The relationship of 4-vinylcyclohexene diepoxide toxicity with cell death, oxidative stress, and gap junctions in female rat ovaries

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INTRODUCTION

Vinylcyclohexene diepoxide (VCD) is a metabolite of 4-vinylcyclohexene (VCH), which is frequently used in the industrial field and classified among carcinogens according to the International Agency for Research on Cancer (IARC) classification.1 VCD, a chemical intermediate, is a reactive diluent for epoxy resins and diepoxides.2 VCD, which is used commercially in many industrial areas, itself is also a carcinogenic agent according to Hazardous Substance Data Bank (HSDB) data. In the studies conducted by the National Toxicology Program (NTP) on the carcinogenic potential of VCD, it has been reported that it has ovotoxic effects in addition to...
its carcinogenic effects. VCD can trigger infertility in rodents and humans due to its toxic effects on reproductive organs. According to the National Institute for Occupational Safety and Health (NIOSH) and the American Conference of Governmental Industrial Hygienists (ACGIH) recommended, VCD exposure limit is 10-0.1 ppm averaged over a 10 to 8 hours work shift. These exposure limits are for air levels only. When skin contacts or orally exposure occur, it may be overexposed, even though air levels are less than the limits.

Exposure to commercial or environmental toxins causes serious fertility problems in women. Today, one of the biggest causes of increasing infertility is also the increase in exposure to these toxic agents day by day. The toxic effect of VCD can trigger premature ovarian insufficiency (POI) by causing the depletion of the primordial follicle pool in the ovaries. POI, one of the causes of female infertility, is amenorrhea and hypergonadotropic hypogonadism due to loss of ovarian function in women under 40 years of age. It is characterized by the complete depletion or dysfunction of the follicles in the ovaries before menopause. In vivo and in vitro experiments have shown that VCD can induce oocyte dysfunction and create toxic effects in the ovary. In order to increase annual rice production in Southeast Asia, VCD is recommended for suppression of fertility in rodents due to its toxic effect on fertility.

VCD usually causes loss of ovarian follicles by inducing apoptosis. In experimental studies conducted by many researchers on VCD toxicity in the ovary, it is reported that it causes the death of granulosa cells by activating the caspase-mediated apoptosis pathway. VCD triggers apoptosis by increasing the ratio of Bax/Bcl-XL and by increasing the amount of cytochrome c and caspase 3 expression in the cytoplasm. It is also reported that VCD triggers apoptosis via JNK and p38 molecules. The reason why VCD affects especially primordial and primary follicles is related to kit/kitL molecules. The kit/kitL pathway is important for survival in primordial and primary follicles. VCD toxicity prevents KIT autophosphorylation and blocks it to bind to KITL. As a result of this, the apoptotic pathway in the follicles is activated and follicular atresia occurs.

In some studies, it has been shown that VCD increases ovarian degeneration by increasing oxidative stress with lipid peroxidation and hydrogen peroxide formation and by inhibiting the formation of antioxidants. In studies related to the reproductive system, it has been reported that although a certain amount of ROS is needed during follicle development and ovulation, abnormally increased oxidative stress and inflammation inhibit follicle development and cause degeneration in the ovary. It has been also reported that increased oxidative stress and inflammation in the ovary activate apoptotic pathways in follicular cells.

In the ovary, the main functions of connexins, which are gap junction proteins, are taking charge in folliculogenesis, oogenesis, meiotic arrest, steroidogenesis, and apoptosis. It is known that Cx43, which is the most common type of gap junction protein in the ovary, is expressed in granulosa cells and is responsible for the communication between granulosa cells. There are important studies showing that Cx43 is an effective protein in apoptosis. It has been reported that gap junctions in many tissues and organs have negative roles in the apoptotic pathway. Especially, it has been shown that Cx43 is inversely proportional to apoptosis and is necessary for cell survival. Increased apoptosis in ovarian follicles also reduces Cx43 expression. In studies conducted on different tissue types and cells, it has been stated that with the decrease or inhibition of Cx43 expression, the amount of ROS increases in cells and as a result, apoptosis is induced.

The purpose of this study was to demonstrate the relationship between the oxidative stress marker iNOS, the effector protein of apoptosis Casp3, and the gap junction protein Cx43 in rats exposed to VCD toxicity by histopathological, immunohistochemical, and RT-qPCR analyses.

