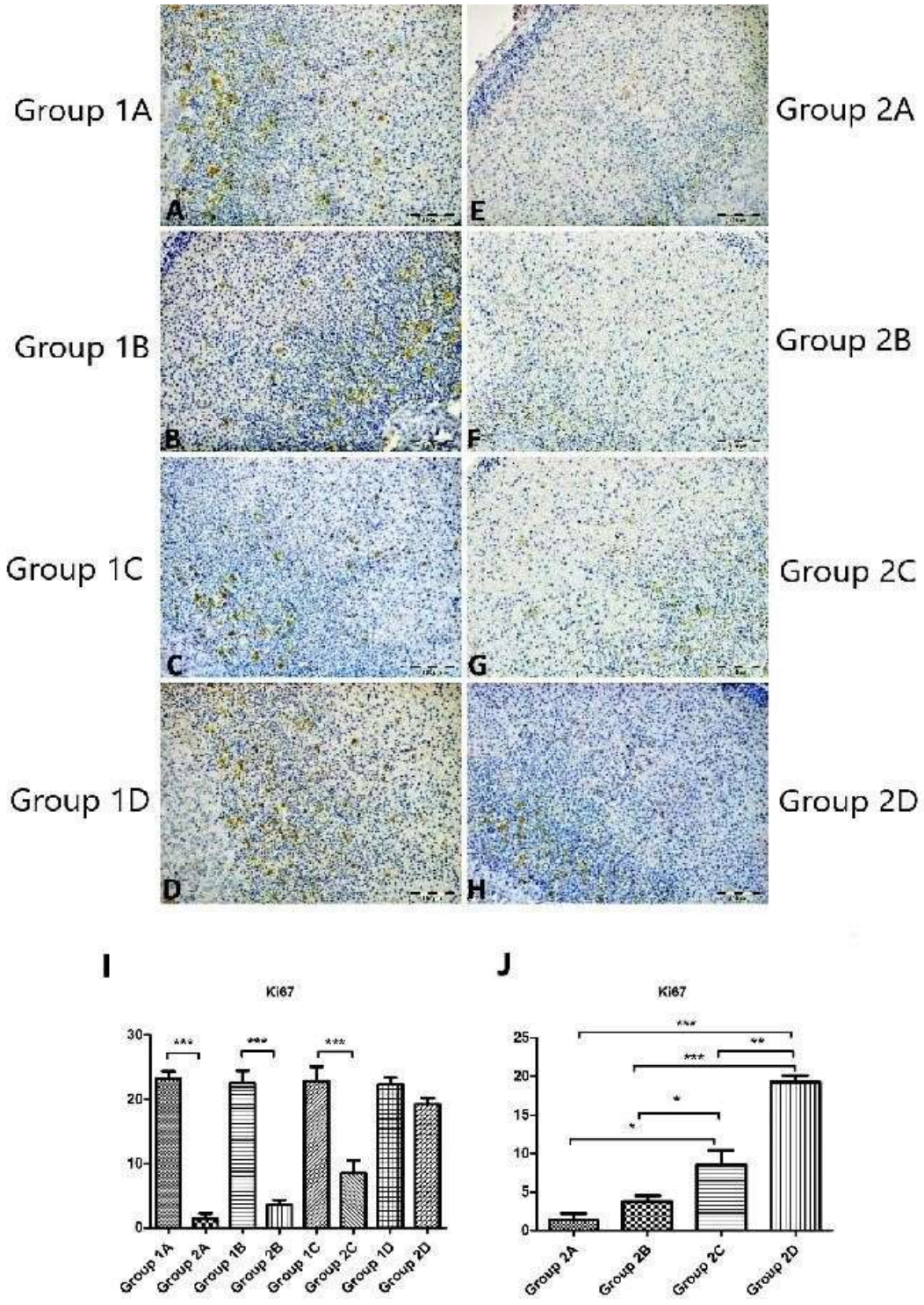

Figure 3. Ki67 immunoreactivity of adrenal gland tissue sections. **(A–D)** High Ki67 expression was observed in all layers of adrenal glands in groups 1A–1D, sacrificed on days 7, 21, 35, and 63 respectively. **(E–G)** Ki67 expression decreased in all layers of the adrenal glands in subjects of groups 2A, 2B, and 2C, sacrificed on days 7, 21, and 35 respectively. **(H)** High Ki67 expression was seen in the ZR layer of the adrenal glands in the subjects of group 2D, sacrificed on day 63. **(I–J)** Statistical evaluation of Ki67 immunoreactivity scores of adrenal gland tissue sections. **(I)** Differences between Ki67 immunoreactivity scores of adrenal glands in subgroups of group 1 and group 2. **(J)** Differences between Ki67 immunoreactivity scores of subgroups in group 2, according to sacrificed days. $***p < 0.0001$; $**p < 0.001$; $*p < 0.01$.

