

The protective effect of esculetin against aluminium chloride-induced reproductive toxicity in rats

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Abstract

One of the prominent health problems caused by Aluminium was the decrease in male fertility rates. In the study, the protective effect of Esculetin (ESC) against the reproductive toxicity induced by Aluminium chloride (AlCl_3) was investigated. For this purpose, AlCl_3 was administered to Wistar Albino rats at a dose of 34 mg/kg and ESC was administered at a dose of 50 mg/kg for 70 days. It was determined that AlCl_3 treatment reduced sperm motility and concentration, increased dead/live rate and abnormal sperm rate. It decreased serum testosterone level, and co-treatment of ESC significantly regulated these values. In the AlCl_3 -treated group, MDA level increased and GSH level, GPx and CAT activities decreased compared with those of the control group. However, co-treatment of ESC showed an amelioratory effect on the values except for CAT activity. It was observed that the expression level of NRF-2 increased in the ESC and AlCl_3 + ESC groups, and NF- κ B increased in the AlCl_3 group with the control group. It was determined that Caspase-3 expression decreased, and Bcl-2 expression increased in AlCl_3 + ESC group compared to AlCl_3 group. It was also determined that AlCl_3 -induced tissue injury was significantly prevented by ESC co-treatment.

KEYWORDS

aluminium chloride, esculetin, oxidative stress, reproductive toxicity, sperm quality

1 | INTRODUCTION

The metals are ubiquitous in nature and they have a wide range of uses in industry. They remain in nature for a long time and it is very difficult for mankind to protect himself from metal exposure (Pizent et al., 2012). Aluminium is one of the most common elements in the earth's crust and takes only the value of +3 (Mayyas et al., 2005). It becomes inevitable for people to be exposed to

Aluminium because of their presence in dust particles in the air, food additives, phosphate binders, antacids, buffered analgesics, antiperspirants, cooking containers, canned boxes, baby formulations, cosmetics, mining, drinking water and some herbal products (Akinola et al., 2016; Guo et al., 2005; Hirata-Koizumi et al., 2011; Mayyas et al., 2005).

Mechanisms such as increasing blood-brain barrier permeability, disrupting phosphorylation-dephosphorylation reactions,

interfering with iron metabolism and disruption of the secondary messaging system have been discussed related to toxic effect of Aluminium (Agarwal et al., 1996). The pro-oxidative, excitotoxic, immunogenic, proinflammatory and mutagenic effects of Aluminium have been reported (Klein et al., 2014). It has been determined by various studies that exposure to aluminium in a dose of 100 mg Al^{3+} /kg body weight/day causes inflammation, free radical formation and lipid peroxidation (Liang et al., 2019). In another study, it was indicated that AlCl_3 exposure orally caused a decrease in GPx, CAT activities and GSH level in rats and an increase in MDA level in a dose of 34 mg $\text{kg}^{-1} \text{ day}^{-1}$ (Güvenç et al., 2020). Mai and colleagues reported that high doses of AlCl_3 (200 mg $\text{kg}^{-1} \text{ day}^{-1}$) increased proinflammatory cytokine levels (Mai et al., 2016). The effect of metals on the male reproductive system has been one of the main health problems as a result of increased industrialisation and excessive growth in the city population (Mathur et al., 2010). High intake of AlCl_3 , considered as a systemic toxicant, may cause damage by accumulating in target organs including testis in humans and animals (Guo et al., 2001). In some studies conducted with experimental animals, it has been reported that AlCl_3 exposure impairs sperm quality and spermatogenesis even at levels found in the human diet, and it induces oxidative stress and inflammation in the testis and thereby reduces adult reproductive ability (Miska-Schramm et al., 2017).

Natural compounds have been used in the treatment of some diseases since ancient times (Choi et al., 2019). Metals can influence the male and female reproductive system by increasing the production of reactive oxygen species (ROS). Therefore, it has been suggested that antioxidant therapy may stop metal-induced toxicity in the light of current studies (Jamalan et al., 2016). ESC (6,7-dihydroxycoumarin), also called 6,7-dihydroxycoumarin, is a coumarin derivative, and many medicinal plants such as *Artemisia capillaries*, *Citrus limonia*, *Euphorbia tathyrius*, *Fraxinus rhyncho-phylla* Hance, *Fraxinus chinensis* Roxb and *Fraxinus aboana* Lingelsh contain ESC (Chen et al., 2018; Subramaniam & Ellis, 2011). In the studies conducted, many biological activities of ESC such as anti-inflammatory, anti-fibrotic, antioxidant (Ozal et al., 2018), anti-viral, anti-asthma (Chen et al., 2018), anti-tussive, expectorant (Xu et al., 2017), anti-cancer, anti-anxiety (Zhen et al., 2019), anti-nociceptive (Jeong et al., 2018), anticoagulant, antibacterial, antidiabetic, adipogenesis suppressor effects have been reported (Liang et al., 2017). It has been reported that ESC has a potential protective role in noncommunicable diseases, and it has been associated with its characteristics to be a potent antioxidant and intracellular ROS extinguishing agent (Kadakol et al., 2016). However, in the literature review, no study was found to determine the effects of ESC on the male reproductive system. Therefore, within the scope of this study, the effects of ESC against AlCl_3 -induced reproductive toxicity in rats were determined through immunohistochemical and histopathological examinations. Also, some antioxidant and inflammation parameters and testosterone levels

were determined. Besides, the protective effects of ESC on the reproductive system were determined for the first time.

2 | MATERIALS AND METHODS

2.1 | Chemicals and reagents

All chemicals and solutions used in the study were purchased from Merck Millipore (Darmstadt, Germany) unless stated otherwise.

