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The effect of Terebinth oil on Ezrin and Moesin expression levels in rats with ovarian ischemia-reperfusion Injury

Efecto del aceite de terebinto sobre los niveles de expresión de ezrina y moesina en ratas con lesión por isquemia-reperfusión ovárica

Gülsüm Pektanç Şengül[1], Seydi Ahmet Şengül[2], Engin Deveci[3]*

[1]*Technology and Research & Development Center, Hatay Mustafa Kemal University, Hatay, Turkey.*
[2]*Department of Pharmacology and Toxicology, Faculty of Veterinary Medicine, Hatay Mustafa Kemal University, Hatay, Turkey.*
[3]*Department of Histology and Embryology, Medical Faculty, Dicle University, Diyarbakır, Turkey.*
[*]*Corresponding author: seydiahmetsengul@mku.edu.tr, tel.: +90-534-8386831*

ABSTRACT

The objective of this study is to examine the expression levels of ezrin and moesin proteins from a molecular and immunohistochemical standpoint following the administration of terebinth oil in rats with ovarian ischemia-reperfusion injury. A total of 32 female Sprague-Dawley rats were utilized in the study. The rats were randomly assigned to one of four groups, with eight rats in each group: control, ischemia, I/R, and I/R+terebinth oil. Following the induction of torsion, the treatment group received 2 $ml.kg^{-1}$ of terebinth oil orally via gavage once daily for 28 d. At the conclusion of the experiment, ovarian tissues were obtained for immunohistochemical and molecular analysis. The immunohistochemical evaluation demonstrated a positive ezrin expression in epithelial cells within the I/R+terebinth oil group, in comparison to the I/R group. Conversely, a negative reaction was observed in the vicinity of blood vessels. The expression of moesin was observed to be positive in granulosa cells and stromal areas. Additionally, a notable decline in the expression levels of ezrin and moesin proteins was observed in the treatment group in comparison to the damage group. Moreover, the administration of terebinth oil was observed to result in protein expression levels that were more closely aligned with those observed in the control group. The present study has demonstrated the impact of terebinth oil administration on the expression levels of ezrin and moesin proteins in a model of ovarian ischemia-reperfusion injury.

Key words: Terebinth oil; ezrin; moesin; ovary; ischemia-reperfusion.

RESUMEN

El objetivo de este estudio es examinar los niveles de expresión de las proteínas ezrina y moesina desde un punto de vista molecular e inmunohistoquímico tras la administración de aceite de terebinto en ratas con lesión por isquemia-reperfusión ovárica. Se utilizaron un total de 32 ratas Sprague-Dawley hembras en el estudio. Las ratas fueron asignadas aleatoriamente a uno de cuatro grupos, con ocho ratas en cada grupo: control, isquemia, I/R e I/R+aceite de terebinto. Después de la inducción de la torsión, el grupo de tratamiento recibió 2 $ml.kg^{-1}$ de aceite de terebinto por vía oral mediante sonda una vez al día durante 28 d. Al finalizar el experimento, se obtuvieron tejidos ováricos para realizar análisis inmunohistoquímicos y moleculares. La evaluación inmunohistoquímica demostró una expresión positiva de ezrina en las células epiteliales dentro del grupo I/R+aceite de terebinto, en comparación con el grupo I/R. Por el contrario, se observó una reacción negativa en las proximidades de los vasos sanguíneos. Se observó que la expresión de moesina era positiva en las células de la granulosa y las áreas del estroma. Además, se observó una disminución notable en los niveles de expresión de las proteínas ezrina y moesina en el grupo de tratamiento en comparación con el grupo dañado. Además, se observó que la administración de aceite de terebinto dio como resultado niveles de expresión de proteínas que estaban más estrechamente alineados con los observados en el grupo de control. El presente estudio ha demostrado el impacto de la administración de aceite de terebinto en los niveles de expresión de las proteínas ezrina y moesina en un modelo de lesión por isquemia-reperfusión ovárica.

Palabras clave: Aceite de terebinto; ezrin; moesin; ovario; isquemia-reperfusión.