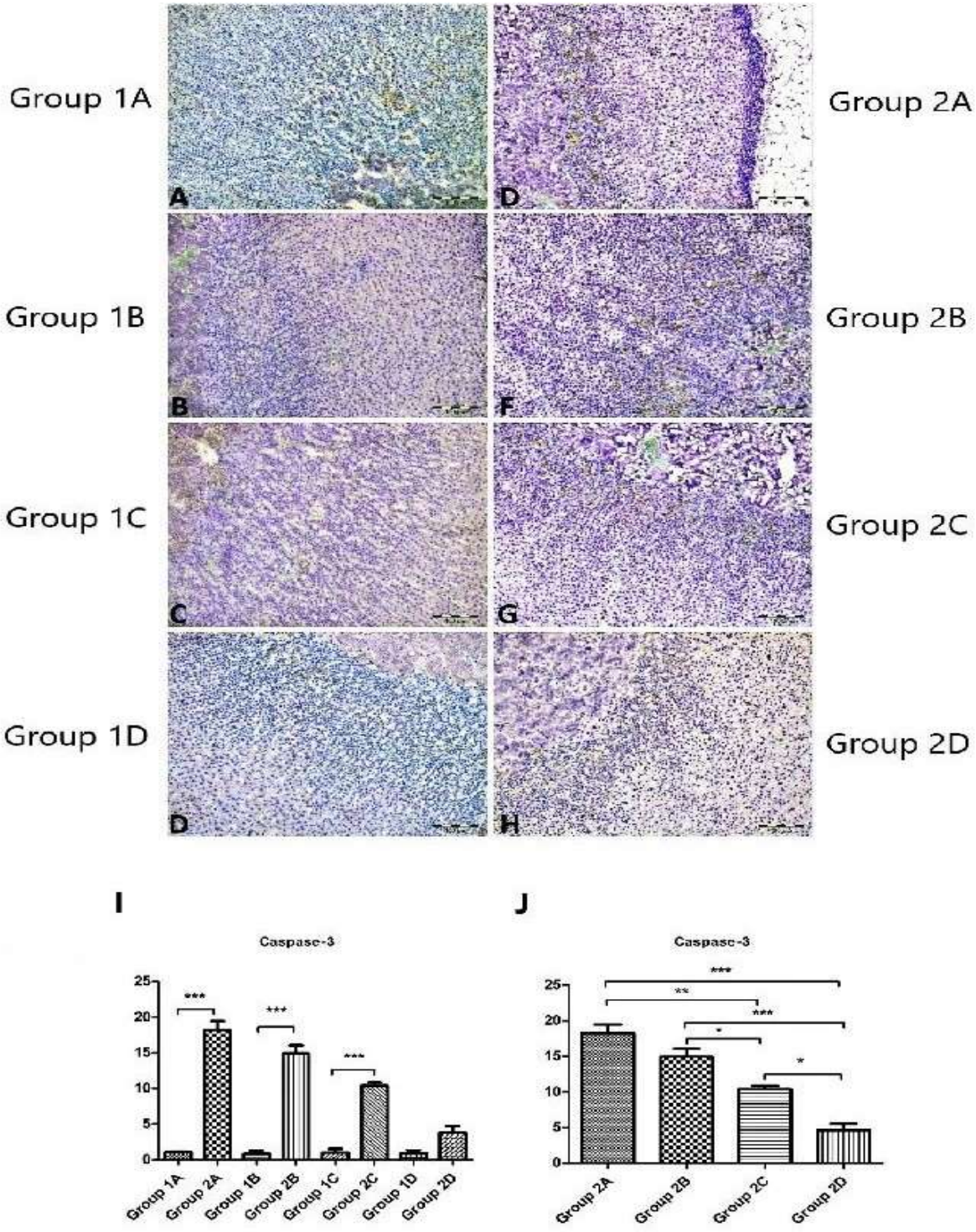

Figure 4. Caspase-3 immunoreactivity of adrenal gland tissue sections. **(A–D)** Low Caspase-3 expression was observed in all layers of adrenal glands in groups 1A–1D, sacrificed on days 7, 21, 35, and 63 respectively. **(E–G)** Increase in Caspase-3 expression was seen in the ZR layers of the adrenal glands in the subjects of groups 2A, 2B, and 2C, sacrificed on days 7, 21, and 35 respectively. **(H)** Low Caspase-3 expression was detected in the ZR layer of the adrenal glands in subjects of group 2D, sacrificed on day 63. **(I–J)** Statistical evaluation of Caspase-3 immunoreactivity scores of adrenal gland tissue sections. **(I)** Differences between Caspase-3 immunoreactivity scores of adrenal gland in subgroups of group 1 and group 2. **(J)** Differences between Caspase-3 immunoreactivity scores of subgroups in group 2, according to sacrificed days. $***p < 0.0001$; $**p < 0.001$; $*p < 0.01$.

