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ARAŞTIRMA / RESEARCH

Protective effect of daidzein on ovarian ischemia-reperfusion injury in rats

Daidzeinin ovaryum iskemi reperfüzyonu hasarındaki koruyucu etkisi

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Abstract

Purpose: In this study, our aim was to investigate the potential effects of strong antioxidant daidzein (DZ) on ovarian ischemia and reperfusion injury.

Materials and Methods: A total of 42 female Sprague-Dawley rats were randomly divided into seven groups. For the experimental model, the clamps were removed after 3 hours of ischemia, and blood flow was provided again. Then, reperfusion process was terminated for 3 hours. Daidzein was orally administered to animals at doses of 35 and 70 mg/kg 30 minutes before ischemia (I) and ischemia and reperfusion (I/R) procedures.

Results: Severe immunoreactivity of the IL-1β, IL-6 and Caspase-3 were detected in I and I/R groups. Moderate immunoreactivity of IL-1β, IL-6 and Caspase-3 was detected in I+DZ35 and I/R+DZ35 groups, and slightly positivity was detected in I+DZ70 and I/R+DZ70 groups. The SOD activity level increased in the groups treated with Daidzein, while MDA levels decreased. In addition, hemorrhage areas and inflammatory cell migration decreased in I/R+DZ70 and I/R+DZ35 groups, when compared to I/R group in a dose dependent manner.

Conclusion: Daidzein has a strong protective role in the treatment of ovarian ischemia-reperfusion injury and can be used as a therapeutic agent.

Keywords: Daidzein, ovary, ischemia-reperfusion injury, rat.

Öz

Amaç: Bu çalışmada, güçlü antioksidan daidzeinin (DZ) over iskemi ve reperfüzyon hasarı üzerindeki potansiyel etkisini araştırdık.

Gereç ve Yöntem: Toplam 42 adet dişi Sprague-Dawley sıçan rastgele yedi gruba ayrıldı. Deney modeli için, 3saatlik iskeminin ardından klempler çıkarıldı ve tekrar kan akışı sağlandı. Reperfüzyon 3 saatin sonunda sonlandırıldı. Daidzein, iskemi (I) ve iskemi ve reperfüzyon (I/R) işlemlerinden 30 dakika önce hayvanlara ağızdan 35 ve 70 mg/kg dozlarında uygulandı.

Bulgular: I ve I/R gruplarında IL-1β, IL 6 ve Caspase 3'ün şiddetli immünoreaktivite görüldü. I+DZ35 ve I/R+DZ35 gruplarında IL-1β, IL 6 ve Kaspaz 3'ün immünreaktivitesi orta düzeyde iken, I+DZ70 ve I/R+DZ70 gruplarında hafif pozitiftir. Daidzein ile tedavi edilen gruplarda SOD aktivite seviyesi artarken, MDA seviyeleri azaldı. Ayrıca I/R + DZ70 ve IR+DZ35 gruplarında I/R grubuna göre doza bağlı olarak kanama alanları ve inflamatuar hücre göçü azaldı.

Sonuç: Daidzein, over iskemi-reperfüzyon hasarının tedavisinde güçlü bir koruyucu role sahiptir ve terapötik bir ajan olarak kullanılabilir.

Anahtar kelimeler: Daidzein, ovaryum, iskemisi ve reperfüzyon hasarı, sıçan

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INTRODUCTION

Ovarian ischemia is a clinical emergency with a prevalence of 2.7% in the general population. Pregnancies, ovarian cysts, overactivity of the adnexa are some of the factors that cause ovarian ischemia[1]. It occurs a result of partial or complete interruption of blood flow after the rotation of the ovarian ligaments due to reasons such as adolescence, pregnancy, trauma, ovarian cyst[2]. The ischemia process interrupts blood flow to related tissues, often causes irreversible ischemic damage[3]. Early diagnosis is very important; otherwise, late diagnosis leads completely lost the organ. It is urgently required to restore blood flow to the ovary by surgical operation[4]. Although the blood flow is restored to the tissue by surgical operation, reactive radicals formed during ischemia transform into superoxide radicals (SOR) in the presence of oxygen. The formation of radicals that are more aggressive exacerbates the damage[5]. The reperfusion process following ischemia causes cellular damage and organ dysfunction known as "reperfusion injury"[6].