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INTRODUCTION

Ovarian torsion, observed in 2.7% of gynecological emergency cases [1], typically affects women of reproductive age but can occur in females of all age groups. Ovarian torsion results from a complete or partial twisting of the ovary around its supporting ligaments [2]. The twisting of the ovary around its supporting ligaments causes compression in the vascular system, leading to ovarian ischemia. The resulting ischemia can lead to potential adverse outcomes such as sepsis, peritonitis, thrombophlebitis, adhesions, bleeding, and even death [3]. Due to the longer length of the right infundibulopelvic ligament, ovarian torsion is more commonly observed on the right side [4]. If ovarian torsion is not diagnosed early, it can result in reduced blood flow to the ovary, necrosis, irreversible tissue damage [5], and decreased follicular reserve [1]. Reperfusion occurs with the restoration of blood flow after ischemia [6] and leads to an increase in reactive oxygen species, disruption of cellular integrity, and apoptosis [4]. Ezrin, one of the ezrin-radixin-moesin (ERM) family proteins, is mainly involved in the protection of cell structure by providing cross-linking between the cortical actin cytoskeleton and the plasma membrane [7 , 8 , 9]. In addition, it plays a role in various cellular processes such as apoptosis, cell migration, cell adhesion, invasion, cell motility, metastasis, carcinogenesis, intercellular communication, and membrane signal transduction. It mediates signal transduction mechanisms by providing connections with membrane molecules to maintain cell morphology and cell polarity [7 , 8]. Moesin, which is a binding protein of the submembranous cytoskeleton and is involved in various physiological processes such as cell motility, development, invasion, and differentiation, is another member of the ERM protein family [7 , 10 , 11]. *Pistacia terebinthus*, a member of the Anacardiaceae family and one of the 20 Pistacia species, is commonly known as the turpentine tree [12]. *Pistacia terebinthus* is rich in proteins, minerals, fats, dietary fibers, carotenoids, unsaturated fatty acids, tocopherols, and phenolic compounds [13 , 14]. Its high antioxidant, antimicrobial, anti-inflammatory, and cytotoxic properties have made it a preferred plant for various diseases. Terebinth oil has been reported to be effective in conditions such as cancer, wound healing, and diabetes mellitus [15 , 16 , 17]. Many medicinal plants are used to prevent complications resulting from ischemic damage [18]. However, the effect of terebinth oil on ovarian torsion is not well understood. In this study, the activity of terebinth oil administration on the expression levels of ezrin and moesin proteins at the molecular and immunohistochemical levels in ovarian ischemia-reperfusion injury was investigated.

MATERIALS AND METHODS

Animals

Experimental procedures were carried out in accordance with the permission of the Local Ethics Committee of Animal Research of Dicle University, number 240638. Thirty-two adult female Sprague-Dawley rats (*Rattus norvegicus*) (approximately 250-300 g) were divided into four groups, with 8 animals per group. The rats were housed in temperature-controlled rooms (21-24°C), with humidity maintained at 40-60% and a light/dark cycle of 12 h each. They were provided with water and food ad libitum. In the study, general anesthesia was administered using xylazine hydrochloride (10 $mg.kg^{-1}$) (Rompun 2%, Bayer) and ketamine hydrochloride (100 $mg.kg^{-1}$) (Keta-Control, 100 $mg.ml^{-1}$, Doğa İlaç). Heart rate and respiratory rates were monitored during euthanesia.

Experimental protocols

Control group: Animals underwent no procedures were sacrificed under general anesthesia at the end of the experiment and their ovarian tissues were collected.

Ischemia group: The ovarian tissues were exposed under general anesthesia and ischemia was performed for 2 h.

Ischemia/reperfusion group: The ovarian tissues were exposed under general anesthesia.The ovaries were first perfomed given 2 h of ischemia and then 2 h of reperfusion.

I/R+Terebinth oil group: After I/R process, 2 $ml.kg^{-1}$ of terebinth oil was administered to the animals via oral gavage for 28 d. Then, the animals were sacrificed under general anesthesia and ovarian tissues were collected.Terebinth oil dose was used based on the study conducted by Uyar and Abdulrahman [17].