2.2 | Animals

In the study, 250–300 g male rats, Wistar Albino race, were used. Experimental applications were carried out under the conditions of care and use of laboratory animals (12 hr light-12 hr dark and $24 \pm 1^\circ\text{C}$). During the experimental applications, rats were supplied with standard commercial feed (pellet feed) and tap water ad-libitum. No active ingredients were given to the animals in the control group. The ESC active ingredient was dissolved in the carrier of solvent (0.5% carboxymethylcellulose). In all groups, the active substances were dissolved in 0.5% carboxymethylcellulose, and a standard volume (1 ml) of the respective solution was administered to all animals in groups via the oral gavage. In the study, the dose of ESC (50 mg/kg p.o.) (Anand et al., 2013) and the dose of AlCl_3 (34 mg/kg p.o.) (Yousef & Salama, 2009) were determined according to the literature. In arrange to permit for the completion of the spermatogenic cycle and the development of the spermatozoa within the epididymis, the experiment was carried out for up to 70 days for all groups (Güvenç et al., 2020; Sarkar et al., 2003; Yüce et al., 2013).

2.3 | Experimental design

The rats used in the study were divided into 4 groups, 8 in each group. Groups were created as follows. The practices were conducted in accordance with the ethical rules approved by the Local Ethics Committee of Animal Experiments in Hatay Mustafa Kemal University (Protocol No: 2018/ 9-1).

1. Group (Control): Carrier of the active substance - p.o daily
2. Group (AlCl_3): AlCl_3 (34 mg/kg) - p.o daily
3. Group (ESC): Esculetin (50 mg/kg) - p.o daily
4. Group (AlCl_3 + ESC): AlCl_3 (34 mg/kg) + Esculetin (50 mg/kg) - p.o daily

The results were not significantly different between the two control and solvent groups and we considered data from the carrier of solvent group as the control group.

TABLE 1 Forward and reverse sequences of primers used in the study

GENES*	FORWARD SEQUENCES	REVERSE SEQUENCES
GAPDH	5'-AGTGCCAGCCTCGTCTCATA-3'	5'-TCCCGTTGATGACCAGCTTC-3'
NRF-2	5'-TTGTAGATGACCATGAGTCGC-3'	5'-TGTCTGCTGTATGCTGCTT-3'
NF-κB	5'-CTGCGATACCTTAATGACAGCG-3'	5'-AATTTTGGCTTCCTTCTTGGCT-3'
TNF-α	5'-GGCATGGATCTCAAAGACAACC-3'	5'-CAAATCGGCTGACGGTGTG-3'

*(Rio et al., 2010).

2.4 | Tissue sample collection, processing and storage

24 hr after the last application, the rats were killed under xylazine (10 mg/kg) -ketamine (60 mg/kg) anaesthesia, and testis samples were taken. The blood sample was taken from the left ventricle of the heart by cannula into the serum tubes. Halves of the testicular tissue samples taken were kept in 10% formol for the use in pathological studies, while the other halves were stored at -80°C until the remaining analyses were performed. After serum was obtained from the blood tissues, testosterone levels were measured.

2.5 | Spermatological analyses

All spermatozoal analyses were done using the methods reported in the study of Turk and colleagues (Türk et al., 2007). The spermatozoa concentration in the right caudal epididymal tissue was determined with a haemocytometer. Freshly isolated left caudal epididymal tissue was used for the analysis of spermatozoal motility. The percentage of spermatozoal motility was evaluated using a phase contrast microscope with a heating plate (37°C) (Nikon E 200). To determine the percentage of morphologically abnormal spermatozoa, the slides stained with eosin-nigrosin (1.67% eosin, 10% nigrosin and 0.1M of sodium citrate) and live/dead spermatozoa, stained with hancock solution, were prepared. The slides were then viewed under a light microscope at 400x magnification. A total of 400 spermatozoa were examined on each slide (2,800 cells in each group), and the total abnormality rates of spermatozoa were expressed as percentages.

2.6 | Determination of oxidative stress parameters

Tissue samples were homogenised in 1/10 ratio with 1.15% KCl, while MDA analysis was done in half of the homogenate and the other half was centrifuged at 5,000 g for 1 hr (at $+4^{\circ}\text{C}$). Then, their supernatants were separated and GSH, GPx and CAT analyses were done. Protein analysis in homogenate and supernatant was carried out by Lowry method (Lowry et al., 1951). In the study, the determination of MDA was done according to the methods specified by Placer et al. (Placer et al., 1966), GSH level was specified according to Sedlak and Lindsay (Sedlak & Lindsay, 1968), CAT enzyme activity was measured according to Aebi (Aebi, 1984), and GPx enzyme

activity was detected according to Lawrence and Burk (Lawrence & Burk, 1976).

2.7 | RNA isolation and cDNA synthesis

RNA isolation was done according to the TRIzol method (Rio et al., 2010). For this purpose, approximately 1 ml TRIzol was used for 50 mg tissue (ThermoFisher Scientific, USA). The purity and concentration values of the total RNA obtained were checked in the nucleic acid metre (Merinton SMA 1000, USA). Isolation was repeated from the samples that did not have the appropriate purity and concentration. After the spectrophotometric measurements, samples were evaluated in terms of quality in 1% agarose gel electrophoresis to control RNA quality (100V and 25 min). Samples were treated with DNase I for possible DNA contamination after RNA isolation (DNase I, RNase-free, Thermo Scientific, USA, Cat no: EN0525). DNA was converted to cDNA according to the High-Capacity cDNA Reverse Transcription kit protocol (Thermo Scientific, USA Cat no: 4368814) using 2000 µg total RNA from the samples after the digestion step. In thermal cycler (Biorad T100, USA), the reaction was carried out for 10 min at 25°C , 120 min at 37°C and 5 min at 85°C . The obtained cDNAs were completed to 200 µl with nuclease-free water and kept at -86°C until gene expression studies were performed.

2.8 | RT-qPCR application

NRF-2, NF-κB and TNF-α target genes were amplified with the kit containing SYBR Green I dye, using 10 µl of each cDNA sample (Power SYBR® Green PCR Master, ThermoFisher Scientific, USA, Cat no: 4367659). Duplicates of each sample were analysed. The reaction carried out in RT-qPCR (Biorad CFX96 Touch Real Time PCR, USA) was arranged for 10 min at 95°C followed by 15 s at 95°C , 60 s at 60°C and 40 cycles. GAPDH reference gene was used as the internal control. At the end of the qPCR phase, the region amplified by the primers was checked by melting curve analysis. The sequences of primers used are shown in Table 1 (Güvenç et al., 2019).

