ORIGINAL ARTICLE

The effect of oleanolic acid on ischemia-reperfusion damage in the rat inferior epigastric artery skin flap model

Efecto del ácido oleanólico en el daño por isquemia-reperfusión en un modelo de colgajo cutáneo de la arteria epigástrica inferior en ratas

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Abstract

Objective: *The present study aimed to investigate the effects of oleanolic acid (OA) on I/R injury in a rat groin flap model.* **Method:** *21 male Wistar albino rats were randomly divided into three groups (n = 7): Group I (sham), Group II (I/R), and Group III (OA+I/R). OA was administered intraperitoneally for five pre-operative days. Flap elevation was performed on the 6th day, and the subjects were exposed to 2 h of ischemia followed by reperfusion. Biochemical and histopathological parameters were evaluated.* **Results:** *Group 3 presented a significantly diminished mean total oxidant stress relative to Group 2 (p = 0.002). Furthermore, a significant increase in total antioxidant capacity levels was observed in Group 3 compared to Group 2 (p = 0.048). There was no statistically significant difference between the groups in terms of inflammation and edema (p > 0.05). Necrosis did not develop in any of the samples.* **Conclusion:** *This study revealed that OA significantly reduces oxidative stress and increases antioxidant capacity, potentially benefiting therapies for I/R injuries.*

Keywords: *Oleanolic acid. Ischemia. Reperfusion injury.*

Resumen

Objetivo: *Investigar los efectos del ácido oleanólico (AO) sobre el daño por isquemia-reperfusión (I-R) en un modelo de colgajo inguinal en ratas.* **Método:** *Se dividieron aleatoriamente 21 ratas albinas Wistar macho en tres grupos (n = 7): grupo I (simulado), grupo II (I-R) y grupo III (AO + I-R). Se administró AO por vía intraperitoneal durante 5 días preoperatorios. La elevación del colgajo se realizó el sexto día y los animales fueron expuestos a 2 horas de isquemia seguida de reperfusión. Se evaluaron los parámetros bioquímicos e histopatológicos.* **Resultados:** *El grupo 3 presentó una media significativamente disminuida de estrés oxidante total en comparación con el grupo 2 (p = 0.002). Además, se observó un aumento significativo de los niveles de capacidad antioxidante total en el grupo 3 en comparación con el grupo 2 (p = 0.048). No hubo diferencias estadísticamente significativas entre los grupos en cuanto a inflamación y edema (p > 0.05). No se desarrolló necrosis en ninguna de las muestras.* **Conclusiones:** *Este estudio reveló que el AO reduce significativamente el estrés oxidativo y aumenta la capacidad antioxidante, beneficiando potencialmente las terapias para lesiones por I-R.*

Palabras clave: *Ácido oleanólico. Isquemia. Daño por reperfusión.*

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Introduction

Ischemia is a complex event caused by the interruption of local blood flow to a tissue or organ. Reperfusion, defined as the restoration of blood flow to reverse ischemic damage, is expected to reintroduce oxygen, remove metabolic waste products, and deliver essential nutrients to the cells. However, reperfusion can paradoxically exacerbate tissue damage, a phenomenon known as ischemia-reperfusion (I/R) injury[1].

In plastic, reconstructive, and esthetic surgery, tissues often experience ischemia due to crush injuries, circumferential burns, and traumatic amputations, with revascularization achieved through fasciotomies, revascularization, and replantation surgeries. During free tissue transfers for soft tissue defect repair or reconstructive surgeries, as well as in limb and facial transplantation, tissues are inherently subjected to ischemia and reperfusion processes, which can compromise tissue survival and reduce surgical success[2,3].

Oleanolic acid (OA) is a biologically active pentacyclic triterpenoid primarily derived from the fruit of the olive tree (Olea europaea L.) and over 1,620 other plant species. Pentacyclic triterpenoids, known for their bioactive properties, such as antitumor, antiviral, antidiabetic, and anti-inflammatory effects, hold significant therapeutic potential. In China, OA has been used as an over-the-counter hepatoprotective drug for decades[4]. OA and its derivatives exhibit a wide range of biological activities supporting their pharmaceutical use in various diseases, including potential anti-tumor activity against several cancer cell lines[5,6]. OA has been reported to induce apoptosis by modulating the regulation of anti-apoptotic proteins and reducing oxidative stress in ischemic conditions, demonstrating protective effects in liver, brain, kidney, and heart tissues under I/R injury[7-12].

