Aesth Plast Surg (2022) 46:3047–3062
https://doi.org/10.1007/s00266-022-03048-6

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ORIGINAL ARTICLE BASIC SCIENCE/EXPERIMENTAL

The Novel Role of *Crocus sativus* L. in Enhancing Skin Flap Survival by Affecting Apoptosis Independent of mTOR: A Data-Virtualized Study

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Received: 22 May 2022 / Accepted: 25 July 2022 / Published online: 31 August 2022

Abstract

Background Despite the improvements to enhance skin flap viability, the effects of ischemia-reperfusion (IR), oxidative stress, necrosis, and apoptosis are still challenging. *Crocus sativus* L. (Saffron) is highly noticeable due to its tissue-protective and antioxidant properties. So, we aimed to investigate its effects on skin flap viability, oxidative stress, apoptosis markers, histopathological changes, and mTOR/p-mTOR expression.

Materials and Methods 40 Sprauge-Dawley rats, weighting 200–240 g, were divided into four groups including: (1) Sham (8 × 3 cm skin cut, without elevation); (2) Flap Surgery (8 × 3 cm skin flap with elevation from its bed); (3) Saffron 40 mg/kg + Flap Surgery; and (4) Saffron 80 mg/kg + Flap Surgery. Saffron was administrated orally for 7 days. At day 7, flap necrosis percentage, histopathological changes, malondialdehyde level, Myeloperoxidase and superoxide dismutase activity, Bax, Bcl-2, mTOR, and p-mTOR expression were measured. Protein expressions were controlled by β-Actin.

Results Saffron administration decreased flap necrosis percentage ($p < 0.01$), which was not dose-dependent. Treatment groups showed significant histological healing signs (Neovascularization, Fibroblast migration, Epithelialization, and Epithelialization thickness), decreased MDA content ($p < 0.01$), increased SOD ($p < 0.01$) and decreased MPO activity ($p < 0.01$). Bax and Bcl-2 expression, decreased and increased respectively in treated groups ($p < 0.0001$). mTOR and p-mTOR expression were not changed significantly in Saffron treated groups.

Conclusion Saffron could increase skin flap viability, alleviate necrosis, decrease oxidative stress and decrease apoptotic cell death, after skin flap surgery, but it acts independent of the mTOR pathway. So, Saffron could potentially be used clinically to enhance skin flap viability.

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Keywords Skin flap · *Crocus sativus* L.,Saffron · Oxidative stress · Reactive oxygen spices (ROS) · Ischemia-reperfusion (IR)

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Introduction

Skin flaps are a widely-used major strategy of plastic, aesthetic, and reconstructive surgeries for treatment of skin defects. This method is used to overcome traumatic skin damages, refractory wounds, cancer excisions, burn injuries, and lower limb vascular ulcers due to its convenience and resilience [1–4]. Nevertheless, rate of flap tissue loss, as the main challenging issue, varies from 25 to 50% of flap length [5]. Skin flap survival (SFS) is threatened by a triad including mal-perfusion, inflammatory mechanisms, and oxidative stress (Fig. 1) [6, 7]. Mal-perfusion triggers extensive damage of cell membrane in ischemic flap tissues which results in increased levels of arachidonic acid metabolites. It promotes recruitment of inflammatory cells, substantially the neutrophils. Immune cells produce more reactive oxygen species (ROS) and inflammatory mediators in ischemic tissue. ROS threatens tissue viability, causing DNA and cell membrane destruction, lipid peroxidation, cell apoptosis, and protein changes. In addition, ischemia–reperfusion injury (IRI) occurs due to consequent neovascularization. Blood reperfusion triggers a burst of ROS is the cardinal culprit of IRI pathogenesis which is accompanied by impaired cellular redox homeostasis and extensive cellular apoptosis. ROS accumulation and apoptosis of functional cells are major factors resulting from necrosis of skin flaps [8–10].

Systemic or local administration of various therapeutic agents with the aim of preventing distal flap necrosis, has recently attracted growing attention. Treatments include angiogenesis promotion, oxidative stress/apoptosis/inflammation attenuation, administration of agents such as blood viscosity diminishers, calcium channel blockers (CCB), vasodilators, anti-thrombotics, anti-coagulants, anti-inflammatories, anti-oxidants, steroids and hyperbaric oxygen therapy. However, Surgical delay could be used to control distal necrosis but it requires at least one additional surgery [11–14].