# 2 MATERIALS AND METHODS

## 2.1 Animals

In this study, twelve-week-old adult female Wistar rats, weighing 180 ± 50 g and obtained from Manisa Celal Bayar University Experimental Animal Application and Research Center, were used. Rats were kept for 3 days (for adaptation) against any signs of health problems. Animals were kept under stable conditions (22°C temperature, 30%-70% humidity, light/dark cycle 12/12 hours) throughout the study. The rats were fed with dry pellets and tap water. This study was conducted with the approval of the Local Ethics Committee of Animal Experiments of Manisa Celal Bayar University Faculty of Medicine, dated 13/03/2018 and numbered 77.637.435.

## 2.2 Experimental design

Rats were divided into 3 groups as control (n = 5), sham (n = 7), and VCD (n = 7). In the control group, the rats were fed with sufficient food and water for 24 days, without any application. In the sham group, DMSO, used as the diluent of VCD, was given to rats i.p for 5 days. In the VCD group, 240 mg/kg VCD was diluted in DMSO and it was given to rats via i.p for 5 days.

## 2.3 Hormone assay

The cardiac blood taken from the rats was kept for 15 minutes and sedimented. Then, it was centrifuged at 4000 rpm for 12 minutes, and serum was separated. ELISA analysis was performed using Bioassay Technology Laboratory AMH ELISA Kit (BT-Lab, E0456Ra) according to the protocol of the manufacturer company.

## 2.4 Histopathological analysis

Left ovary tissues taken after dissection were fixed in 10% formalin for at least 48 hours. Tissues kept in running water for 1 night and
were passed through increasing alcohol series (60%, 70%, 80%, 90%) for dehydration processes. Tissues passed through toluene for transparency were embedded in paraffin blocks using the Leica HistoCore Arcadia. Then, 5-μm-thick sections were taken from the paraffin blocks by the microtome device (Leica RM 2245). Sections were taken from all rats belonging to whole groups and stained with H&E and Masson trichrome for histopathological examinations. Primordial, primary, secondary, Graaf follicles, atretic follicles, and corpus luteums in the ovary were detected by light microscope (DM 750 Leica) and photographed with a camera (DP71 Olympus). Follicles were counted in every 20th serial section to avoid double counting of small follicles. Only follicles with a distinct oocyte nucleus were counted. The classification of ovarian follicles is as follows: primordial follicles, a single layer of squamous follicular cells around the oocyte; primary follicles, a single or more than two layers of follicular cells; secondary follicles, two or more layers of granulosa cells with a small antrum; graaf follicles, a fluid-filled antrum with a distinct cumulus oophorous and corona radiata; and atretic follicle, follicle structure misshapen, condensed chromatin in oocyte nuclei, or contact lost between the oocyte and granulosa cells.\(^5\)\(^,\)\(^2\)\(^3\) In all groups, to indicate ovarian damage, criteria such as hemorrhage and congestion, follicular cell degeneration, and vacuolization were determined. Five different areas in each ovary tissue of each group were evaluated. Histopathologic findings in ovarian tissues of all subjects of each group were scored between 0 and 3 for the mentioned criteria: (0: none, 1: mild, 2: moderate, and 3: severe).\(^2\)\(^4\) Finally, statistical analysis of scores was performed for evaluation.

### 2.5 Immunohistochemical staining

In immunohistochemical staining, 5-μm-sections were incubated for overnight in a 60°C incubator to dissolve excess paraffin. Then, the tissues kept in xylene were passed through the decreasing alcohol series for rehydration and washed with distilled water and PBS, respectively. 3% hydrogen peroxide was added for the endogenous peroxide inactivation. Then, sections were incubated with 0.5% trypsin at 37°C for 15 minutes at room temperature. Blocking solution was added onto the sections washed with PBS. Sections were incubated with primary antibodies anti-NOS2 (sc-7271, Santa Cruz), anti Cx43 (sc-271837, Santa Cruz), and anti-Caspase3 (sc-56053, Santa Cruz) at +4°C for overnight. The next morning, the tissues were washed with PBS, and biotinylated secondary antibody was added and waited for 10 minutes. The tissues were then washed three times with PBS again, and streptavidin peroxidase was added. After this step, the tissues were washed three times with PBS again and incubated with DAB to observe the immune reaction, and counterstaining was performed with Mayer’s hematoxylin. Finally, the sections were washed with distilled water, passed through the increasing alcohol series, and covered with entellan. For all antibodies, tissues were evaluated considering the staining intensity in 10 ovary sections of each animal, in 30 areas in the X40 objectives.