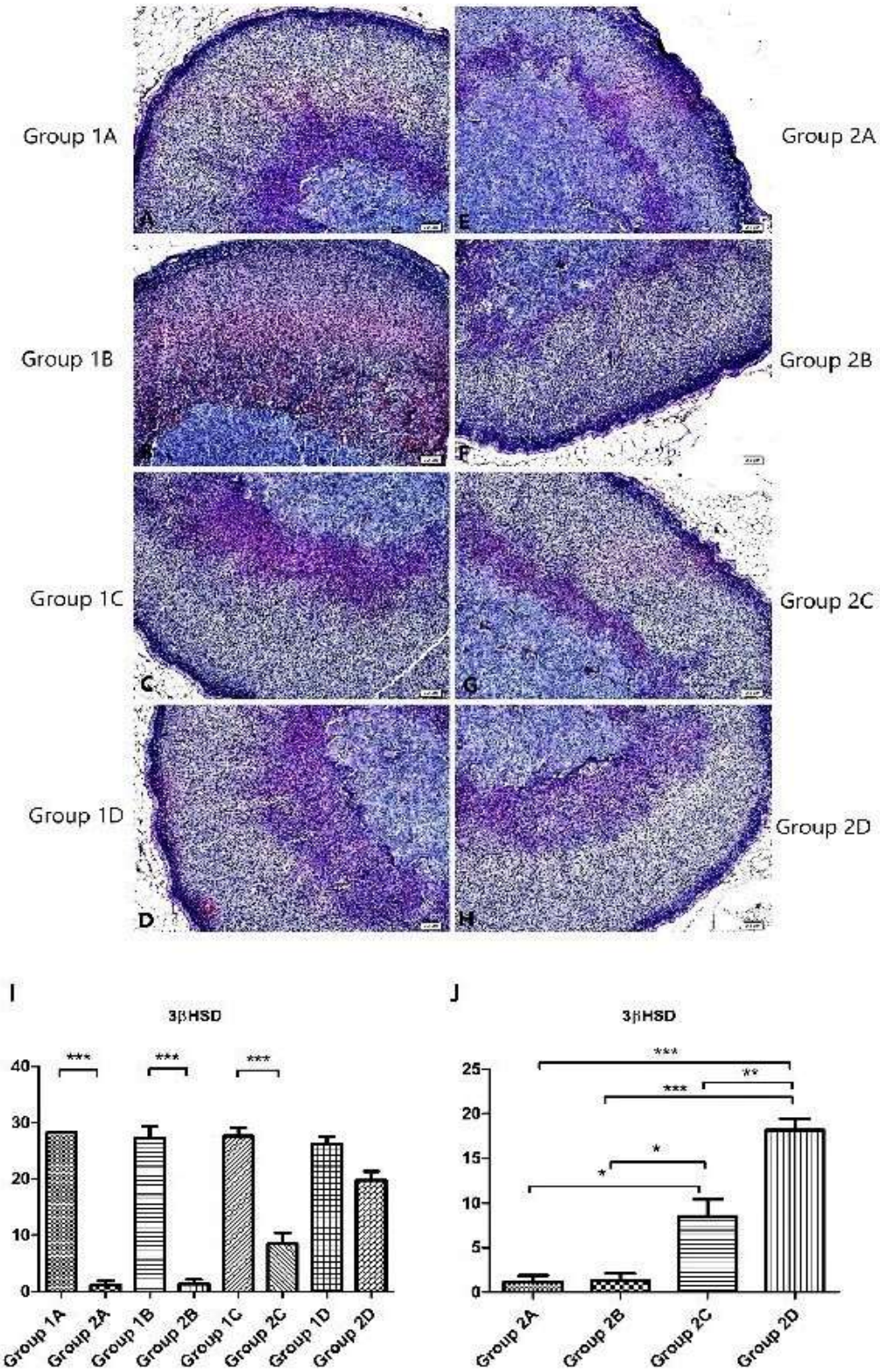

Figure 5. 3βHSD immunoreactivity of adrenal gland tissue sections. **(A–D)** High 3βHSD expression was observed in all layers of adrenal gland in groups 1A–1D, sacrificed on days 7, 21, 35, and 63 respectively. **(E–G)** 3βHSD expression decreased in all layers of adrenal gland in the subjects of groups 2A, 2B, and 2C, sacrificed on days 7, 21, and 35 respectively. **(H)** High 3βHSD expression was seen in the ZR layer of the adrenal glands in subjects of group 2D, sacrificed on day 63. **(I–J)** Statistical evaluation of 3βHSD immunoreactivity scores of adrenal gland tissue sections. **(I)** Differences between 3βHSD immunoreactivity scores of adrenal glands in subgroups of group 1 and group 2. **(J)** Differences between 3βHSD immunoreactivity scores of subgroups in group 2 according to sacrificed days. ∗∗∗p < 0.0001; ∗∗p < 0.001; ∗p < 0.01.

administration (Figs. 4G and 4H). Caspase-3 immunoreactivity scores of the EDS group significantly increased in adrenal gland tissue sections of those sacrificed on days 7, 21 and 35 after EDS administration ($p < 0.0001$ respectively), when compared with those obserevd in the control group. We observed that Caspase-3 immunoreactivity scores of the EDS group significantly decreased according to sacrificed days (Figs. 4I and 4J). As a steroidogenic cell marker, the 3βHSD expression was high in all layers of the adrenal glands in the control group (Figs. 5A, 5B, 5C, and 5D). The Ki67 expression significantly decreased in those sacrificed on the 7th, 21st and 35th days after EDS administration (Figs. 5E and 5F). However, theKi67 immunoreactivity in subjects sacrificed on the 63rd day increased, especially in the ZR layer when compared to the immunoreactivity observed in those sacrificed on the 7th, 21st, and 35th days after EDS administration (Figs. 