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EFFECTS OF ALBUMIN ADMINISTRATION ON CYTOCHROME C-1 (CYC1) IN ISCHEMIA-REPERFUSION DAMAGED RAT OVARY

İSKEMİ-REPERFÜZYON HASARLI RAT OVARYUMUNDA ALBÜMİN UYGULAMASININ SITOKROM C-1 (CYC1) ÜZERINE ETKİLERİ

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ABSTRACT

This study aimed to examine the effects of albumin administration on ischemia-reperfusion in the rat ovary by using biochemical, histological, and immunohistochemical methods. Thirty-two *Wistar albino* rats were used in the study, and they were divided into 4 groups: control, albumin, placebo, and ischemia-reperfusion. Healthy ovaries were taken from the first group. In the other three groups, 2-hour ischemia and 2-hour reperfusion were applied to the bilateral ovaries. In the albumin group, intraperitoneal albumin (2.5 g/kg, 20% human albumin) was administered 30 minutes before reperfusion, and in the placebo group, the same volume of intraperitoneal saline was administered instead of albumin 30 minutes before reperfusion. Ovarian damage scores, cytochrome C-1 immunoreactivity, total oxidant status, total antioxidant status, and oxidative stress index levels were evaluated. In the statistical analysis performed between the groups, it was seen that the results of the control group were significantly lower than the ischemia-reperfusion group in terms of total oxidant status values ($p=0.001$), and the results of the ischemia-reperfusion group were significantly higher than the control and albumin groups in terms of oxidative stress index values ($p<0.001$ and $p=0.004$, respectively). In histological examinations, the total damage score obtained by evaluating follicular degeneration, edema, vascular congestion, and hemorrhage was found to be significantly higher in the ischemia-reperfusion group than in the control group ($p=0.003$). According to the immunohistochemical examination results, cytochrome C-1 immunoreactivity in the ischemia-reperfusion group was significantly stronger than the control and albumin groups ($p<0.001$). We think that albumin administration reduces cytochrome C-1, reactive oxygen species, and oxidative stress levels, therefore it will play a helpful role in the ischemia-reperfusion treatment process.

Keywords: Cytochrome C-1, human serum albumin, immunohistochemistry, ischemia-reperfusion, Ovary.

ÖZ

Bu çalışma, albumin uygulamasının rat ovaryumunda iskemi-reperfüzyon üzerine etkilerini biyokimyasal, histolojik ve immune histokimyasal yöntemlerle incelemeyi amaçladı. Çalışmada 32 adet *Wistar albino* rat kullanıldı. Hayvanlar 4 gruba ayrıldı: kontrol, albümin, placebo ve iskemi-reperfüzyon. Birinci gruptan sağlıklı overler alındı. Diğer 3 grupta overlere bilateral 2 saatlik iskemi ve 2 saatlik reperfüzyon uygulandı. Albümin grubuna reperfüzyondan 30 dakika önce intraperitoneal albumin (2.5g/kg, %20 human albumin), placebo grubuna reperfüzyondan 30 dakika önce albumin yerine aynı hacimde intraperitoneal salin verildi. Yumurtalık hasar skorları, Sitokrom C-1 immüno reaktivitesi, toplam oksidan durumu, toplam antioksidan durumu ve oksidatif stress indeksi değerleri değerlendirildi. Gruplar arasında yapılan istatistiksel analizlerde toplam oksidan durumu değerleri açısından control grubu sonuçları iskemi-reperfüzyon grubuna gore belirgin olarak düşük olduğu ($p=0.001$) ve oksidatif stress indeksi değerleri açısından ise iskemi-reperfüzyon grubu sonuçlarının control ve albumin gruplarına gore anlamlı olarak yüksek olduğu görüldü ($p<0.001$ ve $p=0.004$, sırasıyla). Histolojik incelemelerde folikül dejenerasyonu, ödem, damar tıkanıklığı ve hemoraji değerlendirilerek elde edilen toplam hasar puanı IR grubunda control grubuna gore anlamlı olarak yüksek bulundu ($p=0.003$). İmmüno histokimyasal inceleme sonuçlarına gore iskemi-reperfüzyon grubundaki sitokrom C-1 immün reaktivitesinin control ve albumin gruplarına gore anlamlı derecede daha güçlüydü ($p<0.001$). Albümin uygulamasının sitokrom C-1, oksijen radikalleri ve oksidatif stress düzeylerini düşürdüğünü, bu nedenlei skemi-reperfüzyon tedavi sürecinde yardımcı bir rol oynayacağını düşünüyoruz.