### 2.6 RNA extraction and RT-qPCR analysis

RNA was isolated from the right ovary tissues using the RiboEX total RNA isolation kit according to the manufacturer’s protocol. Amount and purity of RNAs were measured with NanoDrop ND-1000 Spectrophotometer (Thermo Fisher Scientific). WizScript cDNA synthesis kit (W2211, Wizbiosolutions) was used to synthesize cDNA from the obtained RNAs. cDNA synthesis was carried out using cDNA synthesis kit by adding RNA from the obtained RNA into the kit in a way that the final concentration would be 1 μg/μL. Caspase 3 and connexin 43 mRNA levels from the obtained cDNAs were analyzed by qPCR using appropriate primers. Sequences for specific primers are listed in Table 1. The experiment was carried out using the WizPureTM qPCR Master (SYBR) (W1711, Wizbiosolutions) kit in accordance with the manufacturer's protocol. At the end of the experiment, Ct value (threshold cycle, number of cycles passing the threshold) was obtained for each sample. ΔCt values were calculated by normalizing the Casp3 and Cx43 mRNA levels of each sample according to the reference gene GAPDH mRNA expression. The values obtained were shown in the graph as fold change values using the 2\(^{-\Delta\Delta Ct}\) formula. Each sample of each group was studied in triplicate.

### 2.7 Statistical analysis

For comparisons between all groups, the normality test (Shapiro-Wilk) was applied to the groups using the GraphPad® Prism 8.3.1 program. After the data showed normal distribution, one-way ANOVA test was used to statistically compare the difference between the groups. The significance level of \(P ≤ .05\) value was considered as significant in statistical evaluation results. Tukey’s test was used as a post hoc test for multiple comparisons.

### 3 RESULTS

#### 3.1 Morphological evaluation

H&E and Masson trichrome staining was performed for the morphological evaluation of VCD toxicity in the ovary. Thus, ovarian follicles in all groups were counted and histopathological findings were evaluated. In the VCD group, a significant reduction and degeneration was observed in all follicle types, especially in the
primordial and primary follicles, compared to the control and sham groups (Figure 1). Although there was a decrease in the number of corpus luteum in the VCD group compared to the control and sham groups, this decrease was not statistically significant. In the H&E staining performed for histopathological evaluation of ovarian tissue, the cortex and medulla had a regular organization in the ovary sections of the control group. In the cortex, germinal epithelium consisting of single-layered cubic cells at the outermost and just under it, tunica albuginea consisting of tight connective tissue were observed. Under the tunica albuginea, ovarian follicles, and ovarian stroma, formed by connective tissue around it, were seen (Figure 2A-C). The results obtained from the sham group ovary tissue were similar to the control group (Figure 2E-G). Table 2 shows histopathological scores for all groups. Significantly ($P < .033$) higher scores were found in the VCD group in terms of hemorrhage and congestion, follicular cell degeneration, vacuolization, and increased collagen fibers compared to the control and sham groups (Table 2). Very few hemorrhagic areas were observed in the control and sham groups. In the VCD group, hemorrhages and congestions were observed in many areas in the cortex and medulla (Figure 2I,J). Follicular cell degenerations were found less in the control and sham groups. In the VCD group, significantly increased follicular cell degeneration was observed in each follicle type (Figure 2K). Increased vacuolization was observed in follicles and stromal cells in the VCD group compared to the control (Figure 2L). In the Masson's trichrome staining, stained collagen fibers were seen in the tunica albuginea and in theca layers of the developing follicles in the control and sham groups (Figure 2D,H). There were also small amounts of stained collagen in connective tissue areas in the medulla. Significantly increased collagen fibers were observed in both cortex and medulla in the VCD group compared to the control (Figure 2L).