Intracellular antioxidant defense systems are insufficient to clean the SOR in this type of damage[7,8]. Ischemic reperfusion injury activates neutrophil cells within a few hours. The cells secrete mediators such as tumor necrosis factor (TNF) and interleukin-1 (IL-1), then inflammation occurs[9]. Finally, oxidative stress and inflammation may cause permanent or temporary ovarian dysfunction[10].

Antioxidant and anti-inflammatory pharmacological agents can be used as therapeutic and protective drugs for treatment of ischemia and reperfusion damage. The patients can be treated antioxidant and anti-inflammatory agents to protect from ovarian reperfusion injury before restore blood flow to the ovary by surgical operation. Although there are lots of usable antioxidant and anti-inflammatory agents all over the world, ovarian ischemia and reperfusion injury could not sufficiently be treated, then infertility can be develop in many patients. Therefore, new strong antioxidant and anti-inflammatory agents are needed to be investigated for treating the ovarian ischemia and reperfusion injury.

Daidzein (DZ) is a compound isoflavonoid with antioxidant, anti-inflammatory, and anti-cancer properties. Especially, it has been found in Chinese herbs such as soybeans, red clover, kwao, krua and kudzu[11]. Previous studies have demonstrated that daidzein is more effective than quercetin which is well-known strong antioxidant agent[12]. In addition, it is converted into equal and O-desmethylangolensin (O-DMA) forms in our body by gut bacteria[13] and these metabolites of daidzein, especially O-DMA, have strong antioxidant capacity[14]. Furthermore, Li et al. showed that daidzein inhibited TNF-induced proinflammatory and chemokine secretions in vitro to show its anti-inflammatory effects[15]. Daidzein also down-regulated the IL-6, IL-12, and TNF-α expression levels on the LPS-induced inflammation in human monocyte cells[16]. In this regard, daidzein and its metabolites may be an effective option in the prevention of ischemia and reperfusion injury. In current literature, it has been shown that daidzein has a protective role against ischemia damage in organs such as brain and heart. However, anti-inflammatory effect and antioxidant potential of daidzein on ovarian ischemia-reperfusion injury have not been investigated yet.

Our aim was to investigate the potential protective effect of daidzein on ovarian ischemia and reperfusion injury in rats by histopathological, immunohistochemical and biochemical methods.

MATERIALS AND METHODS

Animals

The Institutional Animal Care and Use Ethics Committee of Kafkas University approved the study on 26.12.2019, which was conducted in accordance with protocol number 2019/154. In this study, 42 Sprague-Dawley female rats with 200-250 gram and 10-12 weeks-old were used. Total animal counts were determined according to 3R principle (Replacement, Reduction, Refinement) of the Institutional Animal Care and Use Ethics Committee of Kafkas University. Rats were kept in at temperatures ranging between 19°C and 22°C, with a standard 12-h light–dark and were feed ad-libitum. This study was carried out in the Animal Experiments Research and Application Center of Kafkas University.

Surgical procedure and experimental animal model

The animals were randomly divided into seven groups. Group 1: Sham, Group 2: Ischemia (I), Group 3: I+Daidzein 35 mg/kg given orally (DZ35), Group 4: I+Daidzein 70 mg/kg given orally (DZ70), Group 5: I/R (Ischemia ve Reperfusion), Group 6: I/R+DZ35 given orally and Group 7: I/R+DZ70

given orally. On the day of the experiment, the animals were anaesthetized with Ketamine (Ketalar, Phizer) and xylazine (Rometar, Bioveta) mixture (in ratio of 9/1). An incision was made in the abdominal area of each animal to detect the ovaries. Vascular clamps were applied to the ovaries for 3 hours to induce ischemia. After 3 hours, the ischemia groups were terminated. For the reperfusion groups, the clamps were opened and blood flow was restored to ovaries for 3 hours, then reperfusion groups were terminated. The rats in control groups underwent laparotomies without the induction of I and I/R injuries. To investigate the protective effect of daidzein I/R injury, DZ (Alfa Aesar, 1 g, Lot No: B22877) dissolved in distilled water and was orally given at doses of 35 and 70 mg/kg[17] during both I and I/R (30 minute prior to surgery)[18].

Ovaries were removed at the end of the experiment. They were stored in 10% formaldehyde solution for histopathological and immunohistochemical examinations and at -80 ˚C for biochemical analysis.