The aim of this study is to evaluate the effects of pre-operatively administered OA on I/R injury in a rat ischemia-reperfusion model using an inferior epigastric artery skin island flap (groin flap).

Method

This experimental study was approved by the University of Health Sciences, Gülhane Local Ethics Committee for Animal Experiments at our university (Protocol number: 2022-2020). A total of 21 male Wistar albino rats, which weighed 350-550 g, were kept in individual special standard cages for rats, at a temperature of 21-24°C, with 50% air humidity throughout the day. All the rats were fed ad libitum with food and water, and a 12-h automatic light/dark cycle was maintained. All of the surgical procedures were performed at the same center according to the guidelines for the care of laboratory animals published by the United States National Institutes of Health.

The rats were randomly divided into three groups, with seven rats in each group: group I (sham), group II (I/R), and group III (OA+I/R). Over 5 days, animals were treated without anesthesia as follows: group 1 was maintained under standard housing and feeding conditions without intraperitoneal intervention. On the 6th day, under general anesthesia, a 3 × 6 cm epigastric flap was elevated and immediately replaced without ischemia or reperfusion. After a 4-h waiting period, 1.5 × 1.5 cm biopsies were taken from the distal flap for histopathological and biochemical analysis. Group 2 received intraperitoneal phosphate-buffered saline at a dose of 1 mL/kg body weight for 5 days. On the 6th day, under general anesthesia, a 3 × 6 cm epigastric flap was elevated, and microvascular clamps were applied to induce a 2-h ischemia period, followed by 2 h of reperfusion. After reperfusion, animals were sacrificed under general anesthesia, and 1.5 × 1.5 cm biopsies were taken from the flap for analysis. Group 3 was treated with intraperitoneal OA, with a purity of ≥ 97% (Sigma Aldrich, USA), at a dose of 8 mg/kg/day for 5 days (Fig. 1). On the 6th day, under general anesthesia, a 3 × 6 cm epigastric flap was elevated, and ischemia was induced for 2 h using microvascular clamps, followed by 2 h of reperfusion (Fig. 2). After reperfusion, animals were sacrificed under general anesthesia, and 1.5 × 1.5 cm biopsies were taken from the flap for analysis (Table 1).

Intraperitoneal injections were performed using a 30G needle at a 30-45° angle into the right lower quadrant of the abdomen, following negative aspiration, for 5 days. General anesthesia was administered intraperitoneally with a mixture of 80-90 mg/kg ketamine (Ketalar, Pfizer Warner-Lambert, Turkey) and 5-10 mg/kg xylazine (Alfazyne, Alfasan International BV, Netherlands), and the depth of anesthesia was assessed by skin or toe pinch responses. For the surgical procedure, animals were shaved from the costal arch to approximately 7 cm below, and the inferior epigastric artery skin island flap was elevated by making a midline incision and dissecting the flap medially to laterally, preserving the pedicle formed by the inferior epigastric artery, vein, and nerve. The surgical field was aseptically prepared using 10% povidone-iodine. All rats were operated on under general anesthesia by the same surgeon, who did not