Nowadays, there is a special focus on reducing oxidative stress and increasing tissue defense mechanisms to deal with ROS injury. Major antioxidants like superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) and glutathione (GSH) are important for cellular protection due to their ability to detoxify free radicals, such

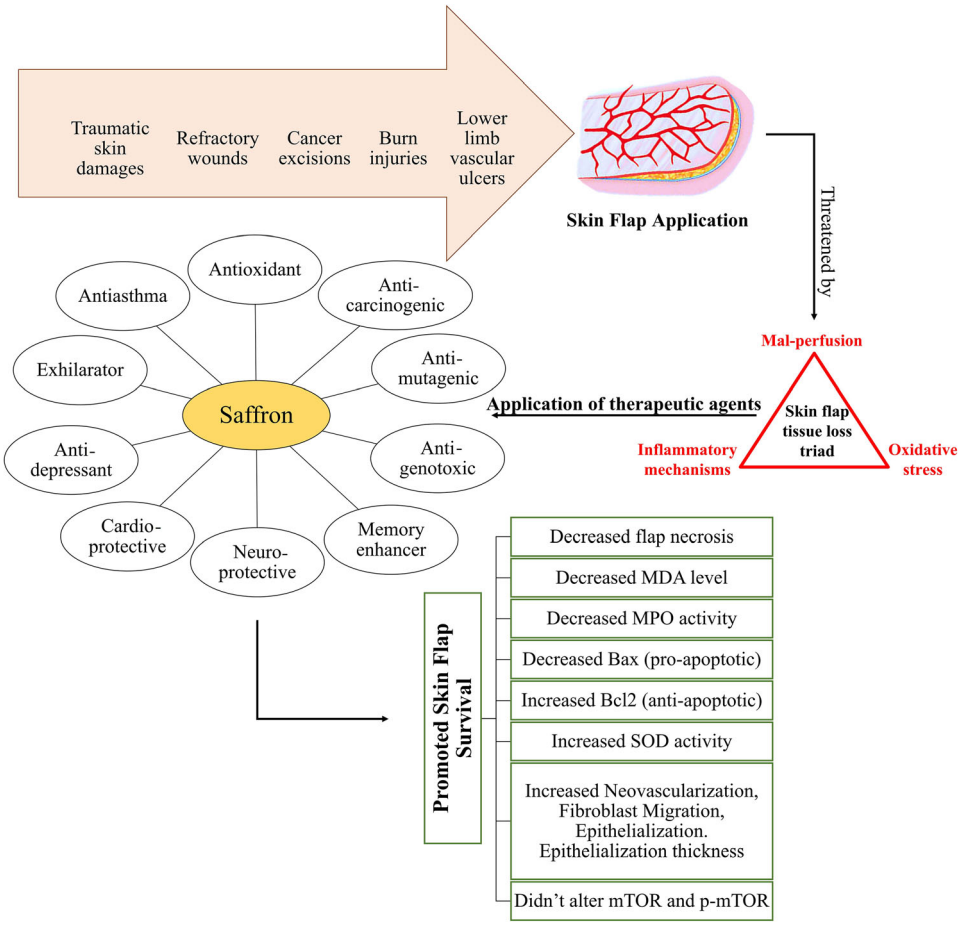

Fig. 1 Skin flaps are used as a major technique in reconstructive surgery but the survival of flaps decreases under the influence of threatening factors. Saffron increases the survival of skin flaps and has the potential for clinical use

as ROS. At the same importance of ROS elimination, apoptosis control is noticeably mentioned enhance SFS. It would be a challenging issue that except decrease in ROS effects, necrosis, and inflammation, we could increase anti-apoptotics and decrease pro-apoptotics. Bcl-2 is an important protein of BCL2 family that prevent cell death through apoptosis. This family also contains pro-apoptotics such as Bax. By decreasing Bax, or increasing Bcl-2, SFS could be promoted (Fig. 2) [15, 16].