Histopathological analysis

All tissue samples were fixed in 10% formalin solution. Then, routine tissue processing procedure was performed. According to this protocol; tissues were firstly washed for 20 minutes in running water and formalin was removed. Then, they were dehydrated in graded alcohol series (50, 60, 70, 80, and 96-1 hour). Finally, the tissue was embedded in paraffin wax (2x1h) and tissue samples were blocked. The paraffin blocks were sectioned using a microtome (Leica RM2125 RTS). For histopathological examinations, 5-μm sections were stained with Mallory's triple stain modified by Crossman.

For scoring, ovary sections were examined approximately in 5 randomly and were evaluated in terms of the severity of changes in inflammatory cells, edema, and hemorrhage. The scores were derived semi-quantitatively using light microscopy on the preparations from each animal and were reported as follows: none (-), none or rare (-/+), mild (+), moderate (++) and severe (+ + +) (Table 1).

Table 1. Histopathological scoring findings and immunohistochemical Scores of IL-1-β, IL-6 and Caspase-3;

Groups	HE	EA	IC	IL-1-β	IL-6	CAS-3
Sham	-	-	-	-/+	-	+
Ischemia	+++	++	-	++	+	++
I+DZ35	++	-	-	++	+	++
I+DZ70	+	-	-	+	-	+
I/R	+	+	+++	+++	++	+++
I/R+DZ35	-/+	-	+	+	-/+	+
I/R+DZ70	-	-	-	-	-	-

The immunoreactivity scale of Table 1 is as following; no or very little (-/+), mild (+), moderate (++), and severe (+++);(HE: Hemorrhage, EA: Edematous Areas, IC: Inflammatory Cells, IL-1-β: Interleukin-1-Beta, IL-6: Interleukin-6, CAS-3: Caspase-3 (DZ: Daidzein, I: Ischemia, I/R: Ischemia and Reperfusion)

Immunohistochemical staining procedure was performed on IHC Automatic Staining Module (Ventana Medical Systems Inc., Tucson, AZ)[19]. The sections were kept at 65°C for 20 minutes for deparaffinization. After washing with reaction buffer, they were kept in citrate buffer solution for 60 minutes at 95°C for antigen retrieval. After re-washing, they were incubated in inhibitor solution for 4 minutes to block endogenous peroxides and protein. The primary and secondary antibodies were applied for 60 and 8 minutes, respectively. Then, DAB chromogen and H_2O_2 were applied. After washing procedure, reaction was stopped with the application of copper and counterstaining was done with hematoxylin. Caspase-3, IL-6 and IL-1-ß (Santa Cruz Biotechnology Inc., Dallas, TX) monoclonal primary antibodies were used at a 1: 100 dilution. All immunohistochemical stainings were evaluated with an image processing system (Nikon Eclipse 600).

In our study, semi-quantitative scoring was used for immunohistochemical staining findings. Each specimen was examined approximately in 5 randomly selected areas. The results were reported as follows: no or very little immune positivity - (0%), mild + (0-30%), medium ++ (30-60%) and severe +++ (60-100%) (Table 1)[20].

Biochemical analysis

For biochemical analysis, 100 mg of ovary tissues from each rat were grinded in liquid nitrogen using a TissueLyser II grinding jar set (Qiagen, Hilden, Germany). Then, they were centrifuged. Subsequently, SOD activity and MDA level from each supernatant were measured in duplicate with highly sensitive kits (BT LAB -E0168Ra, E0156Ra (Chine), respectively) according to the manufacturer's instructions. The protein concentrations were determined by the Lowry method using commercial protein standards (Sigma Aldrich, Total protein kit-TP0300-1KT-(USA). All data were presented as the mean ± standard deviation (S.D.) results based on per mg of protein[18].

Statistical analysis

The data were statistically analyzed with IBM 20.00 SPSS program. MDA and SOD parameters were compared between the groups using one-way ANOVA–Duncan's post-hoc test. Mean and SD values were used for some analyses and the differences between the groups were defined as statistically significant when the p values were less than 0.05.

RESULTS

In the study, the cortex and medulla in the ovarian tissue were microscopically examined. Primary, secondary, graff follicles and corpus luteum which were in a healthy-looking were observed in the sham group. Blood vessels were normal in the medulla (Figure 1).

In the ischemia group, dense hemorrhage areas were observed in the cortex. In addition, vascular congestions were especially seen in the medulla (Figure 1). When ischemia treatment groups were examined; while edema and hemorrhage areas were observed in the I+DZ35 group in cortex, minimal hemorrhage and edema areas were observed in the I+DZ70 group. Inflammatory cell migrations were not detected in those groups (Figure 1).