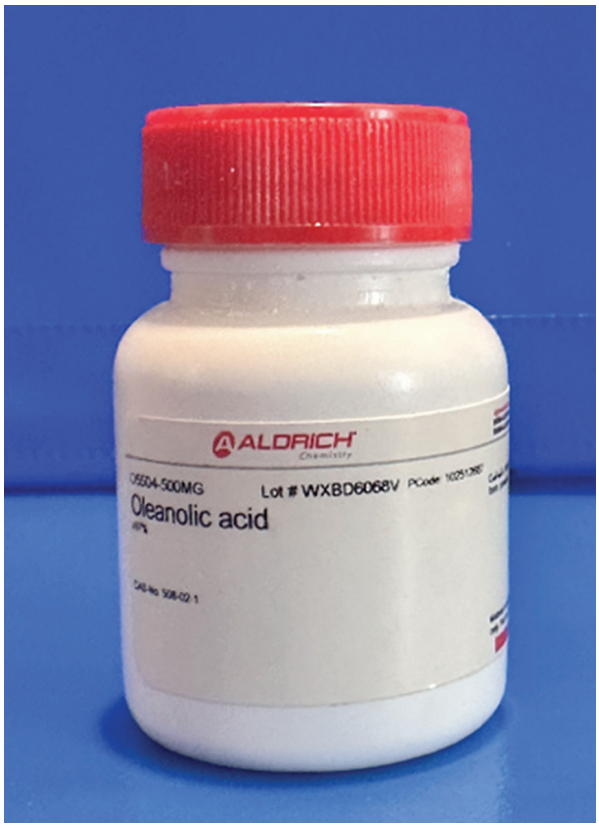

Figure 1. *The commercial form of oleanolic acid.*

know which groups the rats belonged to. Preparation and dissection of the epigastric artery island flap was based on the description of Petry and Wortham[13].

For biochemical analysis, the samples were centrifuged, transferred to Eppendorf tubes, and stored at –80°C. After measuring their weights, the samples were homogenized in a cocktail of Triton X solution and protease inhibitors at a volume 4 times their weight. The total oxidant stress (TOS) and total antioxidant capacity (TAC) levels were determined using Rel Assay Diagnostics test kits from Gaziantep, Turkey. TOS was measured using the Rel Assay Diagnostics test kit (Catalog No: RL0024), which quantifies oxidants based on their ability to oxidize a ferrous ion-chelator complex. The color intensity of the formed complex is proportional to the total oxidants present and is reported as micromolar H_2O_2 equivalents per liter (μmol H_2O_2 Eq/L)[14]. TAC was assessed using the Rel Assay Diagnostics test kit (Catalog No: RL0017), which measures the reduction of the blue-green ABTS.+ radical to colorless ABTS. The absorbance change at 660 nm indicates the TAC of the sample and is expressed as trolox equivalent mmol/L[15].

For histopathological analysis, tissue samples were fixed in 4% formaldehyde and embedded in paraffin. The paraffin-embedded blocks were sectioned at 4 μm

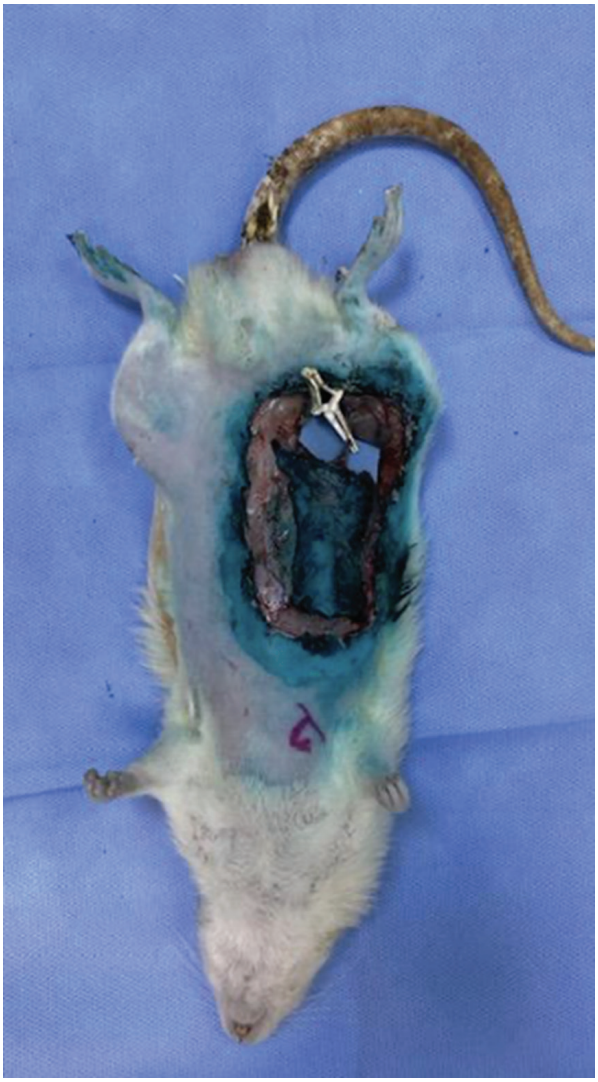

Figure 2. *Creation of ischemia with a microvascular clamp following the design and elevation of the flap.*

thickness, stained with Hematoxylin-Eosin, and examined semiquantitatively under a light microscope by a pathologist. The analysis focused on inflammation, including polymorph nucleated leukocytes (PMNL) count and lymphocyte density, as well as edema and necrosis in the papillary dermis. All histopathological evaluations were conducted in a blinded manner at the Medical Pathology Department of the University of Health Sciences, Gulhane Faculty of Medicine.