Autophagy as intracellular degradation/recycling of dysfunctional proteins and organelles, has a great control on cell signaling, homeostasis, cell survival, and apoptosis prevention. It can alleviate ROS production and suppress apoptosis via the mTOR signaling pathway. It is reported that autophagy has attenuated liver IRI, inhibited apoptosis induced by intestinal ischemia-reperfusion, and had important role in disc degeneration due to alleviating mitochondrial dysfunction. As a consequence, research suggest that autophagy induction could enhance angiogenesis, decrease apoptosis and oxidative stress. Altogether it might be beneficial for skin flaps survival (Fig. 2) [17, 18].

Dried stigmas of *Crocus sativus* L. (Saffron), a worthy plant of Iridaceae family is mainly made up of carbohydrates including starch, reducing sugars, gums, pectin, pentosans, and dextrins. It is a plant with a vast range of pharmacological properties such as anti-carcinogenic, anti-mutagenic, and anti-genotoxic [19, 20], anti-oxidant [21], memory enhancer [22], neuro-protective [23, 24], anti-depressant, exhilarator, antiasthma, and cardio-protective effects (Fig. 1) [25]. Even it is declared that Saffron is a potential drug-supplement for severe acute respiratory syndrome coronavirus (COVID) management [26, 27]. Components such as Crocin (digentiobiosyl8, 8′-diapocarotene-8, 8′-oate; $C_{44}H_{64}O_{24}$) [28] and Crocetin are more responsible for cardio-protective and anti-atherosclerotic properties [29, 30]. Saffron has been used as an effective factor on neuro-degenerative disease and related memory impairment, ischemic retinopathy and age-related macular degeneration, coronary artery disease, blood pressure abnormalities, acute and chronic inflammatory disease, mild to moderate depression, seizure, Parkinsonism [31, 32]. It is reported that oral administration of Saffron extract, has inhibited cisplatin, cyclophosphamide, mitomycin-C and urethane induced genotoxicity in male Swiss albino mice [33, 34].

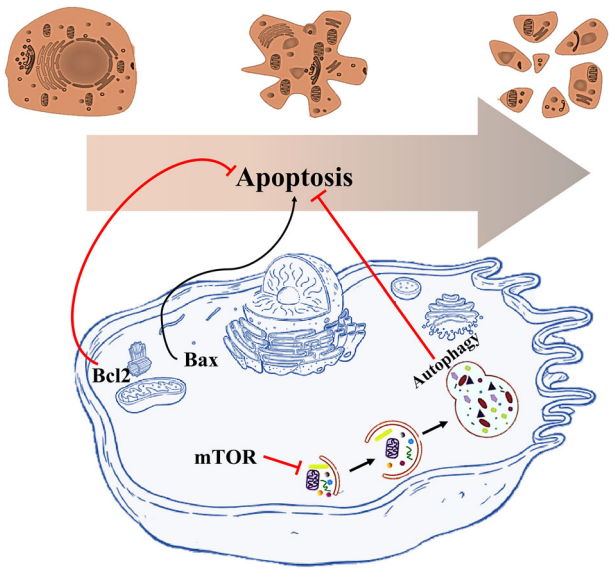

Fig. 2 Apoptosis promotes by increasing the Bax/Bcl-2 ratio. mTOR (especially mTORC1) inhibits autophagy. During autophagy, dysfunctional parts degrades and this results in apoptosis alleviation

Skin flap survival is a process that depends heavily on micro-environment of the tissue. Considering this issue and the properties of Saffron, it seems to be possible to manage oxidative stress and cell death of skin flap by Saffron administration. Saffron have received growing attention as potential preventive agent that scavenges ROS and detoxify them, inhibit inflammatory pathways, and finally decrease cell death. The present study was designed to investigate the modifying effects of Saffron extract on survival, antioxidant status, inflammation, apoptotic markers, histopathologic changes, and its probable effect on mTOR pathway of skin flap model in Sprauge-Dawley albino rats.