Anahtar kelimeler: Sitokrom C-1, insan serum albümini, immünohistokimya, iskemi-reperfüzyon, ovaryum, .

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INTRODUCTION

Ovarian torsion is the sixth most frequent gynecological emergency. The therapy is to execute detorsion to restore blood flow.[1-3]Delayed diagnosis and treatment of ovarian torsion in women results in necrosis and organ loss in the ovaries.[4] Cell damage is caused by reactive oxygen species (ROS) generated during torsion-induced ischemia.[5] The ROS is one of the major causes of tissue damage.[6] Reperfusion is required to repair ischemia-induced tissue damage and to eliminate harmful metabolites from the organ. However, it is well known that reperfusion causes further damage.[7,8] Detorsion causes the movement of polymorph nuclear leukocytes and platelets toward ischemic tissue, follicular cell degeneration in the ovaries, and interstitial and intrafollicular hemorrhages. Furthermore, this condition accelerates the production of ROS.[1,2]

One of the most essential components that triggers apoptosis is Cytochrome C (Cyt-C), which initiates apoptosis by moving from the mitochondria to the cytoplasm. CYC1, which is also known as isoform-1 cytochrome C, is the homolog of cytochrome C1. Because of this reason, CYC1 is a marker for the existence of the apoptotic pathway.[9] ROS, which is created in excess as a result of ischemia-reperfusion (IR), disrupts mitochondrial functioning.[10] The release of CYC-1 from the mitochondria into the cytoplasm is increased when the mitochondrial functioning is disrupted.[9]

Albumin, which also possesses antioxidant capabilities, is the most abundant protein in the plasma and is responsible for the majority of the ROS capture. Albumin also protects against damage caused by lipid peroxidation and peroxyl radicals.[11] In addition, it reduces reperfusion-induced inflammatory response by improving microcirculation and preventing organ dysfunction.[12]

Here, we aimed to examine the effects of albumin administration on ischemia-reperfusion in the rat ovary by using biochemical, histological, and immunohistochemical (IHC) techniques.

MATERIALS AND METHODS

Animals

This study was imposed in accordance with the "Care and Use of Laboratory Animals Guidelines" in the Animal Experiments Laboratory of Kafkas University. The permission for the experiment was given from the Animal Experiments Local Ethics Committee of Kafkas University (decision date/numbered; 23-09-2021 / 2021-138). For the study, 32 female *Wistar albino* rats (8-12 weeks aged, weighing 180-260 g) were utilized. They were fed ad libitum and kept in a room that had a 12-hour day-night cycle and 22 ± 2.

Groups

In this study, thirty-two rats were randomly and equally divided into four groups.

Control group: After anesthesia, healthy ovarian tissues were bilaterally excised.

Albumin group: After anesthesia, 2-hour ischemia and 2 -hour reperfusion were done. 30 minutes before the initiation of reperfusion, 2.5 g/kg 20% human albumin (12.5 ml/kg) was injected intraperitoneally.[13] The ovarian tissues were extracted bilaterally at the end of the reperfusion period.

Placebo group: After anesthesia, 2-hour ischemia and 2-hour reperfusion were done. 30 minutes before the initiation of reperfusion, 12.5 ml/kg saline was injected intraperitoneal. The ovarian tissues were extracted bilaterally at the end of the reperfusion period.

IR group: After anesthesia, 2-hour ischemia and 2-hour reperfusion were done. The ovarian tissues were extracted bilaterally at the end of the reperfusion period.