Dense hemorrhage, edema and inflammation areas were observed in I/R groups. On the other hand, while minimal hemorrhage, edema and inflammatory cell migrations were observed in I/R+DZ35 group, minimal hemorrhage areas were remarkably decreased in I/R+DZ70 group and the general appearance was close to Sham group. Inflammatory cell migration were not detected in this group (Figure 1).

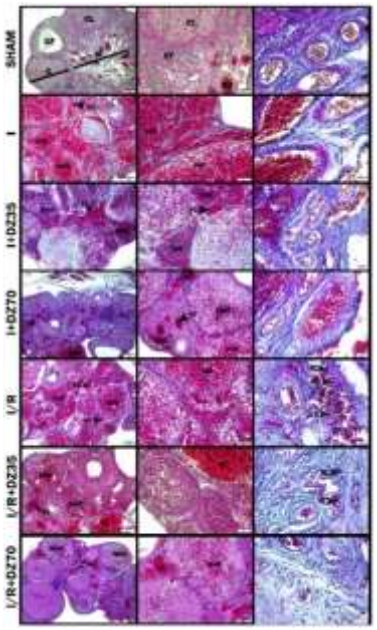

Figure 1. Histopatological findings

(C: Cortex, M: Medulla, CL: Corpus Luteum, ST: Stroma, BV: Blood Vessels, GF: Grafian Folicule, DHE: Dense hemorrhage, MHE: Minimal hemorrhage, VC: Vasculer Congestion, IC: Enflamatuary Cell- I: Ischemia, I/R: Ischemia and Reperfusion, DZ: Daidzein.)

Histopathological scoring was performed according to hemorrhagic and edematous areas and accumulation of inflammatory cells in ovarian tissue as follows: very little (-), mild (+), medium (++) and severe (+++) (Table 1).

In this study, the levels of MDA and SOD activity were investigated in I and I/R damage models in rat ovarian tissues. The MDA level increased in I and I/R groups compared to Sham group ($p<0,05$), The highest level of MDA was measured in I/R groups, while it was considerably reduced in daidzein treatment groups. The MDA activity level was lower in I+DZ70 and I/R+DZ70 groups than I and I/R groups (Figure 2-A).

The SOD activity level was detected higher in sham group, unlike the I and I/R groups. It was also observed that the SOD activity level increased in experimental groups treated with Daidzein, especially in high dose (I/R+DZ70 and I/R+DZ70) ($p< 0,05$) (Figure 2-B).

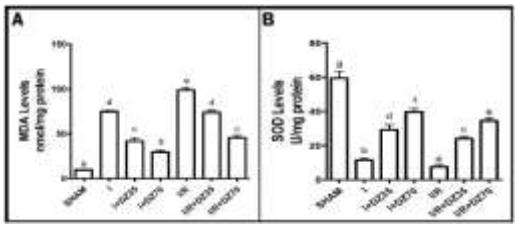

Figure 2. IL 1β, IL 6 and Caspase 3 Immunohistochemical Findings

(IL 1β: Interleukin 1 Beta, IL 6: Interleukin 6, I: Ischemia, I/R: Ischemia and Reperfusion, DZ: Daidzein) and Histopathology Staining Findings.

In terms of IL-6 positivity, there was severe immunoreactivity both in I and I/R groups, mild immunoreactivity in I+DZ35 and I/R+DZ35 groups, little or no immunoreactivity in I/R+DZ70 and I/R+DZ70 groups, and immune negativity in Sham group (Figure 3).

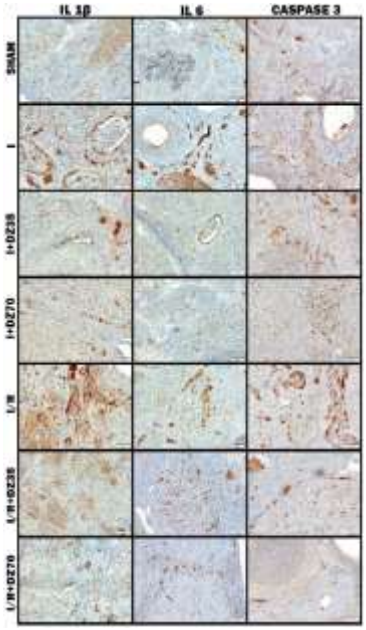

Figure 3. A; SOD levels of all experimental groups: B; MDA levels of all experimental groups

(I: Ischemia, I/R: Ischemia and Reperfusion, DZ: Daidzein).