Immunohistochemical analysis for ezrin and moesin

Immunohistochemical analysis was performed to determine the levels of ezrin and moesin proteins in ovarian tissues. Ovarian tissue sections, 4-6 µm thick, were incubated at 60°C (Nüve, EN 500, Turkey) for 6 h. The sections were first deparaffinized in xylene and then dehydrated through a series of decreasing alcohol concentrations. The sections were first placed in distilled water and then washed with Phosphate Buffered Saline (PBS). For antigen retrieval, the sections were transferred to an Ethylenediaminetetraacetic acid solution and heated in a microwave oven for 3x6 min. After washing with PBS, the sections were treated with 3% hydrogen peroxide. The sections washed with PBS incubated with blocking solution. The sections were treated with primary antibodies against ezrin and moesin and incubated overnight at 4°C. After another round of PBS washing, biotinylated secondary antibodies were applied and incubated for 14 min, followed by additional washing. Subsequently, streptavidin-peroxidase was applied for 15 min, and 3,3'diaminobenzidine was added to the sections, with color development observed. The sections washed with PBS counterstained with Harris hematoxylin. The sections washed with tap water were clarified by passing through an increasing alcohol series and xylene. Microphotographs (Nikon, Y-TV55, Japan) of the sections covered with coverslips by dropping entellan were taken under a Zeiss Imager A2 light microscope (Axio Imager, A2, Germany).

Western blot analysis

Ovarian tissues stored at -80°C (Daihan, Uni Freeze U700, Korea) were thawed and homogenized in liquid nitrogen. Cold RIPA buffer containing protease-phosphatase inhibitor cocktail and nucleases was then added to the samples. The total cellular protein concentration of the resulting lysates was determined using a bicinchoninic acid protein assay kit. Proteins (approximately 20 µg) were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Bio-Rad, Mini-Protean Tetra Cell, USA). The separated proteins were transferred to a polyvinylidene fluoride (PVDF) membrane (Bio-Rad, USA). The membranes were incubated with ezrin and moesin antibodies for 2 h at room temperature. β-actin was used as a loading control. RP-conjugated secondary antibodies specific to the primary antibodies were used.Proteins were treated with ECL (LI-COR Biosciences, USA) substrate and visualized using G:-Box Chemi XRQ (Syngene, USA).

Statistical analysis

Statistical analysis was performed using SPSS® 11.5 (SPSS Inc.; Chicago, IL, USA). Data with normal distribution among multiple groups were analyzed using One Way ANOVA, and post hoc analysis was conducted using Tukey HSD test. A value of $P<0.05$ was considered statistically significant.

RESULTS AND DISCUSSION

Immunohistochemical results

In the control group, negative ezrin expression was observed in the connective tissue cells and capsules under the epithelium, as well as in the granuloza cells. However, a widespread positive ezrin expression was noted in the corpus luteum cells (FIG. 1A). In the ischemia group, positive ezrin expression was observed in some cells of the hyperplastic ciliated columnar epithelium and in cells along the developed antral follicle, attributed to degenerative changes. Conversely, negative ezrin expression was detected in the endothelial cells of the expanded vessels resulting from ischemia (FIG. 1B). In the I/R group, positive ezrin expression was seen in most of the epithelial cells on the luminal surface and in aggregate inflammatory cells. Negative ezrin expression was found around the vessels (FIG. 1C). In the I/R+terebinth oil group, positive ezrin expression was observed in most cells of the epithelial structure and non-developing follicles, whereas negative expression was noted in the underlying connective tissue and around the vessels (FIG. 1D).