Anesthesia

According to the body weight, 10 mg/kg xylazine (Vetaxyl®, Vet-Agro) and 90 mg/kg ketamine (Keta-Control®, Doa Pharmaceuticals) were administrated by intramuscular injection for anesthesia.

Surgical method

After anesthesia, all rats were found supine, and a 2 cm abdominal incision was performed after the surgical site was shaved and sterilized. The abdomen was closed after the adnexa including the tuba and ovarian arteries was ligatured with a 3/0 silk suture.[14] The abdomen was opened again, the ligature around the adnexa was removed for reperfusion, and the abdomen was closed again. After reperfusion, the abdomen was opened and tissue and blood samples were taken.

Biochemical analysis

The blood serum was used to test albumin, calcium, total oxidant status (TOS), total antioxidant status (TAS), and oxidative stress index (OSI). TOS and TAS were determined using Erel's automated colorimetric technique (Rel Assay Diagnostics®, Mega Tıp, Türkiye).[6] TOS values are reported in μmol H_2O_2Eq/L units. TAS levels are reported in mmol Trolox Eq/L. OSI is the ratio of TOS to TAS in μmol [(TOS/(TAS*1000))*100], and it is an indicator of oxidative stress levels. Albumin and calcium were determined using a colorimetric technique (Otto Scientific®, Mega Tıp, Türkiye) with a spectrophotometer. Albumin levels are reported in g/dL. Calcium levels are reported in mg/dL.

Histological analysis

After follow-up procedures, the ovarian tissues were preserved in 10% formalin and embedded in paraffin blocks.[3] Serial sections at 5 μm thickness were taken from these blocks by using a microtome (Leica RM2125RTS).Haematoxylin-eosin (H&E) was applied and taken photos by using a light microscope (Olympus Bx53, Tokyo, Japan). Two slides of every subject and five- fields in each slide were examined and scored at 10x magnification to determine the ovaries' tissue damage levels and scored, which were scored by considering follicular cell degeneration, hemorrhage, vascular congestion, and edema (0=none, 1=weak, 2=moderate, 3=strong).The total score of tissue injury was calculated by adding these scores.[1]The follicle degeneration was evaluated in the primordial, primary, secondary, and graff follicles.

Avidin-biotin-peroxidase complex (ABC) staining was used for IHC staining. We employed a 1:100 dilution of a monoclonal CYC1 primary antibody (Elabscience, E-AB-40271). CYC1 IHC was applied and taken photos by using a light microscope (Olympus Bx53, Tokyo, Japan). Two slides from each subject and five fields on each slide were examined at 10x magnification and scored to determine the intensity of CYC1 immunoreactivity(0=none, 1=weak, 2=moderate, 3=strong).[3]

Statistical Analysis

The data from our study were analysed using the IBM SPSS Statistical version 25 (IBM®, USA). The means and standard deviations of the datawere calculated. Then, a one-way ANOVA test was used to compare biochemical data between groups. Post-hoc Türkiye test was used for multiple comparisons in one-way ANOVA. Median, minimum and maximum values were determined in histological and immunohistochemical score evaluations. Then, the Kruskal-Wallis test was used for comparisons between groups, and the Mann Whitney-U test was used for pairwise comparisons of groups. In comparisons between groups, statistical significance was accepted as $p<0.05$. In pairwise comparisons of groups, the p value was considered significant at $p\leq0.008$ (p value/number of tests=0.05/6) by applying Bonferroni correction to avoid type 1 error. Since follicle degeneration values of both control and albumin groups were zero, they were excluded from the analysis.

RESULTS

Biochemical findings

The results of biochemical tests are shown in the table (Table 1). TOS values of the control group were significantly lower than those of the placebo and IR groups (p=0.008, p=0.001, respectively). Again, the OSI values of the control group were significantly lower than the placebo and IR groups (p=0.019, p<0.001, respectively). TOS values of the albumin group were significantly lower than the IR group (p=0.018). Again, the OSI values of the albumin group were significantly lower than those of the IR group (p=0.004). Albumin and placebo groups were similar in terms of TOS and OSI scores. Calcium, albumin, and TAS values did not show a statistically significant difference between the groups.