Data were analyzed using IBM SPSS Statistics version 21 (IBM SPSS Inc, Chicago, IL). Statistical significance was set at $p < 0.05$. Descriptive statistics for continuous variables included means ± standard deviations, medians, and ranges. Normality of the data was assessed with the Kolmogorov-Smirnov test, and homogeneity of variances was evaluated using Levene's test. For non-normally distributed data with

Table 1. Study groups

Procedures	Group I (sham)	Group II (I/R)	Group III (OA+I/R)
Care and treatment	Standard care for 5 days ↓	PBS administered intraperitoneally for 5 days (1 mL/kg/day) ↓	OA administered intraperitoneally for 5 days (8 mg/kg/day) ↓
Flap elevation	Flap elevation on the 6th day		
I/R time	Immediate replacement of the flap without ischemia ↓	Two hours of ischemia and 2 h of reperfusion were induced in the flap ↓	Two hours of ischemia and 2 h of reperfusion were induced in the flap ↓
Sample collection	1.5 × 1.5 cm samples were taken from the flap for biochemical and pathological analysis		

I/R: ischemia reperfusion; OA: oleanolic acid; PBS: phosphate-buffered saline.

Table 2. Comparison of TOS, TAC, inflammation, edema, and necrosis data across groups

Parameters	Group I (sham)	Group II (IR)	Group III (OA + R)	p
TOS (μmol H_2O_2 equivalent/L (Mean ± SD)	0.55 ± 0.24	3.53 ± 0.42	1.13 ± 0.52	**0.001 (Group 1-2: 0.002, Group 1-3: 0.018, Group 2-3: 0.002)**
TAC (mmol/L) (Mean ± SD)	0.16 ± 0.07	0.49 ± 0.2	0.71 ± 0.11	**0.001 (Group 1-2: 0.003, Group 1-3: 0.002, Group 2-3: 0.048)**
Inflammation (Mean ± SD)	1 ± 0.58	1.57 ± 0.79	1.14 ± 0.38	0.254
Edema (Mean ± SD)	0.57 ± 0.53	1.43 ± 0.98	0.71 ± 0.49	0.120
Necrosis (Mean ± SD)	0 ± 0	0 ± 0	0 ± 0	1.000

Bold values indicate statistically significant p values ($p < 0.05$) in pairwise comparisons (Mann-Whitney U test) following Kruskal-Wallis analysis. IR: ischemia reperfusion; OA: oleanolic acid; SD: standard deviation; TAC: total antioxidant capacity; TOS: total oxidant stress.

more than two groups, the Kruskal-Wallis test was used to compare distributions, and the Mann-Whitney U test was applied to identify significant differences.

Results

The mean TOS levels were 0.55 ± 0.24 μmol H_2O_2 equivalent/L in the sham group, 3.53 ± 0.42 μmol H_2O_2 equivalent/L in the I/R group, and 1.13 ± 0.52 μmol H_2O_2 equivalent/L in the OA+I/R group, with statistically significant differences among all groups ($p = 0.001$). For TAC, the mean values were 0.16 ± 0.07 mmol/L in the sham group, 0.49 ± 0.2 mmol/L in the IR group, and 0.71 ± 0.11 mmol/L in the OA+I/R group, also showing significant differences ($p = 0.001$). There were no statistically significant differences in inflammation or edema among the groups ($p > 0.05$), and no necrosis was observed in any samples (Table 2, Figs. 3 and 4).