5G and 5H). However, the 3βHSD immunoreactivity scores were significantly lower in the adrenal gland tissue sections of those sacrificed on the 7th, 21st and 35th day after EDS administration ($p < 0.0001$ respectively), when compared with those observed in the control group. The EDS group was evaluated according to sacrificed days, and 3βHSD immunoreactivity significantly increase daily in the group (Figs. 5I and 5J). H-score data of Ki67, Caspase-3, and 3βHSD immunohistochemical stainings are summarized in table 2.

Biochemical Results

Serum testosterone levels significantly reduced in subjects sacrificed on days 7, 21, and 35 after EDS administration. However, these levels were similar in those sacrificed on the 63rd day after EDS administration across both groups (Fig. 6A). According to sacrificed time, serum testosterone levels of the EDS subgroups significantly increased daily (Fig. 6B). Serum testosterone levels of the rats are summarized in table 3.

Table 2. H-score was calculated on the basis of immunohistochemical staining of Ki67, Caspase-3 and 3βHSD.

H-score		**Ki67**		**Caspase-3**		**3βHSD**	
The intensity category		**H-score < 2**	**H-score ≥ 2**	**H-score < 2**	**H-score ≥ 2**	**H-score < 2**	**H-score ≥ 2**
Control group	7th day	165(150-180)	50(28-70)	17(15-35)	6(1-9)	152(145-182)	68(45-80)
	21st day	170(150-190)	44(32-66)	22(15-35)	4(2-7)	145(130-158)	72(48-85)
	35th day	162(148-190)	46(36-58)	18(10-32)	3(1-5)	142(135-160)	70(50-90)
	63rd day	156(136-182)	45(35-60)	19(16-40)	3(1-7)	135(125-150)	65(55-78)
EDS group	7th day	8(5-15)	-	55(45-70)	21(15-35)	5(2-10)	-
	21st day	12(7-15)	-	44(36-50)	17(11-32)	7(5-10)	-
	35th day	35(20-40)	18(10-25)	31(25-50)	15(10-25)	29(15-45)	17(10-30)
	63rd day	135(128-150)	38(30-55)	27(20-45)	10(4-15)	54(40-70)	42(35-60)

H-score was presented as the median with a quantile range.