Histological findings

In the control group, hemorrhagic patches and edema were seldom found surrounding the follicles. In the cortex, there was little vascular congestion. The control group showed no evidence of follicle degeneration. In the medulla region, there were minor hemorrhagic patches and vascular congestion (Figure 1, A-B).

Hemorrhagic areas and minor vascular congestion were found in the cortex and medulla regions of the albumin group at a similar level to the placebo group. Edema ranged from moderate to extensive throughout the tissue. The albumin group did not have follicle degeneration (Figure 1, C-D).

The placebo group experienced follicle degeneration. There were somewhat more hemorrhagic spots throughout the tissue than in the control group, and there was modest vascular congestion in the cortical regions. Furthermore, there was mild to severe widespread edema throughout the tissue. The medulla slice showed moderate hemorrhage regions and modest vascular congestion (Figure 1, E-F).

Follicle degeneration was detected in the IR group to a lesser extent. Mild hemorrhagic patches were seen in the cortex and medulla. Throughout the tissue, there was some vascular congestion and moderate edema (Figure 1, G-H).

Histopathological evaluation results are shown in the table (Table 2). There was a statistically significant

Table 1. Comparisons of biochemical findings between groups (mean ± SD).

Groups	Calcium (mg/dL)	Albumin (g/dL)	TAS (mmol/L)	TOS (µmol/L)	OSI
Control	9.32 ± 0.45^{a}	3.42 ± 0.23^{a}	1.60 ± 0.15^{a}	8.46 ± 4.46^{a}	0.52 ± 0.26^{a}
Albumin	9.92 ± 0.65^{a}	3.50 ± 0.15^{a}	1.58 ± 0.20^{a}	11.51 ± 4.25^{ab}	0.74 ± 0.30^{ab}
Placebo	10.07 ± 0.36^{a}	3.31 ± 0.21^{a}	1.53 ± 0.06^{a}	18.89 ± 8.47^{bc}	1.22 ± 0.50^{bc}
IR	9.73 ± 0.72^{a}	3.33 ± 0.29^{a}	1.43 ± 0.51^{a}	20.96 ± 5.64^{c}	1.56 ± 0.59^{c}
p-value*	0.071	0.336	0.639	0.001*	<0.001*

**: p<0,05 (Oneway ANOVA). SD: Standard Deviations.^{a,b,c}: Different superscripts in the same column indicate statistical differences between groups (post-hoc Tukey p<0.05).*

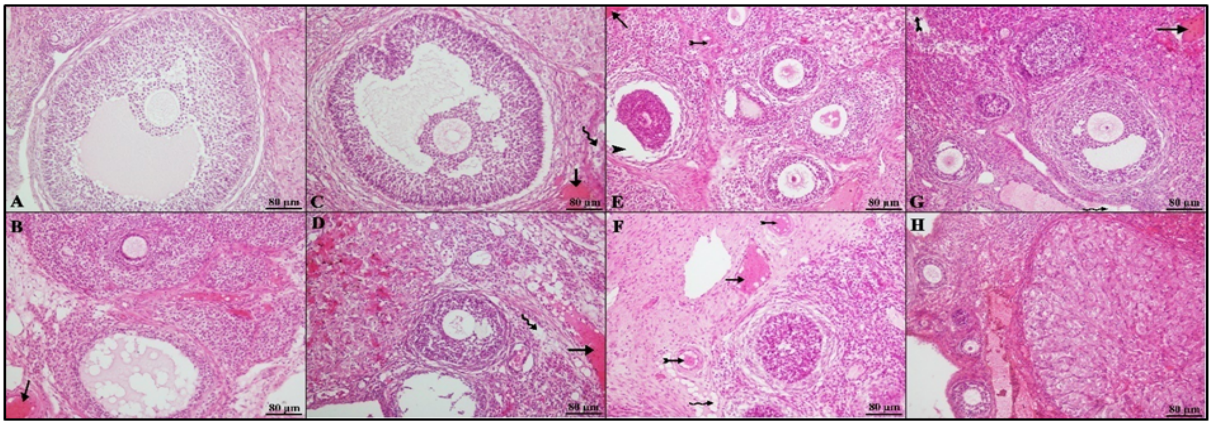

Figure 1.H&E staining, 20x magnification (80µm). **A-B**. Control group,**C-D.** Albumin group, **E-F.**Placebo group, **G-H.** IR group. (Straight arrow: Hemorrhage, Curved arrow: Edema, Arrowhead: Follicular degeneration, Vest arrow: Vascular congestion.)