3.2 | Immunohistochemical evaluation

The toxic effect of VCD on the ovary was evaluated by immunohistochemical staining performed for Casp3, iNOS, and Cx43. In the current study, the oxidative stress marker iNOS immunoreactivity showed a statistically significant increase in the VCD group compared to the control and sham groups (Figure 3B). There was no statistically significant difference between control and sham groups. VCD group had strong positive cells in all follicle types, corpus luteums, and stroma (Figure 3A). Casp3 expression was significantly increased in VCD group, especially in primordial and primary follicles, compared to the control group (Figure 4A). The increase in secondary and Graaf follicles was also statistically significant (Figure 4B). In the VCD group, especially in primordial and primary follicles, Casp3 expression was found to be very intense in both granulosa cells and oocytes. The immunoreactivity of the gap junction protein Cx43 was found to be strong in the control and sham groups, especially in antral follicles, and also in preantral follicles. In the VCD group, Cx43 immunoreactivity was observed slightly in primordial, primary, secondary, Graaf follicles, corpus luteum, and atretic follicles (Figure 5A). This decrease in the VCD group was found to be statistically significant (Figure 5B). When looking at all groups, Cx43 expression was observed more locally (at cell membrane) in small and developing follicles, while an intense staining was observed in all areas in the control and sham groups due to the high expression of Cx43 in preovulatory follicles. However, in the VCD group with less Cx43 expression, in small and developing follicles, which are the direct target of VCD, and in preovulatory follicles that are not to be the direct target of VCD, Cx43 expression was found to be more local. In addition, when the data sheet of the antibody used in this study was examined, such cytoplasmic stainings were observed.

3.3 | Hormone assay and RT-qPCR analysis

There was no difference in serum AMH level in the sham group compared to the control group. On the other hand, it was determined that in the VCD group, serum AMH level was significantly decreased (Figure 6). In the RT-qPCR results, Casp3 and Cx43 mRNA levels were evaluated for all groups. It was observed that the level of Casp3 mRNA increased and the level of Cx43 mRNA

![FIGURE 1](image-url) The number of ovarian follicles among groups was counted. ***$P < .001$, **$P < .002$, and *$P < .033$
It has been shown in our study and in different studies that exposure to VCD, which is an occupational chemical, triggers ovarian damage. The exposure of many women, working in the industrial sector, to this chemical, which is frequently used in the industrial field, causes infertility cases to increase day by day. The fact that VCD, which is known to have carcinogenic effects, also triggers infertility makes VCD a chemical that should definitely be avoided in terms of human health. Studies showing the effects of VCD on fertility have generally reported that apoptosis is the main mechanism, but there are no sufficient studies showing VCD toxicity in the ovary. VCD accelerates the degeneration especially in primordial and primary follicles and may trigger POI as a result of the depletion of ovarian follicles. Kappler et al. 8 state that there is a high amount of KIT expression in primordial and primary follicles and KIT plays a role as a survival factor in these follicles. In studies conducted with VCD, it has been reported that VCD makes an antisurvival effect especially in primordial and primary follicles by reducing BMT expression. 25 The fact that primordial follicles were damaged more than developing follicles in the VCD group was reported in our study and some other studies. 8 It is thought that since complete depletion of the primordial follicle pool is very difficult to reverse compared to developing follicle destruction, overexposure to VCD can trigger irreversible infertility.

Until now, no study has been found to show histopathological changes such as hemorrhage, congestion, vacuolization, and fibrosis caused by VCD exposure in ovarian tissue. Therefore, histopathological findings such as hemorrhage, congestion, vacuolization, and fibrosis triggered by VCD exposure were clearly shown in this study. There is no information about hemorrhage caused by VCD in the ovary. However, increased hemorrhage in some organs such as the liver and kidney as a result of VCD toxicity has been reported. 26 In studies conducted with different agents that cause toxicity in the ovary, it has been stated that the reason for increased hemorrhagic

**TABLE 2** Histopathologic evaluation scores of the ovarian tissues in all groups. Data are presented as mean ± standard deviation (SD)

|                | Hemorrhage and Congestion | Follicular Cell Degeneration | Vacuolization | Increasing Collagen Fibers |
|----------------|---------------------------|------------------------------|---------------|---------------------------|
| Control        | 0.8 ± 0.79                | 0.6 ± 0.7                    | 0.4 ± 0.52    | 1.1 ± 0.88                |
| Sham           | 1.1 ± 0.74                | 0.9 ± 0.74                   | 0.4 ± 0.52    | 1.3 ± 0.74                |
| VCD            | 2.7 ± 0.48<sup>a</sup>    | 2.4 ± 0.84<sup>a</sup>       | 2.5 ± 0.53<sup>a</sup> | 2.4 ± 0.70<sup>b</sup> |