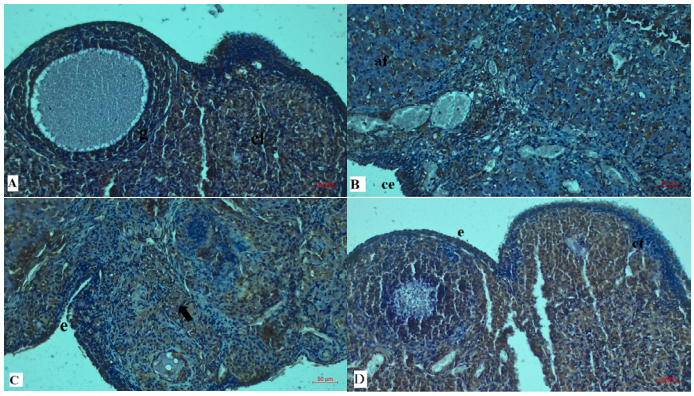

FIGURE 1. Cross sections of ovarian tissues stained with ezrin immunostaining. A. Control group ovarian section. g: granulosa cells, cl: corpus luteal cells. B. Ischemia group ovarian section, ce: ciliated cylindrical epithelium, af: antral follicle. C. I/R group ovarian section. e: epithelial cells, arrow: inflammatory cells. D. I/R+terebinth oil group ovarian section. e: epithelium, ct: connective tissue. (Bar: 50 µm, ezrin immunostaining: A-D, magnification, x200)

In the literature on *in vitro* and *in vivo* studies related to ezrin and moesin proteins is limited. High levels of ezrin expression have been observed in the small and large intestines and the stomach, and it can also be expressed in renal proximal tubules and glomeruli [19]. In a study by Louvet *et al.* [20] ezrin was first characterized in mouse embryos. The study revealed a decrease in ezrin protein levels from the oocyte to the blastocyst stage, with a more pronounced reduction during compaction. Additionally, it was noted that ezrin remained associated with the microvillar pole during the transition from the 8-cell to the 16-cell stage and was present only in the outer cells after division. In another study, it was found that some ezrin molecules in long spermatids in postnatal mouse testes bind to cytoplasmic actin. Ezrin immunoreactivity was observed in the cytoplasm of stage 15 and 16 spermatids from the fifth week after birth until adulthood. The immunostaining results suggested that ezrin expression was only found in the seminiferous tubules and was not detected in the interstitial tissue[21]. Baiocchi et al. [22] investigated tauroursodeoxycholic acid (TUDCA) in a rat liver I/R model and found that TUDCA treatment significantly increased ezrin protein expression compared to normal rats. In a study by Sak [23] it was observed that ezrin expression was at low levels in the syncytiotrophoblast cells and cells in the villous stroma of the placentas of patients with gestational diabetes. In placentas from non-diabetic patients, it has been reported that ezrin expression is positive in both the cytoplasm and cell membranes of maternal decidua, in some nuclei of decidual cells, and in the syncytiotrophoblast cell membranes of the placental villi. In another study, placentas from preeclamptic women who smoked were compared with those who did not smoke. It was found that in the smoking preeclampsia group, syncytial cells carrying microvilli showed negative ezrin expression, whereas in the non-smoking group, syncytial cells at the villus periphery exhibited negative ezrin expression. Additionally, positive ezrin expression was observed in Hofbauer cells, vascular endothelial cells, the trophoblast layer of floating villi, and connective tissue in large villi in the non-smoking preeclampsia group [24]. Fadiel *et al.* [25] indicated in their study on ovarian epithelial cancer (OVCA) cells that long-term ovarian surface epithelial cell culture increased ezrin expression and cytological abnormalities. Additionally, it has been found that epidermal growth factor significantly increases the translocation of ezrin in ovarian surface epithelial cells in a time-dependent manner. In another study, excessive ezrin expression was observed in human OVCA cell lines (Caov-3 and SK-OV-3) compared to human ovarian epithelial cell line (IOSE80) cells [26].

In the control group, slight positive moesin expression was observed in the epithelial layer, while negative expression was noted in the medium-sized antral follicle cells (FIG. 2A). In the ischemia group, the capillaries and sinusoidal-type capillaries in the lower regions were well-developed, and widespread positive moesin expression was observed in the follicular cells. Additionally, positive moesin expression was noted in the endothelium of the expanded thrombotic vessels (FIG. 2B). In the I/R group, positive moesin expression was observed in the cells of the germinal epithelium and some degenerated antral follicle structures in the superficial regions, as well as in the dilated blood vessels and endothelial cells. However, granulosa cells in the parenchymal area and cells in the antral follicle exhibited widespread negative moesin expression (FIG. 2C). In the I/R+terebinth oil group, positive moesin expression was observed in most granulosa cells, vascular endothelia, and stromal areas within the epithelial structure. However, moesin expression was negative in the theca folliculi externa region (FIG. 2D).