Concerning the IL-1-β positivity findings; severe immunoreactivity was calculated in both I and I/R groups; however the immunoreactivity was decreased in DZ treatment groups (moderate positivity in I+DZ35 and I/R+DZ35 groups, slightly positivity in I+DZ70 and I/R+DZ70 groups) (Figure 3).

Finally, severe immunoreactivity was observed in I and I/R groups in terms of Caspase-3 positivity, while mild and very little immunoreactivities were observed in I+DZ35 and I/R+DZ35 and I/R+DZ70 and I/R+DZ70 groups, respectively (Figure 3).

DISCUSSION

In this experimental study, we showed a significant recovery in the ovaries of I+DZ35, I+DZ70, I/R+DZ35 and I/R+DZ70 groups compared to I and I/R groups by histopathological, immunohistochemical and biochemical methods.

The ovaries feed with uterine and ovarian arterial. Reversible or irreversible ischemia damage occurs in the tissue with partial or complete interruption of blood flow to ovary[21]. In case of ischemia damage, sufficient oxygen and nutrients cannot reach the tissue and hypoxia develops. Many chemical events occur with the impairment of cellular function in hypoxic tissue that leading to death[22]. It is necessary to restore blood flow for repairing the ischemic tissue damage. This process is called as "reperfusion or detortion treatment". However, oxidative damage that starts during the ischemia process increases with reperfusion. The exacerbation of the damage after reperfusion of the ischemic tissue, in contrast to expectations, is known as the "oxygen paradox"[23]. As a result of tissue re-oxygenation, toxic compounds such as superoxide (O_2) and hydrogen peroxide (H_2O_2) are formed in the tissue[24,25]. The rise of free radicals in the tissue also initiates inflammation. In addition, the results of inflammatory response initiate the migration of neutrophils into the tissue. At that point, reperfusion damage also develops in tissue in adition to ischemic damage[26]. Many histological changes such as edema, hemorrhage, polymorph nucleated leukocytes (PMNL) infiltration and vascular conjuncture occur in tissue as a result of oxidative damage during I and I/ R[27]. In a study that ovarian torsion was performed with Vardenafil; edema, vascular congestion, and follicular cell degeneration were observed[28]. Ergun et al. evaluated histologically the effects of hypothermia on adnexal torsion/detorsion damage in ovarian tissue and they observed the bleeding, interstitial edema, and PMN infiltrations[29]. Another ischemia-reperfusion study conducted by Altıntaş Ural et al. also showed the hemorrhage, edema, neutrophil infiltration in ovarian tissue[30]. In our study, severe hemorrhage and edema areas were seen in ischemia groups, and the level of this damage was decreased to minimal levels in treatment groups, especially in I/R+DZ70 group. In

the light of these data, it can be said that Daidzein plays a protective role especially on the vascular endothelium, and reduces the migration of erythrocytes into the tissue. On the other hand, it was a pleasing finding that inflammatory cells were detected in I/R group; and were not detected in I/R+DZ70 group. It has been shown in a previous focal cerebral ischemia injury study that daidzein can have such an effect[31].

One of the main biochemical indicators of oxidative stress is the elevation of MDA levels which is the final product of lipid peroxidation[32]. An ovarian ischemia study reported that a significant increase in MDA levels was found in ischemia and ischemia-reperfusion groups compared to control group[33]. Another study investigating the effects of octreotide for preventing I/R damage in rat ovaries showed that an increase of MDA level in ischemia group was observed while a significant decrease was detected in octreotidine-treated groups[34]. In a study conducted with letrozole, it was shown that increased MDA levels were reported in both ischemia and ischemia-reperfusion groups while decreased MDA levels were detected in letrozole treatment group. In our study, we found significant increases in MDA levels in I/R and ischemia groups compared to sham group; however, MDA levels were significantly decreased in I+DZ and I/R+DZ treatment groups. It is known that isoflavonoids directly scavenge ROS produced by human neutrophils[35,36]. Lai et al demonstrated that daidzein inhibited peroxynitrite-mediated LDL oxidation in a dose dependent manner[37]. Also, other scientific studies showed that daidzein caused a reduction in MDA levels which were consistent with our findings. In a previous study, it was observed that MDA levels decreased in the daidzein treatment groups after ischemia[11].