difference between the IR group and placebo groups in terms of follicular degeneration (p=0.004). The difference in edema scores between the placebo and control groups was statistically significant (p=0.007). There was a statistically significant difference between the control group and the placebo and IR groups in the total scoring (p=0.008, p=0.003, respectively). There was no strong difference between the groups in terms of vascular congestion and hemorrhage.

Immunohistochemical findings

In the control group, no significant CYC1 immuno reactivity was seen in follicle granulosa cells and theca layers. Ovarian connective tissue cells showed partial immune reactivity. However, modest widespread immune reactivity was seen in corpus luteum granulosa lutein cells (Figure 2, A-B).

Weak CYC1 immuno positivity was identified in follicular granulosa cells and theca layers in the albumin group, and weak CYC1 immuno reactivity in ovarian connective tissue. In corpus luteum granulosa lutein cells, immune positivity was shown to be somewhat extensive (Figure 2, C-D).

The Placebo group showed moderate CYC1 positivity in follicular granulosa cells and theca layers. Rare immune reactivity was found in cumulus oophoruses. Ovarian connective tissue cells showed somewhat extensive staining. In contrast, moderate and widespread CYC1 immuno reactivity was seen in corpus luteum granulosa lutein cells (Figure 2, E-F).

In the IR group, follicular granulosa cells and theca layers showed moderate CYC1 immuno reactivity. In ovarian connective tissue cells, immune reactivity ranged from moderate to strong. In the corpus luteum, granulosa lutein cells of the IR group, strong widespread staining was seen (Figure 2, G-H).

CYC1 immunohistochemical examination results are shown in the table (Table 3). In terms of CYC1 immuno reactivity, a statistically important difference was identified between the control group and the other groups (p<0.001 in all). Furthermore, there was a statistically significant difference in CYC1 immuno reactivity results between the albumin group and the IR groups (p=0.003). In terms of CYC1 immuno reactivity, there was no statistical difference between the placebo and IR groups.

Table 2. Comparisons of histopathological findings between groups [median (min – max)].

Groups	Follicle Degeneration	Hemorrhage	Vascular Congestion	Edema	Total Score
Control	0(0–0)	1(0–1)^{a}	0(0–1)^{a}	1(0–1)^{a}	2 (0–3)^{a}
Albumin	0(0–0)	1(1–3)^{a}	0 (0–1)^{a}	1.5(1–3)^{ab}	3(2–5)^{ab}
Placebo	0(0–1)^{a}	1.5(0–3)^{a}	0 (0–1)^{a}	2(1–3)^{b}	3.5(2–5)^{b}
IR	1(0–1)^{b}	1(0–2)^{a}	0.5(0–1)^{a}	1.5(0–2)^{ab}	3.5(2–5)^{b}
p-value*	<0.001*	0.126	0.639	0.034*	0.010*

**: p<0,05 (Kruskal wallis). min: Minimum. max: Maximum. ^{a,b}: Different superscripts in the same column indicate statistical differences between groups (Mann Whitney-u, p≤0.008). Note: The comparison was done between placebo and IR groups for follicle degeneration.*