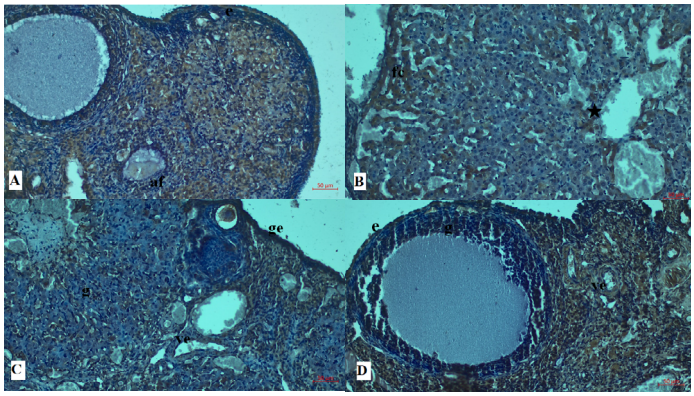

FIGURE 2. Cross sections of ovarian tissues stained with moesin immunostaining. A. Control group ovarian section. e: epithelium, af: antral follicle cells. B. Ischemia group ovarian section. fc: follicular cells, asterisk: thrombosed vascular endothelium. C. I/R group ovarian section. ge: germinal epithelium, ve: endothelial cells of dilated vessels, g: granulosa cells. D. I/R+terebinth oil group ovarian section. e: epithelium, g: granulosa cells, ve: vascular endothelium. (Bar: 50 µm, moesin immunostaining: A-D, magnification, x200)

Multiple tissues and organs can express moesin protein. It has been reported that moesin expression is significantly high in endothelial cells, including those in the spleen, kidneys, lungs, blood vessels, and lymphocytes [19]. In another study, Wakayama *et al*. [21] reported that no immunoreactivity for moesin was detected in the testes of either adult or postnatal mice.

Terebinth oil, which has various biological activities, is reported to possess antimicrobial, anti-inflammatory, cytotoxic, and antioxidant properties due to the richness of secondary compounds found in its fruits and resins [27]. Additionally, in traditional Turkish folk medicine, it is known to have antipyretic, diuretic, antibacterial, and antiviral effects [28]. In the literature, there are *in vitro* and *in vivo* studies that reveal effect of terebinth oil on different tissue injury and organisms. Altunova [29] demonstrated that the antibacterial activity of immature terebinth fruit extract was more effective against methicillin-resistant *Staphylococcus aureus* (MRSA) and vancomycin-resistant *Enterococcus* (VRE) compared to mature terebinth extract. Erdem et al. [30] indicated that terebinth oil used against liver damage resulting from mammary cancer induced by 7,12-dimethylbenz[a]anthracene (DMBA) in rats had a mitigating effect on the damage. However, no study has been found showing the effect of terebinth oil on ovarian ischemia-reperfusion injury and the expression levels of ezrin and moesin proteins in this tissue.

Western blot findings

The expression levels of ezrin and moesin proteins in ovarian tissues were determined using the Western Blot method. The expression levels of ezrin and moesin proteins in ovarian tissues are presented in FIGS. 3 and 4. It was found that the expression levels of ezrin and moesin proteins were significantly higher in the I/R group compared to the control group ($P<0.05$). On the other hand, in the I/R+terebinth oil group, the expression levels of ezrin and moesin proteins were found to be significantly lower compared to the I/R group ($P<0.05$). Additionally, in the I/R+terebinth oil group, it was determined that the expression levels of both proteins were close to the levels in the control group (FIG. 4).