Discussion

OA (3β-hydroxy-olean-12-en-28-oic acid) is a triterpenoid compound that occurs naturally in various plant species[16]. It is a potent antioxidant that neutralizes free radicals. Numerous studies have demonstrated the antioxidant effects of OA by protecting cell membranes and reducing oxidative stress[17]. OA reduces inflammation by inhibiting various inflammatory cytokines and enzymes[18]. Due to its anti-inflammatory, antitumor, antiviral, antidiabetic, antihyperlipidemic, and hepatoprotective properties, OA has been investigated in many medical studies concerning various neurological diseases, diabetes, cardiovascular diseases, cancer, rheumatoid arthritis, ulcerative colitis, asthma, allergic rhinitis, and other inflammatory conditions; it is believed that many of these effects are attributed to the antioxidant properties of OA[17].

With the recent popularity of free tissue transfers and limb and facial transplantations, I/R injury has emerged as a significant parameter affecting tissue survival and surgical success[2,3]. The pathophysiology of I/R injury includes reactive oxygen species (ROS), PMNL, the complement system, and endothelial cells. ROS are continuously produced by normal oxygen utilization in the body, such as respiration, and by certain cell-mediated immune functions[19].

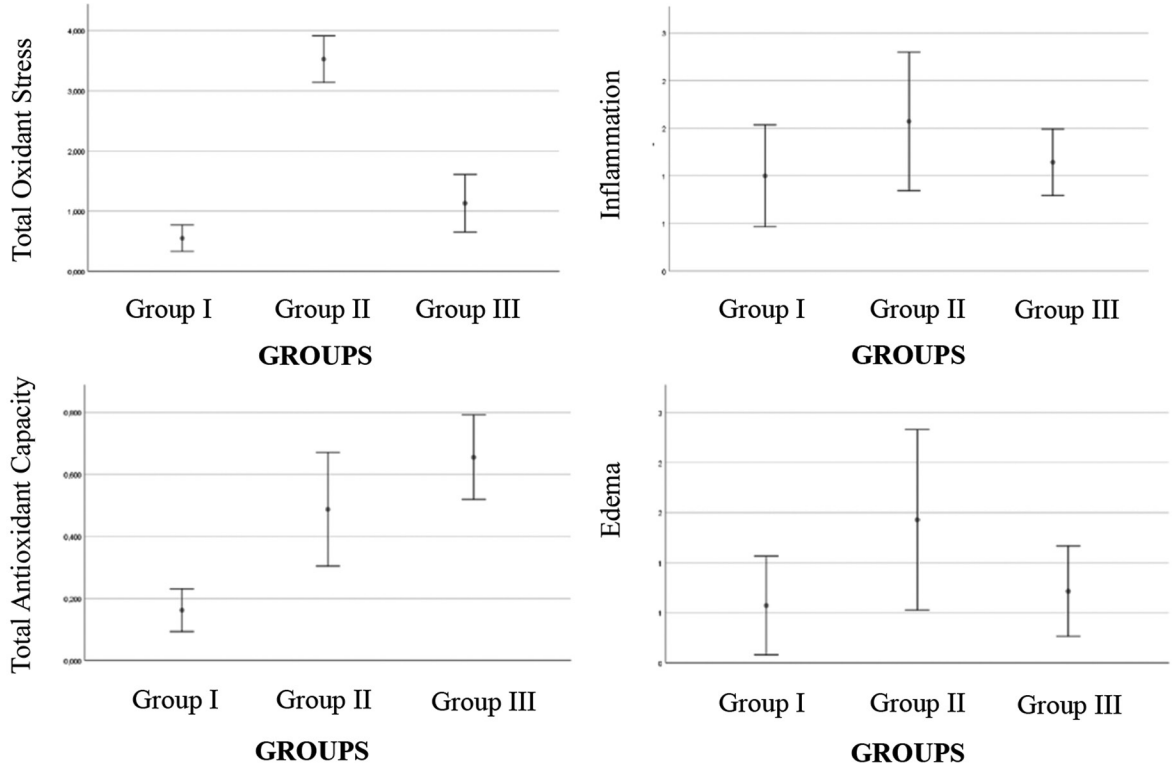

Figure 3. *Comparison of total oxidant stress, total antioxidant capacity, inflammation, and edema data among the groups.*