2.9 | Histopathological investigation

At the end of the study, the testicular tissues taken after necropsies were fixated in a 10% buffered formaldehyde solution for 72 hr.

Within the scope of routine follow-up, the tissue samples were blocked in paraffin after they were dehydrated by passing through a series of alcohol (70, 80, 90, 100%) and became transparent by passing through a series of xylol. Serial sections of 4–5 microns thick were taken from these paraffin blocks by microtome (Leica RM 2135). Haematoxylin-Eosin stained sections of testicular tissue samples taken from each rat were scored according to the Johnsen Testicular Biopsy Score method and expressed in Table 2 after they were examined in the light microscope (Nikon 80i-DS-R12) (Johnsen, 1970). In this method, the average score of the normal testis varies between 8 and 10. Lower values show decreased germ cell

maturation, spermatogenesis and germinal epithelial damage.

2.10 | Immunohistochemical investigation

Caspase-3 expressions were stained using the streptavidin-peroxidase method (ABC), with the streptavidin/biotin immunoperoxidase kit (Histostain-Plus Bulk Kit; Zymed, South San Francisco, CA, USA) in accordance with the staining procedures of the manufacturer companies with minor changes (Yaman et al., 2019).

After the sections taken in 4–5 micron thick by microtome (Leica RM 2135) had been placed on adhesive slides, they were passed through xylene and alcohol series. To remove the endogenous peroxidase activity, the sections were kept in 3% Hydrogen peroxide (H_2O_2) for 20 min after being washed with PBS (phosphate buffer solution). After placing the antigen in the retrieval solution (citrate buffer) and covering it, it was heated twice in the microwave oven for 20 min. Then, it was taken out of the oven and it was left to cool until it reached room temperature. After being washed again with PBS, the sections were blocked by protein blocking (nonimmune serum) for 20 min. Caspase-3 polyclonal antibody (Catalog no: PA5-16335, 1/100 dilution, ThermoFisher Scientific, USA) and Bcl-2 monoclonal antibody (Catalog no: M0887, 1/100 dilution, DAKO A/S, Glostrup, Denmark) were dropped into each tissue and left overnight at $+4^\circ C$. Sections were washed again with PBS and incubated

for 20 min at room temperature with biotinylated secondary antibody. The sections washed again with PBS were left in streptavidin-peroxidase for 20 min and then washed in the same way as PBS. After washing, 3,3'-Diaminobenzidine (DAB) was dropped and left for 1–2 min. Then, all sections were kept in Mayer's haematoxylin (Bio-Optika, 05-06002E) for 1–2 min and washed in tap water. Sections were passed through 70%, 80%, 90% and 96% alcohol for 3 min, respectively, and 100% alcohol for 10 min and the xylol series for 5 min. Negative controls reacted with PBS were used instead of primary antibodies to confirm staining. Sections were examined and photographed under a light microscope (Nikon 80i-DS-R12). The immunoreactivity staining intensities of the testicular samples obtained from the groups with primary antibodies were scored as mild (+), moderate (++) and severe (+++).

2.11 | Statistical analysis

Statistical evaluations were done with SPSS Statistics 23 software. Histopathological examinations were evaluated semi-quantitatively. Shapiro-Wilk normality test was used to determine whether the raw values of all measured parameters showed normal distribution. As a result of the test, it was found that the values in all parameters showed normal distribution. According to the results of this test, one-way analysis of variance (ANOVA) was used to detect differences between the groups and post hoc Duncan test was used for binary comparisons. All values were given as mean $\pm$ standard error ($\pm S.E.M.$), while the results at $p < .05$ were considered as significant.

3 | RESULTS

3.1 | The effect of ESC and $AlCl_3$ on sperm parameters

Sperm motility, concentration, dead/live sperm rate and abnormal sperm rate values were detected in the groups to determine the

10	Germinal epithelium is a multi-layer structure around the central lumen, the lumen is open and contains a large number of spermatozoa
9	Although the germinal epithelium is seen, a small amount of rash that can block the lumen, spermatozoon and rash epithelium are seen in the clogged lumen
8	Germinal epithelium is multi-layered but there are fewer than 10 spermatozoa in the lumen
7	Having a large number of spermatids and no spermatozoon
6	There is no spermatozoon and there are fewer than 10 spermatids
5	There are only spermatocytes, no spermatozoon or spermatids
4	There are no spermatozoon and spermatids, and there are fewer than 5 spermatocytes
3	There is only spermatogonium as a germ cell
2	There are Sertoli cells only and no germ cells.
1	There are no cells in the seminiferous tubule lumen

*(Johnsen, 1970).

TABLE 2 Categorisation of testicular biopsies according to Johnson scoring system*

TABLE 3 Effect of ESC on AlCl_3 -induced changes in sperm quality

Groups	Motility (%)	Concentration (million/right cauda epididymis)	Dead/Live Sperm Rate (%)	Abnormal sperm rate (%)
Control	68.00 ± 2.00^a	167.60 ± 3.50^a	30.40 ± 1.29^a	12.20 ± 0.86^a
AlCl_3	17.00 ± 1.92^c	94.00 ± 6.80^c	93.20 ± 0.86^b	28.60 ± 2.40^b
ESC	77.80 ± 2.22^b	203.40 ± 2.67^b	14.00 ± 1.14^c	10.00 ± 0.70^a
AlCl_3 + ESC	71.00 ± 1.87^{ab}	185.00 ± 2.98^a	23.60 ± 0.68^d	10.40 ± 0.50^a
Significance	$p < .05$	$p < .05$	$p < .01$	$p < .01$

Note: Data are expressed as mean $\pm$ SEM, $n = 8$. Tukey test was used to determine the differences among the groups. Different superscript letters (a-c) within the column show statistically significant differences between the groups. The lowest value is marked with 'a' and the sign letter is changed gradually when the statistical difference occurs in the same column.