<sup>a</sup>P < 0.001.  
<sup>b</sup>P < 0.002.
areas and congestion in the ovary is that oxidative stress triggers the tissue damage. In this study, the increase in iNOS expression as a result of VCD exposure is a marker of the increased oxidative stress in the ovary. It can be said that hemorrhage caused by VCD in ovarian tissue is also due to oxidative stress. Fibrosis in the normal ovary has often been reported as a condition that occurs with old age, and it is said that increased fibrosis may also be associated with inflammation. In different studies related to the ovary, it has been reported that the amount of collagen fiber increases as a result of inflammation. In this study also, it is thought that fibrotic areas increased as a result of VCD exposure are the result of inflammation triggered by increased oxidative stress.

Hormonal evaluations are at the top of the clinical diagnosis criteria of ovarian damage. The fact that VCD toxicity also caused hormonal changes in the ovary was shown with the decreased serum AMH amount in the VCD group. Serum AMH hormone was also evaluated as evidence of VCD toxicity in the ovaries. AMH is one of the most important markers of ovarian reserve produced by granulosa cells of preantral and small antral follicles, and generally, AMH level is associated with primordial follicle numbers. Also, the AMH level is more sensitive than FSH level, and it is an important indicator for early diagnosis of POI since it can be measured at any stage of the menstrual cycle. In a study conducted by Tran et al. on VCD toxicity in the ovary, it was reported that AMH was released from normally developing follicles and protected the primordial follicle pool by suppressing the return from the primordial follicle to the primary follicle. They stated that the inability to release sufficient AMH from the developing follicles leading to atresia after VCD induction caused the depletion of the primordial follicle pool. Steroid hormones also decrease with the effect of VCD. VCD also suppresses steroid hormone production by triggering atresia in ovarian follicles. In the literature reviews, it was reported that progesterone and estrogen decreased with the effect of VCD.

In this study, protein and mRNA expressions of iNOS, Casp3, and Cx43 molecules that are thought to be effective in the formation mechanism of the ovarian follicles damage caused by VCD toxicity were analyzed. Like our study, Özel et al. observed high iNOS expression in rats in the VCD-induced POI model. They stated that despite the increased iNOS expression, the expression of eNOS decreased in the VCD group. Mantawy et al. stated that the increased TNF-α levels in POI triggered the increase in iNOS expression and that follicular atresia occurred because of inflammation. Tokmak et al. found an increase in iNOS, total antioxidant status (TAS), and total oxidant status (TOS) levels of 44 women with POI in their study. In this case, exposure to VCD is also thought to trigger ovarian follicles damage in relation to oxidative stress and inflammation.

Similar to our study, Hu et al. found increased Casp3 expression in the primordial and primary follicles by VCD exposure in rats.
Takai et al.\(^7\) also reported that VCD toxicity in mice is an important agent in the loss of small follicles in the ovary. As stated by Liu et al.\(^25\), the main reason why primordial and primary follicles are targeted in VCD toxicity is that the KIT-KITL interaction, which provides the anti-apoptotic mechanism in these follicles, is suppressed as a result of VCD toxicity and thus induces the apoptotic pathway. The fact that VCD triggers oxidative stress and inflammation in the ovary ensures the activation of the apoptotic pathway.

It has been emphasized in many studies that Cx43 is effective not only as a channel protein that provides intercellular signal transmission, but also in important processes such as folliculogenesis, oogenesis, steroidogenesis, and apoptosis.\(^{17,35}\) The Cx43 protein can directly control cell death on its own, independent of its channel-related function.\(^{19}\) There are important studies showing that Cx43 is an effective protein in apoptosis. In the literature, there is no study on the relationship of Cx43 with the apoptotic pathway in VCD-induced ovarian damage. Krysko et al. reported that Cx43 expression in granulosa cells was inversely proportional to the apoptotic index and that Cx43 played an important role in the survival of granulosa cells. In the same study, the researchers reported that intercellular apoptotic signals were transmitted between each other through gap junctions; therefore, the gap junction protein expressions increased in apoptosis, but Cx43 regulated the survival of cells rather than the channel function through which death signals were transmitted.\(^{20}\) In many different tissue types, the decrease in Cx43 and the increase in ROS formation induced cell death.\(^{21}\) In this study,
with increased Casp3 expression, we showed that increased oxidative stress due to VCD exposure decreased Cx43 expression important for survival in granulosa cells and increases apoptosis in cells. Thus, thanks to this study, the relationship of VCD toxicity to Cx43 oxidative stress and apoptosis in damaged ovary was evaluated for the first time. So far, Casp3 and Cx43 mRNA levels have not been demonstrated in studies conducted with VCD toxicity. In this study, for the first time, Casp3 and Cx43 mRNA levels in ovarian damage induced by VCD toxicity were demonstrated by RT-qPCR analysis. It was determined that the obtained results showed correlation with IHC results.