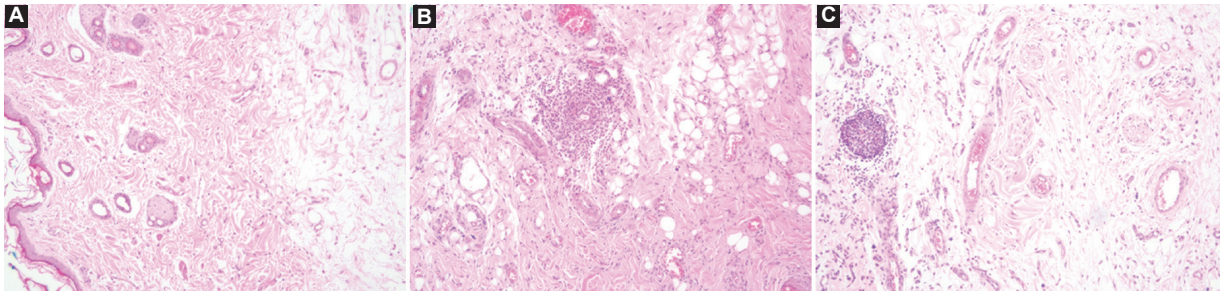

Figure 4. *Histopathological analysis showing inflammation, edema, and necrosis in hematoxylin and eosin-stained light microscopy images:* **(A)** *Group I (Sham),* **(B)** *Group II (I/R),* **(C)** *Group III (OA + I/R). IR: ischemia reperfusion; OA: oleanolic acid.*

While physiological concentrations of ROS are necessary for normal cell function, excess ROS, if not effectively cleared by cellular components, can react with various biomolecules, such as DNA[20], lipids[21], and proteins[22], initiating peroxidation of membrane lipids and leading to the accumulation of lipid peroxides. Consequently, DNA and proteins may sustain damage, resulting in disease states. In fact, ROS have been associated with over 100 diseases[23]. During I/R injury, the amount of ROS increases, thereby exacerbating oxidative stress. A balance between oxidants and antioxidants is important in preventing flap loss. Antioxidants neutralize free radicals in cells, reducing oxidative stress and preventing cellular damage[24]. In this study, we aimed to investigate the protective effects of OA against I/R injury observed during the surgery of skin flaps by utilizing its antioxidant properties. To date, no study has specifically investigated the effects of OA in flap surgery, making our study a pilot investigation in this regard.

Li et al. conducted a study administering OA intraperitoneally, concluding that OA reduces oxidative stress[25]. Han et al. created a subarachnoid hemorrhage model in animals to investigate the antioxidant effects

of OA, observing that oxidative stress was reduced, and neuronal apoptosis was inhibited in treated animals. Moreover, OA's antioxidant effects were shown to activate the nuclear factor erythroid 2–related factor 2 / Heme oxygenase-1 (Nrf2/HO-1)-1 pathway[26]. Wang et al. confirmed that OA enhances antioxidant capacity, reducing oxidative stress and demonstrating potent antioxidant activity[27]. In a review by Fernández-Aparicio et al., the reducing effects of OA on oxidative stress were examined. This review analyzed over 5,000 articles published in the past 20 years and presented results from 13 animal experiments and three cell-level studies investigating the effects of OA on oxidative stress. The review concluded that OA effectively reduces oxidative stress, modulating the IRS/PI3K/Akt/FoxO1 signaling pathway[28]. In our study, the highest oxidative stress was observed in the control group exposed to I/R injury. The group administered OA exhibited significantly lower oxidative stress compared to the control group, clearly attributable to the antioxidant effects of OA.