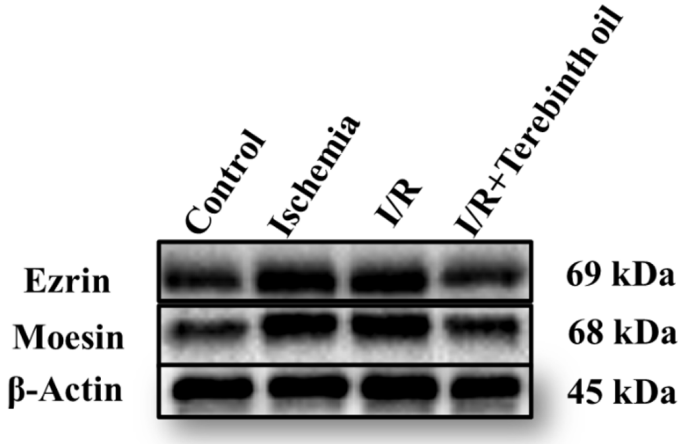

FIGURE 3. Expression levels of ezrin and moesin proteins in ovarian tissues of the control, ischemia, I/R, and I/R+terebinth oil groups. Quantification of protein band optical densities for ezrin, moesin, and ß-actin levels

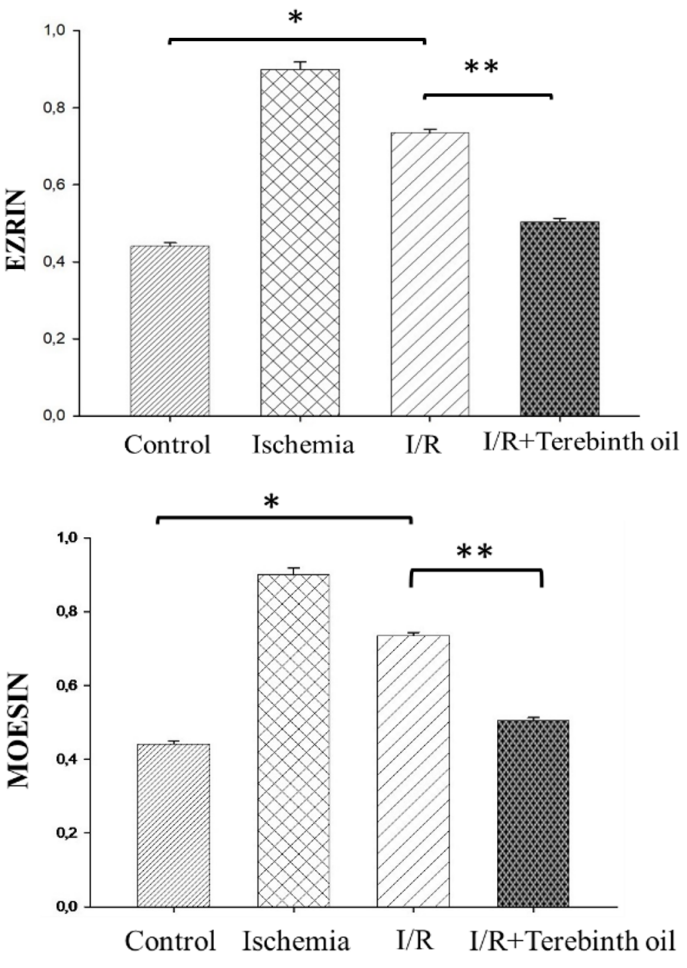

FIGURE 4. Bars represent the mean±SE of experiments. Levels of ezrin and moesin in ovarian tissues of the control, ischemia, I/R, and I/R+terebinth oil groups. (*P<0.05, when the ezrin and moesin proteins are compared with control group) (P<0.05, when the ezrin and moesin proteins are compared with I/R group)**

CONCLUSIONS

The results of the present study suggest that terebinth oil provides a therapeutic effect by reducing the expression levels of ezrin and moesin proteins, which are increased in ovarian ischemia/reperfusion injury. However, further in vivo studies are needed to evaluate its therapeutic mechanisms and role in folliculogenesis.

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Conflict of Interest

The authors declare that they have no conflicts of interest.

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