Materials and Methods

Animals and Ethical Considerations

Male Sprauge-Dawley rats (N = 40, weight = 200–240 g), were kept in 22–25 centigrade degrees and 12-h light, 12-h dark cycles. They were divided into four groups including the (1) Sham group, (2) Flap Surgery (Control) group, (3) Saffron 40 mg/kg, and (4) Saffron 80 mg/kg. After a one-week adaptation, animals were anaesthetized with 50 mg/kg ketamine hydrochloride 10% (i.p.) and 4 mg/kg xylazine 2% (i.p.). A method similar to McFarlane et al. [35] was used to lift the skin flap and assess the necrotic area. Animals were shaved, and the site was disinfected with alcohol. In the Sham group, skin was cut in 8 × 3 cm without skin elevation. In the three other groups, after providing two parallel 8 cm incisions, they were connected caudally with a 3 cm incision and skin was gently separated from its bed using a scalpel, then a sterile plastic film was placed between elevated skin and its supporting fascia. All incisions were sutured. Finally, rats were returned into separate cages. Saffron is administrated orally in flap surgery day and the next six days. Group 3 received Saffron

extract at 40 mg/kg/day dosage and group 4 received Saffron extract at 80 mg/kg/day dosage. Then, on day seven after surgery, the animals were sacrificed by injecting ketamine at a dose of 150 mg/kg. Tissue sampling was performed by excising a full-thickness skin from the intermediate margin between the necrotic area and the grossly healthy area [36]. The flaps were divided into two parts by a precise longitudinal incision, one component used to measure Mean MDA level, MPO and SOD activity, Bax, Bcl-2, mTOR, and p-mTOR expression (kept at − 80 ° C). The other part used to determine pathological damage and parameters (preserved in formalin 10%), Fig. 3.

The study protocol was approved by Research Ethics Committees of School of Medicine—Tehran University of Medical Sciences (Tehran, Iran), Approval ID: IR.TUMS.MEDICINE.REC.1400.1013. All applicable institutional and/or national guidelines for the care and use of animals were followed.

Measurement of Flap Necrotic Area

The percentage of the necrotic area in each flap was calculated on day seven after flap surgery. For this purpose, the flap necrosis rate was computed using the paper pattern method. The boundary between living tissue and necrosis was determined by markers of viability (including skin color, skin softness, warmth, and hair) and signs of necrosis (including dark skin, stiffness, coolness, and hair loss). A mold was drawn from the entire flap, and a transparent paper of equal size was cut and weighed to Calculating the percentage of necrosis. Then, the amount of necrosis was drawn on transparent paper and cut and weighed. By dividing the weight of the part of the transparent paper that showed flap necrosis by the total weight of the translucent paper, the percentage of flap necrosis was obtained.

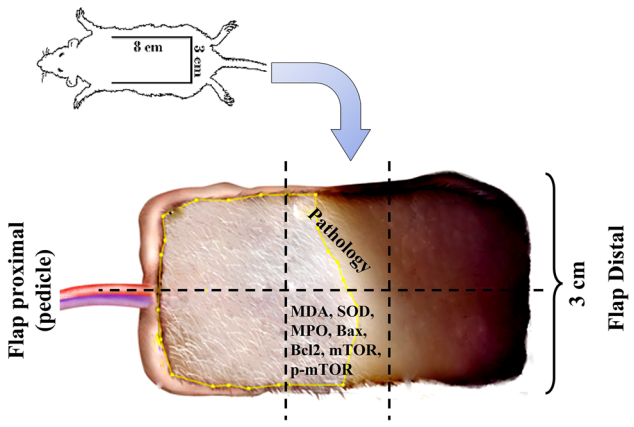

Fig. 3 Skin flap location. Tissue sampling was performed by excising a full-thickness skin from the intermediate margin between the necrotic area and the grossly healthy area

$$\text{Percentage of flap necrosis } (\%) = \frac{\text{Weight of flap necrosic area transparent paper}}{\text{Total weight of the translucent paper}} \times 100$$

MDA Content, MPO and SOD Activity in the Flap Tissues

Tissue samples were obtained from each flap, weighed and then were homogenized in Tris-ethylenediamine ether acetic acid buffer (pH = 7.0 and 4 °C) and diluted to 10% (v/v) in an ice bath. SOD enzyme activity was determined by using the kit method (ab65354, ab83464, Abcam), which was based on the method of Muthuraman et al. [37]. MDA was measured by determining the thiobarbituric acid reactive species (TBARS). The absorbance of the resultant product was measured at 534 nm (UV-Vis spectrophotometer). MPO activity in the flap tissue homogenates was determined based on commercially available MPO activity kit (Sigma-Aldrich; MAK069).