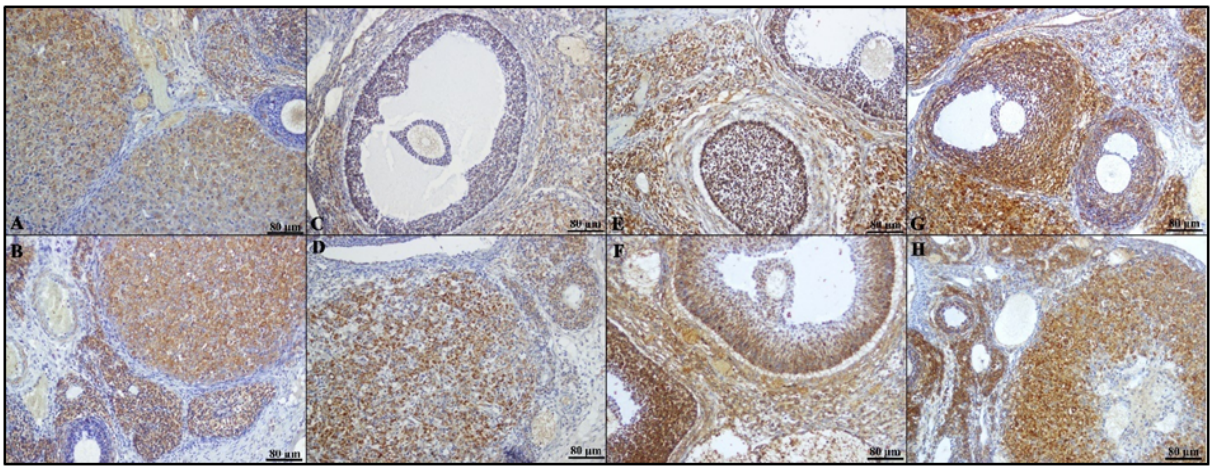

Figure 2. CYC1 IHC staining, 20x magnifications(80μm). **A-B.** Control group, **C-D.** Albumin group,**E-F.** Placebo group,**G-H.** IR group.

Table 3. Comparisons of CYC1 immunoreactivity between groups [median (min – max)].

	Control	Albumin	Placebo	IR	*p-value**
CYC1	1(1–1)^{a}	2(2–2)^{b}	2.5(2–3)^{c}	3 (2–3)^{c}	<0.001*

**: p<0,05 (Kruskal wallis).min: Minimum. max: Maximum.^{a,b,c}: Different superscripts on the same row indicate statistical differences between groups (Mann Whitney-u, p≤0.008).*

DISCUSSION

An experimental bilateral IR model was generated in rat ovaries for this investigation. The impact of intraperitoneal albumin administration on ovarian tissue damage and CYC1 immunoreactivity was studied.

In some experimental IR model investigations, there was no change in serum calcium levels across the groups.[15,16]In our study, there was no significant difference between the groups in terms of serum calcium levels. The comparison of serum calcium levels showed that ovarian IR damage did not produce alterations in serum calcium levels between groups.

Some articles in which it was constructed an experimental ovarian IR model showed that the greatest TOS levels were in IR groups.[2,17,18] In the experimental IR model studies in the ovaries, it was found that TOS levels in the IR groups were greater than those in the control groups.[14,19-21] In this investigation, there was a significant difference in terms of TOS levels between the groups. TOS levels rose considerably in the IR group (20.96±5.64 μmol/L) and placebo group (18.89±8.47 μmol/L) compared to the control group (8.46±4.46 μmol/L). Furthermore, the TOS levels in the IR group increased considerably as compared to the albumin group (11.51±4.25 μmol/L). TOS levels in the control and albumin groups revealed similar findings.

In other studies, it was discovered the lowest TAS levels in IR groups in experimental ovarian IR model investigations.[2,14,17-19,21] In the present study, there was no powerful difference between the groups in terms of TAS levels. Despite this, it was established that the IR group had the lowest TAS levels in this study.

Studies on the experimental ovarian IR model stated that the highest OSI levels were in the IR groups.[17,18] In some studies, they reported that the OSI level in the IR group was higher than that of the control group.[14,19-21] In our study, a statistical difference in OSI levels was found between the groups. OSI levels showed a significant increase in the placebo group (1.22±0.50) and IR group (1.32±0.42) compared to the control group (0.61±0.34). In addition, the OSI levels of the IR group also increased significantly compared to the albumin group (0.74±0.30). OSI levels were similar in the control and albumin groups.