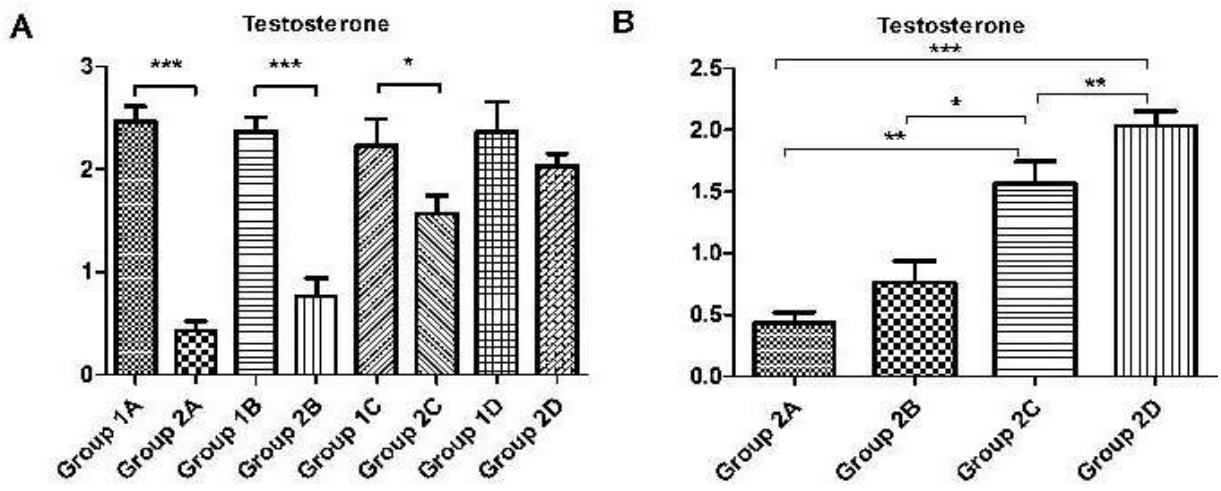

Figure 6. Statistical evaluation of serum testosterone level. **(A)** Comparison of serum testosterone levels of subjects in subgroups of group 1 and group 2. **(B)** Comparison of testosterone levels within the subgroups of group 2. ∗∗∗p < 0.0001; ∗∗p < 0.001; ∗p < 0.01.

Table 3. Serum testosterone levels were compared among the control and EDS groups.

	Control group				EDS group			
Serum Testosterone level (ng/ml)	7th day	21st day	35th day	63rd day	7th day	21st day	35th day	63rd day
	2.4 ± 0.3	2.3 ± 0.1	2.2 ± 0.1	2.3 ± 0.2	0,4 ± 0.1[a,d]	0.7 ± 0.1[a,d]	1.7 ± 0.2[a,b]	1,9 ± 0.3[c,d]

a: p < 0.05, when compared with control and EDS groups.

b: p < 0.05, when compared with group 2A and other EDS subgroups.

c: p < 0.05, when compared with group 2B and other EDS subgroups.

d: p < 0.05, when compared with group 2C and other EDS subgroups.

Discussion

In the present study, the possibility of a parralel regeneration of the adrenal glands and the adult Leydig cell was observed between the 7th and the 63rd day after administering a single dose of 75 mg/kg EDS injection, causing adult Leydig cell elimination. Although complete regeneration did not occur on the 7th, 21st, and 35th days after EDS administration, it was determined that the complete regeneration was observed on the 63rd day after EDS administration.

The most effective method used to examine the development of adult Leydig cells in the post-pubertal period aroccurred within 3–4 days following a single dose administration of EDS. The number of adult Leydig cells, testosterone levels, and other parameters return to their pre-injection state after EDS administration with the regeneration of new Leydig cell population within 7–10 weeks [25-28].

Except for Leydig cells, EDS causes a temporary involution in the adrenal gland, leading to disruption in steroidogenic activity and reduction in adrenal gland weight. Fifteen days after EDS application, while the size of three zones forming the adrenal gland cortex decreased significantly, those of the adrenal medulla did not differ from those from rats in the control group. Furthermore, in the adrenal cortex, the most prominent cell atrophy is in the zona reticularis layer [18,29]. Thus, we observed a significant decline in the ratio of ZR thickness in rats sacrificed on the 7th, 21st, and 35th days after EDS administration. Moreover, it was reported

11β HSD (the steroidogenic enzyme) declines in the first 7 days after EDS administration, followed by a progressive increase in the immunoreactivity by days 14 and 21 [19].