Western Blot Analysis to Determine Bax, Bcl-2, mTOR and P-mTOR Expression in the Flap Tissues

Mice were anesthetized at day 7. Immediately skin flaps were removed and frozen in liquid nitrogen and maintained at − 80 °C until used for western blot. We homogenized tissues in ice-cold RIPA lysis buffer (#9806, Cell Signaling Technology, Italy), containing protease inhibitor, to extract total protein. Later all samples were centrifuged at 13000 g for 20 min at 4 °C, and then the supernatant was removed and protein concentration was quantified by spectrophotometer. Protein samples were boiled for 5 min at 95 °C in sodium dodecyl sulfate loading buffer. Proteins (20 mg) were separated through 10% polyacrylamids SDS gels and transferred onto a nitrocellulose membrane. All membranes were incubated with primary antibodies for β-actin (Abcam; β-actin (ab8226), as loading control). For Bcl-2 analysis, we used rabbit polyclonal anti-rat Bcl-2 (ab59348), and for Bax analysis, we used anti-rat Bax (ab53154). We used mTOR (#2972, Rabbit polyclonal antibody for mTOR Cell Signaling Technology, Italy) and P-mTOR (#2974, Rabbit polyclonal antibody for Phospho-mTOR (Ser2481), Cell Signaling Technology, Italy) for mTOR and P-mTOR analyses. Then membranes were incubated with HRP-conjugated anti-mouse secondary antibody (#7072, Cell Signaling Technology, Italy). Membranes were developed with ECL plus reagent, and protein bands were visualized by exposing X-ray films, and finally quantified and analyzed by the lab work software.

Histological Evaluation

After day 7, flap tissues were collected, and consequently, the tissue preparation procedure for H&E staining was started. Tissue sections have been prepared by H&E staining on day seven and studied by light microscopy. Two expert pathologists blinded to the treatments, have evaluated all slides and they reported the results for Neovascularization, Fibroblast Migration, Epithelialization, and Epithelialization thickness. Scores were defined as follows: 0 = normal, 1 = mild, 2 = moderate, and 3 = severe for Neovascularization, Fibroblast Migration, Epithelialization, and Epithelialization thickness was reported by + or ++.

Statistical Analysis

All analysis was performed using GraphPad Prism 8 (GraphPad Software, San Diego, California, USA). One-way analysis of variance (ANOVA) and Post-Hoc analysis (Tukey test) were applied for comparing the biochemical data between groups. Data are presented as mean ± SD, including 99% confidence interval (CI) ($\alpha = 0.01$).

Results

Percentage of the Flap Necrosis

Skin flap necrosis percentage in all flap samples determined at day seven after flap surgery and average of the necrotic of each group has been reported in Table 1, Supplementary Table 1, and illustrated in Fig. 4A. Sham group had the least flap necrosis (under 1%). Flap elevation led to a significant necrosis (39.71 ± 4.94%) in Flap Surgery (Control) group ($P < 0.0001$ vs. Sham). Saffron administration in 40 mg/kg/day dosage group, significantly decreased the necrotic percentage of the flap tissue to 23.47 ± 3.54% ($P < 0.0001$ vs. Flap Surgery group). Also, in 80 mg/kg/day dosage group, necrosis percentage significantly decreased to 19.77 ± 4.60% ($P < 0.0001$ vs. Flap Surgery group). We did not observe a significant difference in the flap necrosis percentage between Saffron 40 mg/kg/day and 80 mg/kg/day treated groups ($P = 0.162$). Difference between group means is illustrated in Fig. 4B. As shown in this figure, difference of the mean between the Sham group and Flap Surgery group is the most negative one, which indicates that flap surgery had an increasing effect on the flap necrosis percentage. In the other hand, the most positive differences of the means are related to the difference between Flap Surgery group and groups treated with Saffron, which indicates that Saffron treatment had a reducing effect on the flap necrosis percentage. Difference between Flap Surgery group mean, with Saffron 80 and Saffron 40 is 19.94% and 16.23%, respectively. Also, the difference between the two groups of Saffron 40 and Saffron 80 is almost close to the zero line, which shows that the results of these two groups are not significantly different.