It has been shown in the literature that even a single dose of VCD exposure causes irreversible follicle damage in the ovary. In the literature, there are different dosage (80-160 mg/kg doses for 10-15 days) in studies on the gonadotoxic effects of VCD, and these doses are known as low and average doses. In our study, similar to Sahambi et al., accelerated exposure to high dose was applied. Especially in the case of women working in the industrial sector exposed to high VCD toxicity, albeit for a short time, it was aimed to investigate the damage to the ovary. In particular, it was desired to observe the damage caused by exposure to high-dose VCD. In addition, when the experiment was designed, doses higher than 240 mg/kg were planned, but these doses were not safe in preliminary studies, and it was seen that the animals died. Sahambi et al showed in their study, they stated that after 5 days of exposure to 240 mg/kg VCD, a decrease in ovarian follicles (especially primordial and primary follicle) from the 15th day of the experiment. They also expressed that all ovarian follicles except antral follicles disappeared.
on the 100th day of the experiment. In addition, in some different studies, it was determined that VCD caused a significant damage to the ovarian follicles on the 10th day at the earliest. In our study, the reason why Casp3 and Cx43 mRNA and protein levels changed at the end of 20 days compared to the control is evidenced that short-term high-dose VCD exposure triggers almost irreversible ovarian damage. In addition, VCD affects the oocyte as well as the granulosa cells. It shows this effect by downregulating the KIT/KITL pathway, which is especially necessary for the survival of primordial/primary follicles. Thus, it triggers the apoptosis of both oocyte and granulosa cells. The follicle that goes to atresia is also prevented from being ovulated. Therefore, VCD exposure can cause infertility by triggering the depletion of ovarian follicles at an early age. Since changes in granulosa cells were evaluated in our study, the changes in the oocyte were not mentioned much. However, damaged, apoptotic oocytes were observed within the increased atretic follicles observed in the VCD group.

In conclusion, the pathologies caused by VCD toxicity in the ovary were comprehensively shown in this study. In our study and in different studies in the literature, VCD has been reported to increase inflammation and oxidative stress in the ovary. It is known that increased oxidative stress triggers apoptosis in the ovary as in many tissue types. The fact that connexin 43 is responsible for the survival of granulosa cells in the ovary, independent of the canal function, makes it an important molecule in ovarian damage. It has been reported in the literature that oxidative stress suppresses gap junction proteins in different tissue types. As a result of our study, it has been shown for the first time that VCD exposure suppresses cx43, which is an important protein in the survival of granulosa cells, by increasing oxidative stress, may be a cause of increased apoptosis in cells. However, this relationship between gap junction proteins and oxidative stress is still unknown. With further studies, the link between gap junction proteins and oxidative stress and apoptosis needs to be elucidated. Additionally, a toxic agent such as VCD is thought to affect many important molecules in ovarian physiology. Considering that we are knowingly or unknowingly exposed to environmental toxic agents such as VCD in many areas of our lives, there is an urgent need for every study in this area. Therefore, the effects of VCD toxicity in the ovary should be revealed in more detail with comprehensive studies.

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CONFLICT OF INTERESTS
The authors declare no conflict of interests.

HUMAN RIGHTS STATEMENTS AND INFORMED CONSENT
This work does not contain human subjects.
ANIMAL STUDIES AND APPROVAL BY ETHICS COMMITTEE
All institutional and national guidelines for the care and use of laboratory animals were followed. All procedures and protocols were approved by the committee on animal research at Manisa Celal Bayar University, Manisa, Turkey (approval number: 77.637.435).

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