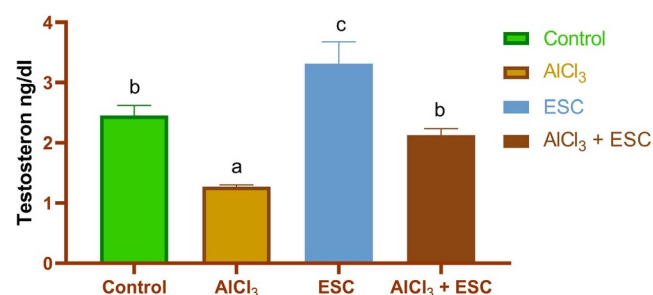


FIGURE 1 Serum testosterone levels. Data represent the means $\pm$ S.E.M. of eight rats in each group. Tukey test was used to determine the differences among the groups. Graphs (a-c) show significant differences ($p < .001$) among each group. The lowest value is marked with 'a' and the sign letter is changed gradually when the statistical difference occurs

protective effect of ESC on the AlCl_3 -induced harmful effect. The results obtained are given in Table 3. Accordingly, it was determined that sperm motility and concentration decreased significantly ($p < .05$), and the ratio of dead/live sperm and the number of abnormal sperm increased in the AlCl_3 group ($p < .01$). Besides, it was observed that sperm mobility and concentration increased in rats treated with solely ESC compared with the control group ($p < .05$), the ratio of dead/live sperm decreased and there was no significant change in abnormal sperm ratio ($p < .01$). Also, the administration of ESC together with AlCl_3 to rats prevented the decrease in sperm

motility and concentration ($p < .05$), and the increase in dead/live sperm rate and abnormal sperm rate, compared with the AlCl_3 group ($p < .01$).

3.2 | The effect of ESC and AlCl_3 on testosterone hormone level

When the changes in the testosterone hormone level were examined, it was observed that the serum testosterone level of the rats in the AlCl_3 -treated group decreased significantly compared with that in the control group ($p < .001$) and increased significantly in the ESC group. Also, it was seen that the detrimental effects of AlCl_3 on testosterone were eliminated in the group administrated ESC together with AlCl_3 completely (Figure 1).

3.3 | The effect of ESC and AlCl_3 on antioxidant parameters

Within the scope of the study, MDA, a lipid peroxidation product and GSH levels, GPx and CAT enzyme activities, which are a component of the antioxidant defence system, were determined to examine the protective effect of ESC against oxidative stress caused by AlCl_3 in the testicular tissue. According to the results obtained, the MDA level was significantly increased compared to that of the control group ($p < .001$), in the testicular tissues in the Al treated

TABLE 4 The contents of MDA, GSH and the activities of GPx and CAT in testicular tissue of rats

Group/Variables	MDA (nmol/gr prot)	GSH (nmol/gr prot)	GPx (IU/gr prot)	CAT (U/gr prot)
Control	3.34 ± 0.34^a	3.45 ± 0.06^a	99.07 ± 1.63^{ab}	100.47 ± 5.31^a
AlCl_3	5.32 ± 0.32^b	2.22 ± 0.13^b	82.97 ± 2.67^c	81.15 ± 3.95^b
ESC	3.50 ± 0.17^a	3.64 ± 0.15^a	104.03 ± 2.46^a	112.07 ± 4.69^a
AlCl_3 + ESC	3.48 ± 0.33^a	2.89 ± 0.17^c	92.09 ± 2.12^b	94.78 ± 4.12^{ab}
Significance	$p < .001$	$p < .001$	$p < .001$	$p < .01$

Note: Data are expressed as mean $\pm$ SEM, $n = 8$. Tukey test was used to determine the differences among the groups. Different superscript letters (a-c) within the column show statistically significant differences between the groups. The lowest value is marked with 'a' and the sign letter is changed gradually when the statistical difference in the same column.

group; however, the GSH level and GPx ($p < .001$) and CAT activities decreased ($p < .01$). Additionally, it was seen that ESC treatment significantly prevented these effects caused by AlCl_3 ($p < .001$), and in spite of this, the protective effect of ESC was limited on CAT activity ($p < .01$). The results obtained are summarised in Table 4.

3.4 | The effect of ESC and AlCl_3 on transcription levels of NRF-2, NF- κ B and TNF- α

At the end of total RNA isolation, samples were determined to be of sufficient purity and concentration ($\text{A}_{260}/\text{A}_{280} = 1.93 \pm 0.01$; Concentration = 916.42 ± 50.13 ng/ μ l). The transcription levels of NRF-2, NF- κ B and TNF- α are given in Table 5. Accordingly, it was determined that NRF-2 transcription increased significantly in the groups of ESC and AlCl_3 + ESC compared with other groups, whereas NF- κ B transcription increased only in the group treated with AlCl_3 , and ESC co-treatment prevented this increase ($p < .05$).

3.5 | Histopathological findings

No change was observed in the macroscopic examination of the testis of the rats in all experimental groups. The average of the results obtained according to the Johnsen testicular biopsy score criteria is presented in Figure 2, and the microscopic images of histopathological findings are presented in Figure 3. According to the Johnsen testicular biopsy score results, there was no statistically significant difference in the ESC and AlCl_3 + ESC groups compared with the control group. However, the Johnsen biopsy score decreased significantly in the group that was given AlCl_3 compared with the control group ($p < .001$). In the microscopic examination of the testis of rats in the control and ESC groups, tubules seminiferous and Leydig cells and spermatogenic cells in tubules seminiferous were found to have a normal histological appearance, and regular spermatogenesis with germ cells at all stages of spermiogenesis was seen (Figure 3a,d). In the microscopic examination of the testis of the rats in the AlCl_3 group, it was determined that some of the tubules' morphology was disrupted and some of them were atrophied, and tubules consisting of only basement membranes were encountered locally. Germinative epithelial cells were mostly separated from the tubular basement membrane. Some germinative cells were accumulated in the lumen. There were many vacuoles in the germinative epithelium.