In this study, 3βHSD (another steroidogenic enzyme) and Ki67 were significantly lower than those obserevd in the control group, especially at the 7th, 21st, and 35th days after EDS administration due to the cytotoxic effects of EDS on steroidogenic cells. Especially on day 63 after EDS administration, the Ki67 expression in the adrenal gland did not significantly differ from that in the control group. When the serum testosterone levels of the same rats were examined, in parallel with the 3βHSD expression, they were lower than in the control group, on days 7, 21, and 35 after EDS administration. However, due to the regeneration of adrenocortical and Leydig cells, the testosterone level on the 63rd day after EDS increased and reached values close to that of the control group. The increase in Caspase-3 expression on days 7, 21, and 35 after EDS, caused a decrease in the 3βHSD expression, which is an enzyme that is responsible for steroid hormone synthesis in ZR cells of the adrenal cortex. Therefore, disruption in the gonadocorticoid metabolism impaired the cholesterol concentration as a steroid hormone raw material, and a decline in testosterone levels was observed. After the regeneration of adrenocortical cells, Ki67 and 3βHSD increased, and testosterone levels returned to nearly normal on day 63 after EDS. Similarly, with the increased expression of the proliferation marker Ki67, a significant increase in the ratio of ZR thickness was determined in subjects sacrificed on the 63rd day after EDS application.

Some tissues have a continuous regeneration capacity during the life of an organism such as epithelial tissues. Perhaps, many internal organs and glands can regenrate after stress of major illness, trauma, or surgery. These organs include the thymus, thyroid gland, intestine, lungs, heart, liver, kidney, bladder, skin, pancreas, bone, and cartilage [30,31]. In the literature, several studies also reported that extensive cell renewal in the adrenal cortex showed sexual dimorphism because of the differences between stem cell dynamics in males and females. Tissue turnover of the adrenal cortex in females is three times higher than that in males estimated to be 9 months. On the other hand, orchiectomy (rather than ovariectomy) induced proliferation of stem cells suggests that androgens regulate adrenal cortex regeneration [29]. Thus, that increase in testosterone levels due to the regeneration of Leydig cells after EDS administration stimulates the regeneration of the ZR layer of the adrenal cortex.

In this study, we analyzed the regeneration in the adrenal gland after EDS administration through a longer periods using Ki67, 3βHSD, and Caspase-3 immunochemical methods. The toxic effects of EDS on the rat adrenal cortex are observed to be transien; therefore, thus the changes in the adrenal cortex induced by EDS are reversible, such as in the testis. Our results fundamentally support the earlier researchers who investigated the early effects of EDS on the adrenal glands. On the other hand, complete morphological restoration was observed, especially in the ZR layer in approximately 63th day after EDS, that is time which is longer than studies in the literature. Complex physiologic interactions between gonads and the adrenal gland are modulated by exogenous toxicants and experimental manipulations that disrupt secretory functions and cause secondary effects in the adrenal cortex, including atrophy. Therefore, EDS -injected animal models could be used in adrenal gland studies as an adrenal insufficiency model according to timing per group, or alternatively, the adrenal cortex

turnover model, considering the parallel effect of EDS on both organs. Since there is only one study that have showed the short-term effect of EDS in the literature, further studies are required to demonstrate the pathophysiological effect of EDS on the adrenal cortex.

Conclusions

EDS, which has been used in Leydig cell development studies for many years, was showed to cause disruption and then regeneration in the adrenal cortex synchronizely with Leydig cells regeneration. Even, we couldn't observe any morphologic or molecular compansative effects of adrenal cortex at the beginning of Leydig cell elimination. Therefore, studies about EDS injected animal models should be also taken into account not only Leydig cells but also adrenal cortex due to effects on the level of testosterone decreasing. In order to eliminate the parallel effect of EDS on Leydig cells and adrenocortical cells, an alternative model can be created in which these two organs do not affect each other.

Limitations

Serum corticosteroid and testicular testosterone levels were not evaluated in this *study and should be measured in future studies.*

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Conflict of interest: *The authors declare that they have no conflict of interest.*

Ethical statement: *The research protocol was approved by the Scientific Ethics Committee of Cukurova University (Dated 16.02.2017).*

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