SOD is an antioxidant enzyme and protects the cell from damage by converting superoxide radicals into water and hydrogen peroxide. SOD and endogenous antioxidant enzymes protect the tissue from ROS and free radical damage by removing free oxygen radicals from tissues[38]. In an experimental study investigating the effect of resveratrol against hepatic I/R damage showed that SOD activity decreased in I/R group. The authors stated that the findings of resveratrol treatment group were similar to control group and as a result of this findings they declared that resveratrol was protective against hepatic I/R injury[39]. Somuncu et al. investigated the effects of trapidil on ischemia-reperfusion injury in experimentally created rabbit ovary and reported lower and higher SOD levels in torsion-detorsion and treatment groups, respectively. As a result of the statistical significance between the groups, they claimed that trapidil had an antioxidant effect[40]. In our study, the SOD activity was lower in I and I/R groups when compared to Sham group. This is the proof that the intracellular antioxidant system is insufficient in conditions such as I and I/R damage. Daidzein which has a high antioxidant properties inspired us against to perform a study about I and I/R ovary damage. Indeed, significant increases in SOD levels in I and I/R groups treated with DZ were supported by the literature. For example, a study revealed that SOD activity was higher due to oxidative stress damage in I and I/R groups and lower in DZ treatment groups[31].

The produced chemicals during ischemia and reperfusion also contributes to aggravation of oxidative stress and inflammation. Cytokines such as IL-1-β and IL-6 from vascular endothelial cells and phagocytic mononuclear cells are excessively synthesized during I and I/R and released into the circulation. Increased cytokine expression initiates the mass migration of PMNL into damaged tissue. A large number of PMNL cells migrating the tissue leads to an increase in oxidative stress[41]. In the light of these data, we performed immunohistochemical staining with IL-1-β and IL-6 antibodies in ovarian tissue to learn the status of oxidative stress and inflammation in I and I/R injury. A decrease in immunoreactivity in treatment groups was detected, while an increase in immunoreactivity was detected in I and I/R groups. These findings shows that DZ can also eliminate oxidative damage due to PNML by reducing the expressions of cytokines such as IL-1 β and IL-6. There are some studies in current literature that support these findings[42,43]. In addition, it has been observed that DZ significantly reduces IL-6 production in sepsis[44]. Another studies have also pointed out that DZ has an anti-inflammatory effect by suppressing pro-inflammatory cytokines and chemokines during inflammation[45-47].

One of the consequences of excessive increase in inflammation and oxidative stress in tissue is the functioning of cell death cascades such as Bcl-2, Caspase-3 and Caspase-9 and the occurrence of cell apoptosis[48]. Caspase-3 is a key enzyme that plays a role in the execution of apoptosis and leads to cell breakdown. Furthermore, it directly plays a role in cell apoptosis by cleaving DNA repair proteins, cytoskeletal proteins and other related caspase

substrate proteins in cerebral ischemia[49]. At that point, measuring the density of Caspase-3 may also give an idea to understand the amount of oxidative stress that can lead the cell to death. Gungor et al. studied the effects of hesperidin on ovarian ischemia and reperfusion and focused on this relationship[50]. In our study, a decrease in the density of caspase-3 immunopositive cells were observed in I and I/R groups in DZ-treated groups, and the results showed that the obtained data were compatible with the literature. Some studies reported that DZ significantly down-regulated by caspase-3 expression and prevented the apoptosis[51,52]. Zhang et al. indicated that DZ administration significantly reduced caspase-3 activity against spinal cord injury[11]. In addition, Liu et al. demonstrated that DZ showed substantially decreased in the number of caspase-3 positive cells[53]. Our findings about caspase-3 were compatible with current literature.

Due to the ethical rules of animal experiments, the low number of animals is one of the most important limitations of the study. For this reason, the effectiveness of DZ on the reproductive potential of rats could not be tested. Another limitation is that detailed molecular analyzes could not be performed due to financial insufficiency.

In conclusion, demonstrating the benefits of DZ which is one of the most important ingredients of soybean and widely consumed all over the world will increase the consumption of soybean in the future. This result reveals that DZ as a powerful antioxidant may be a potential protective agent against ovarian ischemia-reperfusion injury.

Yazar Katkıları: Çalışma konsepti/Tasarımı: ET; Veri toplama: ET, MAG, TBT, ÖÖA, EA, RAU; Veri analizi ve yorumlama: ET, RAU; Yazı taslağı: ET; İçeriğin eleştirel incelenmesi: JS; Son onay ve sorumluluk: ET, MAG, TBT, ÖÖA, EE, RAU, JS; Teknik ve malzeme desteği:ÖÖA; Süpervizyon: RAU, JS; Fon sağlama (mevcut ise): yok.
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