TAC is a test that measures the amount of all free radicals that can be neutralized by antioxidants in a biological sample. It provides information about the presence and activity of antioxidants[15]. TAC is generally measured in plasma, serum, or tissues and is used in studies to evaluate the efficacy of antioxidant supplements. Considering the important roles of ROS and antioxidant molecules during I/R injury, measuring TAC may provide insights into the severity of I/R injury[29]. In a study by Li et al., the antioxidant effects of OA were explored in an animal model. Although TAC was not measured, the functions of superoxide dismutase (SOD) were assessed, revealing a significant increase in SOD activity in animals treated with OA[25]. Wang et al. also demonstrated an increase in antioxidant capacity following the administration of OA in another animal study[27]. Gao et al. observed that the application of OA led to an increase in the activities of antioxidant enzymes, such as SOD and glutathione peroxidase[17]. While TAC was not directly measured in these studies, the increase in enzyme activities illustrated the antioxidant efficacy of OA. In our study, the lowest antioxidant activity was observed in the sham group, while the I/R group exhibited a higher antioxidant capacity compared to the sham group. The highest antioxidant capacity was found in the group treated with OA. Since the sham group did not undergo ischemia, an increase in oxidative stress and a low level of antioxidant capacity were expected. In the control group, where I/R injury occurred, an increase in antioxidant capacity was noted. However, the highest antioxidant capacity was observed in the group administered OA intraperitoneally. These data suggest that OA mitigates oxidative stress by increasing antioxidant capacity. Previous studies have similarly indicated that OA enhances antioxidant capacity[17,27]. Our findings align with the existing literature regarding the elevation of antioxidant capacity in the context of ischemia-reperfusion during flap surgery.

The pathophysiology of I/R injury arises from a series of inflammatory responses. Initially, in response to tissue hypoxia, there is an increase in neutrophils, monocytes, and other immune cells. This leads to the release of pro-inflammatory cytokines (interleukin [IL]-1, tumor necrosis factor-alpha, and IL-6), which bind to receptors on vascular endothelial cells and other immune cells, prompting further migration of neutrophils and monocytes. These cells then migrate to damaged tissues, perpetuating the inflammatory response. While there is no definitive treatment for I/R injury, therapeutic efforts focus on suppressing inflammation[30,31], highlighting the critical role of inflammation in I/R injury. In a cellular study by Yan et al., OA was shown to inhibit cell adhesion by reducing intercellular adhesion molecule-1 (ICAM-1) expression, thereby suppressing inflammation[32]. In a study by Singh et al., OA was demonstrated to reduce both inflammation and edema in rats subjected to inflammation and edema induction[33]. Lee et al. showed that OA effectively reduces inflammation in cellular studies. Their findings suggest that OA possesses anti-inflammatory effects by inhibiting hyperpermeability, the expression of cell adhesion molecules, and the adhesion and migration of leukocytes, indicating its potential as a therapeutic option for inflammation-related diseases[34]. In our study, although no statistical differences were found among groups, the inflammation and edema values in the OA group were found to be closer to those of the sham group. The control group exposed to ischemia exhibited higher levels of both inflammation and edema compared to the other two groups. This difference suggests that OA may suppress both inflammation and edema, likely linked to the reduction of oxidative stress, although the results were not statistically significant.

Despite showing antioxidant and anti-inflammatory effects, these molecules have not yet been therapeutically applied in humans. This is primarily due to the lack of synthesized OA derivatives that meet the requirements for adequate water solubility and

bioavailability[35]. Therefore, it is essential to develop new terpenoid derivatives that not only address solubility issues but also enhance therapeutic effects.

To date, no studies have been found in the literature regarding the protective effects of OA on I/R injury observed in skin flaps. Our study serves as a pioneering investigation in this area. It is a randomized controlled and prospective study, which are strengths of this research. A limitation of our study is that it was conducted on a small animal group without supporting clinical trials.

Conclusions

The findings of this study demonstrate that OA effectively reduces oxidative stress and enhances antioxidant capacity in skin flaps subjected to I/R injury. Although no statistical differences were found in inflammation and edema values, observationally, the inflammation and edema values in the OA group were closer to those of the sham group. These results suggest that OA holds potential as a protective agent against I/R-induced skin tissue damage. Further research, including large-scale, clinically controlled, randomized trials, is warranted to substantiate these findings. In addition, the development of an effective clinical derivative of OA remains a critical goal for translating these results into therapeutic applications.

Funding

The authors declare that they have not received funding.

Conflicts of interest

The authors declare no conflicts of interest.

Ethical considerations

Protection of humans and animals. The authors declare that the procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation and with the World Medical Association and the Declaration of Helsinki. The procedures were authorized by the Institutional Ethics Committee.

Confidentiality, informed consent, and ethical approval. This study does not involve personal patient data, medical records, or biological samples, and does not require ethical approval. SAGER guidelines do not apply.

Declaration on the use of artificial intelligence. The authors declare that no generative artificial intelligence was used in the writing or creation of the content of this manuscript.

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