The tubules basement membrane was thickened and in some areas, the tubules were adjacent to each other and were nonadjacent in some other areas. There was a marked loss or disorganisation in the germinal epithelium. The nuclei of some spermatogonia shrunk and wrinkled with degeneration in germinal cells and that spermatogenesis stopped or decreased to a great extent. Oedema and haemorrhage in the interstitial areas and congestion in the vessels were observed (Figure 3b). It was observed that the tubules in the testis of the AlCl_3 + ESC group rats mostly preserved their morphology, and the germinative epithelial cells belonging to the spermatogenic series continued to spermatogenesis, including spermatozoa. However, there were thickened basement membrane of tubuli, slight oedema in interstitium and vacuoles in some epithelium. The interstitial space, which also includes Leydig cells, had a normal appearance and congestion was not observed in these areas (Figure 3c).

3.6 | Immunohistochemical findings

The microscopic images of immunohistochemical staining using Caspase-3 and Bcl-2 primary antibody were indicated in Figure 4A-D and Figure 5A-D, respectively, and immunoreactivity scores were shown in Figure 6. Accordingly, it was determined that the expression of caspase-3 decreased considerably in the AlCl_3 + ESC group compared with that of the AlCl_3 group, and the Bcl-2 expression increased significantly ($p < .001$). The spermatogenic cells of the tubules showed no Caspase-3 and Bcl-2 immune reactions in the testes of the rats of control and ESC groups. In terms of Caspase 3, moderate/severe (++/ +++) immunoreactivity in the AlCl_3 group and mild (+) immunoreactivity in the AlCl_3 + ESC group were detected. In terms of Bcl-2, mild (+) immunoreactivity was observed in the AlCl_3 group and moderate/severe (++/ +++) immunoreactivity was observed in the AlCl_3 + ESC group.

4 | DISCUSSION

Within the scope of the study, the protective effect of ESC was examined against reproductive toxicity created by oral administration of AlCl_3 to rats for 70 days. It was determined that AlCl_3 treatment decreased sperm mobility and concentration in rats and increased dead/live rate and abnormal sperm rate. It reduced the level of testosterone, which has a key role in spermatogenesis. While increasing

Gene	Control	AlCl_3	ESC	AlCl_3 + ESC
NRF-2	1.00 ± 0.10^a	1.38 ± 0.17^{ab}	1.56 ± 0.17^b	1.41 ± 0.12^b
NF- κ B	1.00 ± 0.18^a	1.44 ± 0.08^b	1.35 ± 0.15^a	1.31 ± 0.08^a
TNF- α	1.00 ± 0.08^a	1.08 ± 0.14^a	0.80 ± 0.09^a	0.98 ± 0.07^a

Note: Data are expressed as mean $\pm$ SEM, $n = 8$. ($p < .05$) Tukey test was used to determine the differences among the groups. Different superscript letters (a, b) show statistically significant differences between the groups. The lowest value is marked with 'a' and the sign letter is changed gradually when the statistical difference occurs in the same line.

TABLE 5 Gene expression findings in testicular tissues

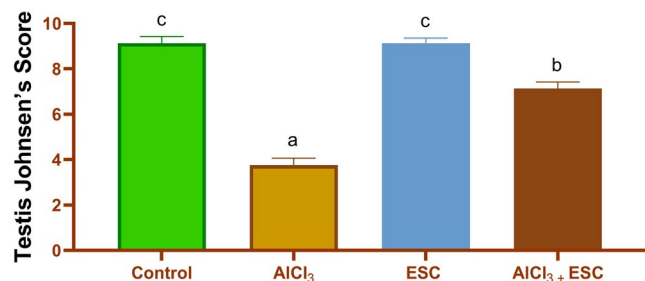
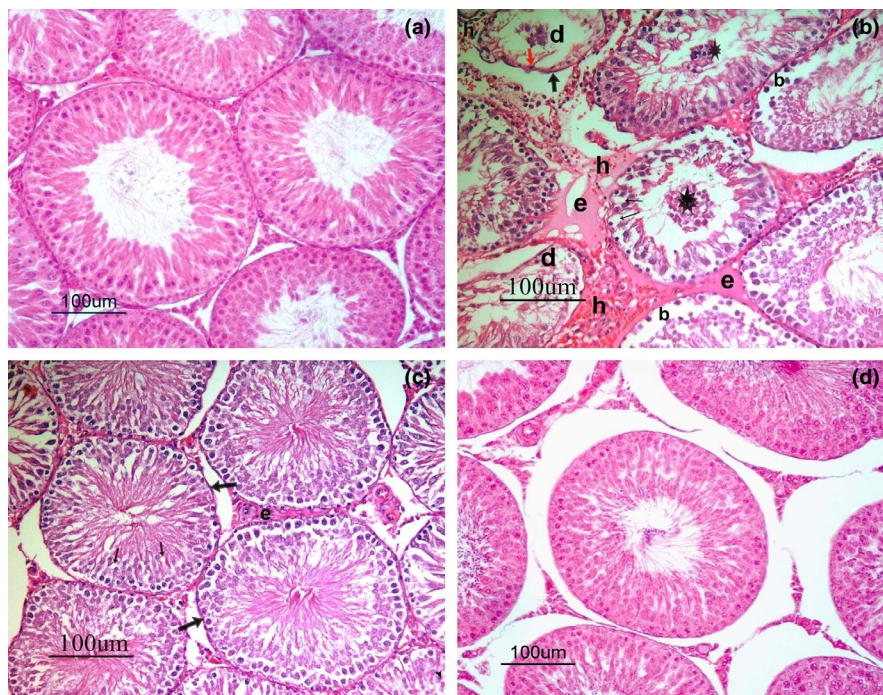


FIGURE 2 Histopathologic variables in testis according to the Johnsen's Score in study groups. Data represent the means $\pm$ S.E.M. of eight rats in each group. Tukey test was used to determine the differences among the groups. Graphs (a–c) show significant differences ($p < .001$) among each group. The lowest value is marked with 'a' and the sign letter is changed gradually when the statistical difference occurs

the level of MDA, it caused a decrease in GSH level, GPx and CAT activities. Compared with the control group, the NF- κ B transcription level increased, and it did not have a significant effect on NRF-2 and TNF- α . However, it caused an increase in Caspase-3 level and a decrease in Bcl-2 level. When histopathological findings were evaluated according to the Johnsen score, a significant decrease was observed. ESC treatment, on the other hand, was found to increase sperm motility, concentration and testosterone levels and to decrease the dead/living ratio compared with the control group. It was determined that ECS co-treatment against aluminium-induced toxicity has a regulatory effect on sperm parameters and antioxidant parameters other than CAT. It raised the testosterone level to normal. It significantly prevented the increase in Caspase-3 level and the decrease in Bcl-2 level. Johnsen score increased significantly compared with the AlCl₃ group.