MDA Level

Lipid peroxidation as an index of oxidative stress in the flap samples was determined based on mean MDA level in the tissue at day seven after flap surgery. MDA level of each group has been reported in Table 1, Supplementary Table 2, and illustrated in Fig. 5A. Sham group had the least MDA level (13.90 ± 10.58 nmol/mg protein). After flap elevation we observed a significant increase in the flap tissue MDA level in Flap Surgery group ($P < 0.0001$ vs. Sham group) and MDA level reached 69.83 ± 7.63 nmol/mg protein in this group. Saffron administration in 40 mg/kg/day dosage group, significantly decreased the MDA level of the flap tissue to 36.46 ± 10.00 nmol/mg protein ($P < 0.0001$ vs. Flap Surgery group). Also, in Saffron 80 mg/kg/day dosage group, MDA level significantly decreased to 36.34 ± 10.34 ($P < 0.0001$ vs. Flap Surgery group). We did not observe a significant difference in the MDA level between Saffron 40 mg/kg/day and 80 mg/

Table 1 Measured necrosis percentage, MDA level, SOD and MPO activity, Bax, Bcl-2, mTOR, and p-mTOR expression

	Unit	Sham	Flap Srg	Saffron 40	Saffron 80
Necrosis Percentage ± SD	%	0.219 ± 1.512	39.714 ± 4.948	23.479 ± 3.545	19.770 ± 4.607
Mean MDA level ± SD	n mol/mg protein	13.903 ± 10.587	69.838 ± 7.638	36.469 ± 10.005	36.345 ± 10.341
SOD Activity ± SD	U/mg protein	69.943 ± 16.887	11.843 ± 3.692	66.963 ± 11.085	70.734 ± 8.578
MPO Activity ± SD	U/mg protein	0.878 ± 0.158	1.962 ± 0.331	1.200 ± 0.180	1.256 ± 0.336
Bax Expression	Relative protein expression	0.515 ± 0.176	1.648 ± 0.405	0.791 ± 0.256	0.621 ± 0.239
Bcl-2 expression	Relative protein expression	1.599 ± 0.373	0.436 ± 0.116	1.709 ± 0.348	1.910 ± 0.247
mTOR expression	Relative protein expression	0.951 ± 0.213	1.048 ± 0.491	0.891 ± 0.310	0.921 ± 0.290
P-mTOR expression	Relative protein expression	1.199 ± 0.357	1.236 ± 0.161	1.309 ± 0.380	1.210 ± 0.271