Although the highest serum albumin values were seen in the albumin group, there was no visible statistical difference in serum albumin levels between the groups. Although serum albumin levels were similar between the groups, there were differences in TOS and OSI values. We considered these results as beneficial effect of administration albumin against IR-induced damage.

Some studies showed that follicle degeneration increased in IR groups compared to control groups in their experimental IR model investigations on ovaries.[1,4,22-25] Some researchers discovered that follicle degeneration was higher in IR groups in experimental IR ovarian model investigations.[26,27] In this study, there were significant variations in follicular degeneration between the groups. Follicle degeneration was observed to be considerably higher in the IR group than in the other groups. This shows that albumin inhibits follicle degeneration.

In some studies that used 3-hour each ischemia and reperfusion in an experimental IR model on ovaries, it was stated that the hemorrhage in IR groups increased compared to other groups.[3,28-31] We found that there was no difference between the groups in terms of hemorrhage in our study which used 2 hours each ischemia and reperfusion. However, it was found that the hemorrhage was partially higher in the IR-applied groups compared to the control group. In this case, we think that the applied ischemia and reperfusion durations are decisive in terms of hemorrhage.

Researchers discovered that vascular congestion increased in the IR-applied groups compared to the control group in their investigation, in which they constructed an ovarian IR model.[2,4,22,23,28] In this investigation, there was no difference in vascular congestion across the groups. As in hemorrhage, we believe that the applicable IR durations are critical in this scenario.

In experimental ovarian IR model investigations, some researchers found that IR groups had the most edema.[17,26,27] The other researchers, on the other hand, observed that edema was greater in the IR group than in the control group in their research in which they produced an IR ovarian model.[3,31] Decently, a substantial difference was identified between the control group and the placebo group in this investigation. The results in our albumin and IR groups were similar to those in the placebo and control groups. This discrepancy is thought to be caused by saline supplied as a placebo, which increases the amount of edema in the placebo group.

Researchers noted that the IR groups had the greatest total score in the ovarian investigations in which they constructed an IR model.[1,24] In comparable IR ovarian experiments, scientists observed that IR treatment significantly raised the total score.[22,32] In this study, there was a significant difference in total scores between the control group and the placebo and IR groups. The fact that the total score in the albumin-treated group was similar to the control group and slightly lower than the other IR-treated groups showed that albumin provided some histological protection.

A study found that CYC1 transcription increased in IR groups in their research of the cerebral IR model.[33] Other studies showed that there was no change in CYC1 immunoreactivity between the IR and control groups in their investigation done in isolated rat hearts.[34]Another study declared that when they analysed the ovaries with fluoride, the CYC1 immunoreactivity was strongerin the granulosa cells in the fluoride groups than in the control group.[35] In our investigation, CYC1 immunoreactivity was considerably higher in the IR-treated groups than that in the control group. However, because the level of CYC1 immunoreactivity in the albumingroup was much lower than in the placebo and IR groups, it is thought that albumin administration protects the tissue by lowering CYC1 immunoreactivity.

CONCLUSION

According to biochemical investigations, oxidative stress is decreased, particularly in the albumin group treated with IR when compared to the IR group. The groups had no differences in blood serum albumin or calcium levels. Histological tests revealed that the size of tissue damage increased considerably in the albu-

min, placebo, and IR groups compared to the control group. The fact is that CYC1, an indication of tissue damage, displayed greater immunoreactivity in the IR and placebo groups than in the control and albumin groups. These results indicated that albumin had a tissue damage-reducing impact. To minimize tissue damage induced by ischemia in ovarian torsion, detorsion should be administered as soon as feasible.

As a result, we believe that albumin, which has been shown to lower elevated CYC1, ROS, and oxidative stress levels, will play a supporting role in the IR therapy process due to its antioxidant properties. More detailed research is needed to back up the impact of albumin, which is a safe antioxidant.

Ethics Committee Approval: Permission for this study was given by Kafkas University Animal Experiments Local Ethics Committee (Decision Date: 23-09-2021) (Decision No: 2021-138).
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