Spermatological parameters such as advanced mobility, sperm cell density, spermatozoa morphology are related to each other. Therefore, one factor that causes one of these to fail also affects other parameters (Bonde et al., 1996). Also, sperm motility is one of the main factors of the reproductive process, and one of the main causes of infertility in humans has been reported to be the deficiency in sperm motility (Jamalan et al., 2016). When the semen samples of rats treated with AlCl₃ at the dose of 34 mg/kg orally during the study are examined, it is seen that sperm motility decreased considerably and concentration decreased greatly. In addition, there was a significant increase in the number of abnormal cells and the proportion of dead/live cells. These results caused by AlCl₃ have also been reported in previous studies (Akhigbe & Ige, 2012; Llobet, 1995; Marzec-Wróblewska et al., 2012). Mouro and colleagues reported that exposure to AlCl₃ reduced sperm motility in rats (Mouro et al., 2018). In another study, the effect of AlCl₃ exposure at 75, 150 and 300 mg/kg dose on sperm parameters in rats was examined, and it was determined that it increased abnormal sperm rate, decreased sperm motility at 150 and 300 mg/kg dose and decreased the sperm count at only 300 mg/kg dose (Falana et al., 2017). It was determined that the administration of ESC together with AlCl₃ resulted in improvement in all sperm parameters compared with the values in the control group and even gave better results in the dead/live ratio parameter than the control group. In rats that were administered ESC only, better results were obtained in all values except abnormal sperm rate compared with the control group. In light of the data obtained, it was determined that ESC not only eliminated the toxic effect of AlCl₃ on sperm parameters but also significantly increased sperm parameters in healthy animals. In previous studies, it has been reported that AlCl₃-induced reproductive toxicity is caused by oxidative stress (Falana et al., 2017), and the decrease in sperm count and mobility may also be caused by tissue

FIGURE 3 Microscopic view of the testis: A: Control group; normal histological appearance. B: AlCl₃ group; thickened basement membrane of tubuli (thick arrow), vacuoles in germinative epithelium (tiny arrow), necrosis (red arrow), germinative cells separated from the basement membrane (b), degenerated and disrupted tubules (d), oedema (e), haemorrhage (h), accumulated germinative cells in the lumen (stars). C: AlCl₃ + ESC group; thickened basement membrane of tubuli (thick arrow), vacuoles in some germinative epithelium (tiny arrow) and slight oedema (e). D: ESC group; normal histological appearance. H.E



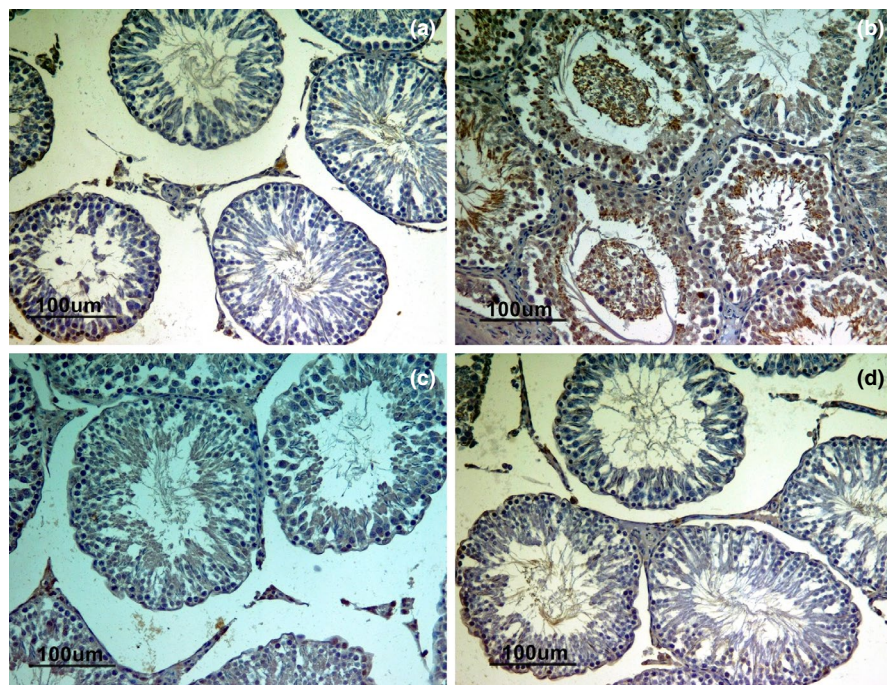


FIGURE 4 Microscopic view of Caspase-3 immunoreactivities: A: Control group, B: AlCl_3 group, C: AlCl_3 + ESC group D: ESC group. ABC peroxidase

damage produced by AlCl_3 (Sajjad et al., 2020). Therefore, it may be said that the protective effect of ESC on sperm parameters is due to its antioxidant properties and reduction of tissue damage.

Testosterone synthesised by Leydig cells as a result of stimulation by LH is essential for the initiation and maintenance of spermatogenesis (Mclachlan et al., 2002). Within this study, it was determined that the serum testosterone level of the rats in the AlCl_3 group was significantly decreased compared with the control group, while ESC co-treatment significantly prevented this decrease. In the ESC-only group, it was observed that ESC significantly increased serum testosterone level compared with the control group.

Testosterone is found in the blood bound to sex hormone-binding globulin or albumin and in a very small amount of free form. The albumin-bound and free forms of testosterone can be used by tissues. Therefore, determining total testosterone can be misleading (Yang et al., 2019). It has been stated in previous studies that the decrease in testosterone level may be due to the blocking of calcium channels by AlCl_3 . Ca^{2+} ions are necessary for the release of gonadotropin from the pituitary. Therefore, it has been reported that a decrease in gonadotropin release may cause a decrease in testosterone level (Akhigbe & Ige, 2012). In another study, the decrease in testosterone level is attributed to damage to Leydig cells as a result of aluminium

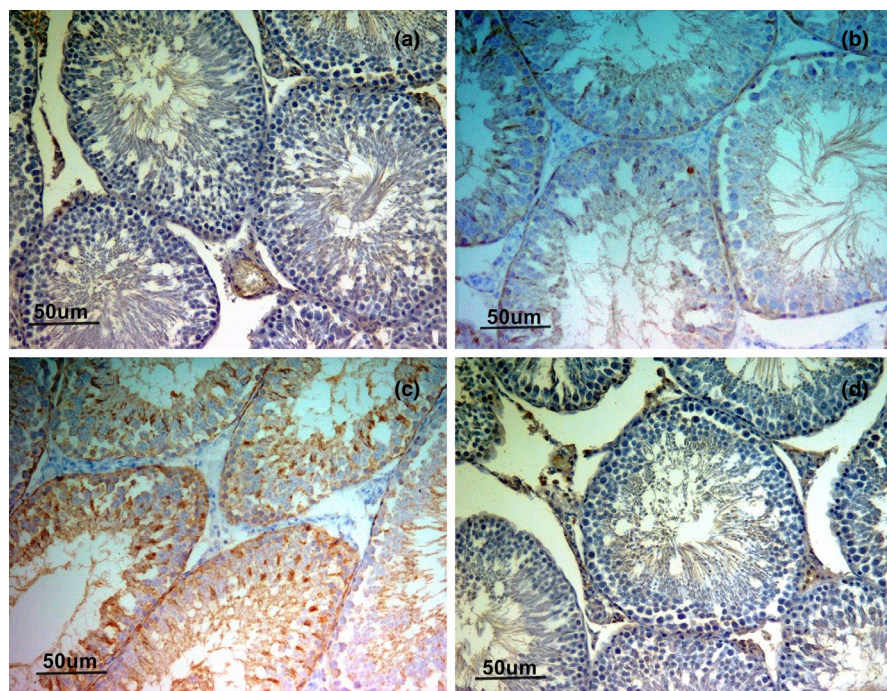


FIGURE 5 Microscopic view of Bcl-2 immunoreactivities: A: Control group, B: AlCl_3 group, C: AlCl_3 + ESC group D: ESC group. ABC peroxidase

accumulating in the testis (Mohammad et al., 2015). Le Person and colleagues determined the metal chelation property of ESC, and it was reported that the tendency to make a complex with Al^{3+} ions was high (Le Person et al., 2014). In this study, it was determined histopathologically that Leydig cells were damaged and ESC treatment resolved the damage in these cells. Therefore, it may be thought that ESC acts against the decreasing of the testosterone level induced by $AlCl_3$ both via protecting Leydig cells and chelating Al^{3+} ions.

The increase in lipid oxidation end products is associated with an increase in the number of free radicals. This indicates a decrease in endogenous antioxidant enzyme and GSH levels. Antioxidants such as GSH, GPx, CAT, SOD play important protective roles against oxidative stress. While GSH controls cellular redox changes related to ROS production, CAT and GPx take part in hydrogen peroxide catabolism (Ballatori et al., 2009; De Freitas et al., 2019; Uyar et al., 2018). In this study, it was determined that GSH level, CAT and GPx enzyme activities were decreased in the testicular tissues of the rats exposed to $AlCl_3$ compared with the control group, while the MDA level increased. It has been determined that exposure of $AlCl_3$ increases MDA level (Moselhy et al., 2012) and reactive oxygen species (Martinez et al., 2017), and it decreases GSH level, CAT and GPx activities in rats (Ghorbel et al., 2016). In previous studies, the increase in the MDA level, the decrease in the GSH level and expression and activities of antioxidant enzymes were inferred as an indicator that free radical-induced oxidative cell damages play an important role in $AlCl_3$ toxicity. It is emphasised that this feature of Al (3+), since its radius is similar to Fe (3+), causes the transfer of Fe (2+) by binding transferrin, and this may increase free radical production (Mohammad et al., 2015). Both oxidative stress parameters and histopathological data obtained from this study support this view. As a result of the administration of ESC to rats with $AlCl_3$, significant improvement was also observed in all data except CAT enzyme compared with the group treated with $AlCl_3$. It was also determined that, as a result of histopathological examination, deterioration in $AlCl_3$ -induced tissue morphology and decreased spermatogenesis were significantly prevented by ESC co-treatment. In diabetic rats, ESC treatment has been reported to increase GPx activity and GSH

levels (Prabakaran & Ashokkumar, 2013), and in another study, it has been reported to decrease MDA levels (Sulakhiya et al., 2016). In the previous studies, it was reported that ESC had a potential protective role in some diseases, which was linked to its potential as an antioxidant and intracellular ROS extinguishing agent (Kadacol et al., 2016). When the findings and information in the literature are evaluated together, it can be said that ESC exerts its antioxidant effect both by regulating enzymatic and nonenzymatic parameters and by acting as a direct ROS extinguisher.

In the study, the transcription levels of NRF-2, NF- κ B and TNF- α genes were examined by performing RNA isolation from testicular tissues in rats exposed to ESC and $AlCl_3$. NRF-2 plays an important role in protecting the cell against oxidative stress (Chang et al., 2020). Pan and colleagues reported that NRF-2 activity has a protective effect against damage to testicular tissue in diabetic rats (Pan et al., 2017). It was determined that NRF-2 gene transcription levels increased significantly in ESC-treated groups compared with the control group. However, a partial increase was also observed in the $AlCl_3$ group. In a study on rats, it was reported that NRF-2 was suppressed in the brain tissue after exposure to $AlCl_3$ (Sadek et al., 2019). Güvenç and colleagues reported that NRF-2 gene expression decreased in testicular tissues of rats exposed to $AlCl_3$ (Güvenç et al., 2020). Although these findings contradict with our results, it can be evaluated that the effect of $AlCl_3$ on NRF-2 expression affects the translation level rather than the transcription stage. NF- κ B is a significant transcription factor involved in many important aspects of cell life such as inflammation, immunity, cell proliferation, cell differentiation (Oeckinghaus & Ghosh, 2009; Turk et al., 2018). The transcription of NF- κ B increased significantly in the $AlCl_3$ group compared with the control group. Similar to our results, Khalifa and colleagues reported that $AlCl_3$ exposure caused an increase in NF- κ B level in rats (Khalifa et al., 2020). Besides, some increase was observed in the ESC and $AlCl_3$ + ESC groups. However, this increase was not statistically significant compared with the control group. In the previous study, it was reported that ESC did not cause a significant change in NF- κ B expression level compared with the control group (Anand et al., 2013). In our study, it was observed that there

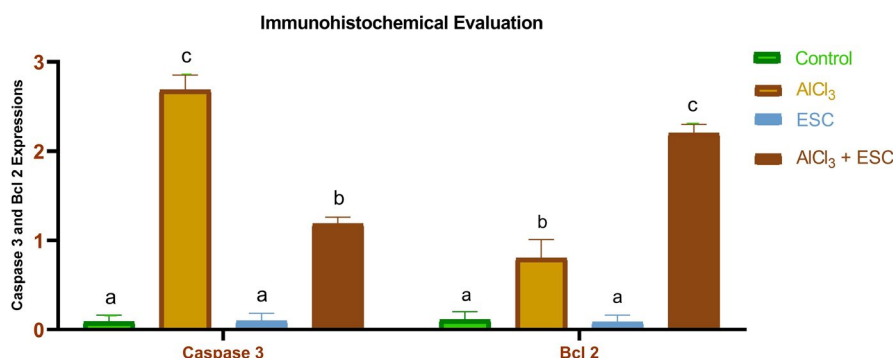


FIGURE 6 Distribution of Caspase-3 and Bcl-2 immunoreactivity scores by groups. Data represent the means $\pm$ S.E.M. of eight rats in each group. Tukey test was used to determine the differences among the groups. Graphs (a–c) show significant differences ($p < .001$) among each group. The lowest value is marked with 'a' and the sign letter is changed gradually when the statistical difference occurs

was a partial increase at the level of transcription. It is thought that this difference in the result obtained may be due to the duration of ESC treatment and the method used in the determination. In addition to this, the NF- κ B transcription level was partially decreased in the AlCl_3 + ESC group compared to the AlCl_3 group. It has been reported in another previous study that ESC has an anti-inflammatory effect partially by reducing NF- κ B expression, which supports the results obtained in this study (Ozal et al., 2018). TNF- α initiates the cascade that enables the activation of some cytokines involved in inflammation (Kuzu et al., 2019). In this study, it was observed that the transcription level of TNF- α decreased partially in the ESC group, while there was no significant change in the other groups. Zhang and colleagues reported that there was no increase in TNF- α transcription level in rats given AlCl_3 for 90 days at 50 mg/kg dose, but there was a significant increase when given at 150 mg/kg dose (Zhang et al., 2020). Besides, it was determined that ECS inhibits the expression of proinflammatory cytokines and decreases TNF- α transcription level in rat models with psoriasis (Chen et al., 2018).

While Caspase-3 leads the cell to apoptosis by causing proteolytic cleavage of most cellular targets (Benzer et al., 2018), Bcl-2 shows anti-apoptotic effect by preventing the opening of mitochondrial membrane pores (Kuzu et al., 2018). Caspase-3 and Bcl-2 expression levels in testicular tissues were examined immunohistochemically to determine the effect of ESC on apoptotic markers. According to the results obtained, it was seen that ESC treatment kept caspase-3 expression at the control group level while the expression of caspase-3 increased significantly in the rats treated with AlCl_3 compared to the control group. Also, it was seen that ESC administration with AlCl_3 significantly increased Bcl-2 expression compared with the AlCl_3 group. In their study, El-Kahtani and colleagues found that rats exposed to 30 mg/kg of AlCl_3 for 21 days increased the expression of caspase-3 and decreased Bcl-2 expression in liver tissues, which was interpreted as inducing apoptosis (Al-Kahtani et al., 2020). Since Caspase-3 is a marker protein in the apoptosis pathway, it has been reported that the increase in its level is a marker of apoptosis. It is emphasised that ESC has been shown to exhibit an anti-apoptotic effect by decreasing the expression of Caspase-3 in rats in which cerebral ischaemia-reperfusion was applied (Xu et al., 2019). In another study, it was reported that DOX-induced Bcl-2 reduction and cleaved caspase-3 increase were decreased by ESC, and ESC reduced apoptosis and ROS formation (Xu et al., 2017). According to these results, it can be said that ESC treatment shows the anti-apoptotic effect by reducing AlCl_3 -induced apoptosis. Although caspase-dependent apoptosis is often used synonymously with programmed cell death (PCD), recently, different PCD pathways from apoptosis have been identified (Kögel & Prehn, 2013). For these reasons, it should not be overlooked that ESC inhibits caspase-dependent apoptosis, however, PCD can occur through caspase-independent pathways.

Within the scope of this study, it was determined that AlCl_3 exposure caused reproductive toxicity in rats by showing toxic effects on sperm parameters, increasing oxidative stress, decreasing testosterone level, inducing apoptosis, causing inflammation and testicular tissue damage. It can also be said that ESC treatment prohibits

AlCl_3 -induced toxicity by preventing the decrease in testosterone level, preventing inflammation and apoptosis, by protecting the testicular tissue architecture, by showing a healing effect on antioxidant parameters, especially sperm parameters. It was also determined that ESC had a positive effect on sperm parameters in healthy rats. However, it is thought that further proven results can be obtained from other studies by applying different doses and times in the treatment of AlCl_3 and ESC, and it is necessary to perform protein level analyses with Western-blot method especially in the detection of inflammation markers.

One limitation of this study that needs to be addressed in later studies can be considered as examining the effect of only one dose of ESC. With further studies using different doses of ESC, the most effective ESC dose can be determined against AlCl_3 toxicity.

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CONFLICTS OF INTEREST

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author.

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