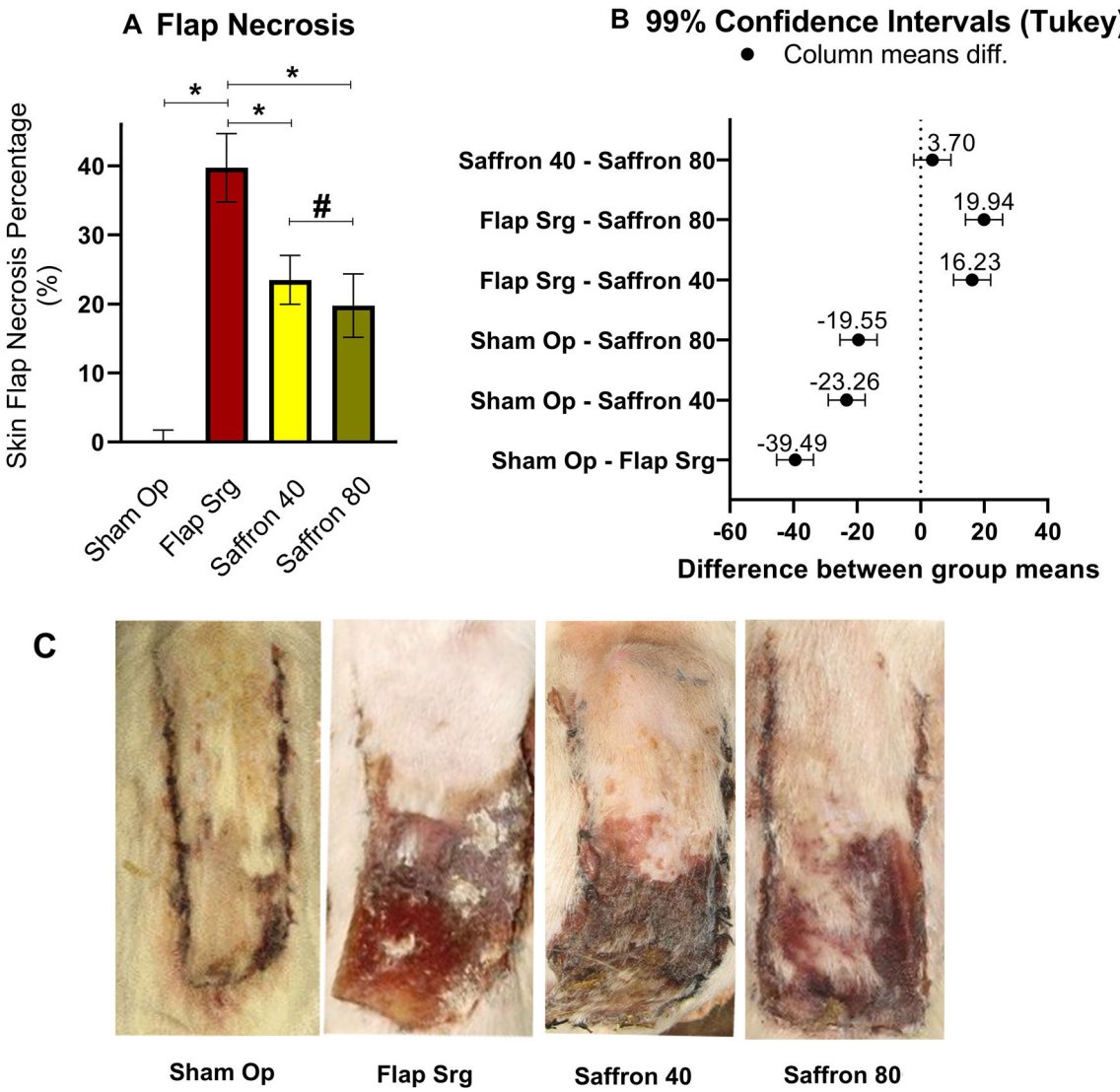

Fig. 4 **A** Flap necrosis percentage 7 days after surgery. Data are represented as Mean ± SD. (*) $P < 0.0001$ and significant. (#) $P = 0.162$ and non-significant. **B** Difference between group means

kg/day treated groups ($P = 0.999$). Difference between group means is illustrated in Fig. 5B. As shown in this figure, difference of the mean between the Sham group and Flap Surgery group is the most negative one, which indicates that flap surgery had an increasing effect on the MDA level. In the other hand, the most positive differences of the means are related to the difference between Flap Surgery group and groups treated with Saffron, which indicates that Saffron treatment had a reducing effect on the MDA level. Difference between Flap Surgery group mean, with Saffron 80 and Saffron 40 is 33.49 nmol/mg protein and 33.36 nmol/mg protein, respectively. Also, the difference between the two groups of Saffron 40 and Saffron 80 is almost close to the zero line, which shows that the results of these two groups are not significantly different.

SOD Activity

SOD activity was used as an indicator of the antioxidant capacity of the flap tissue. SOD activity of each group has been reported in Table 1, Supplementary Table 3, and illustrated in Fig. 6A. Flap Surgery group had the least SOD activity (11.84 ± 3.69 U/mg protein) and we observed a significant decrease in the flap tissue SOD activity in this group ($p < 0.0001$ vs. Sham group). Saffron administration in 40 mg/kg/day dosage group, significantly increased SOD activity of the flap tissue to 66.96 ± 11.08 U/mg protein ($P < 0.0001$ vs. Flap Surgery group). Also, in Saffron 80 mg/kg/day dosage group, SOD activity significantly increased to 70.73 ± 8.57 U/mg protein ($P < 0.0001$ vs. Flap Surgery group). We did not observe a significant difference in the SOD activity between Sham, Saffron 40

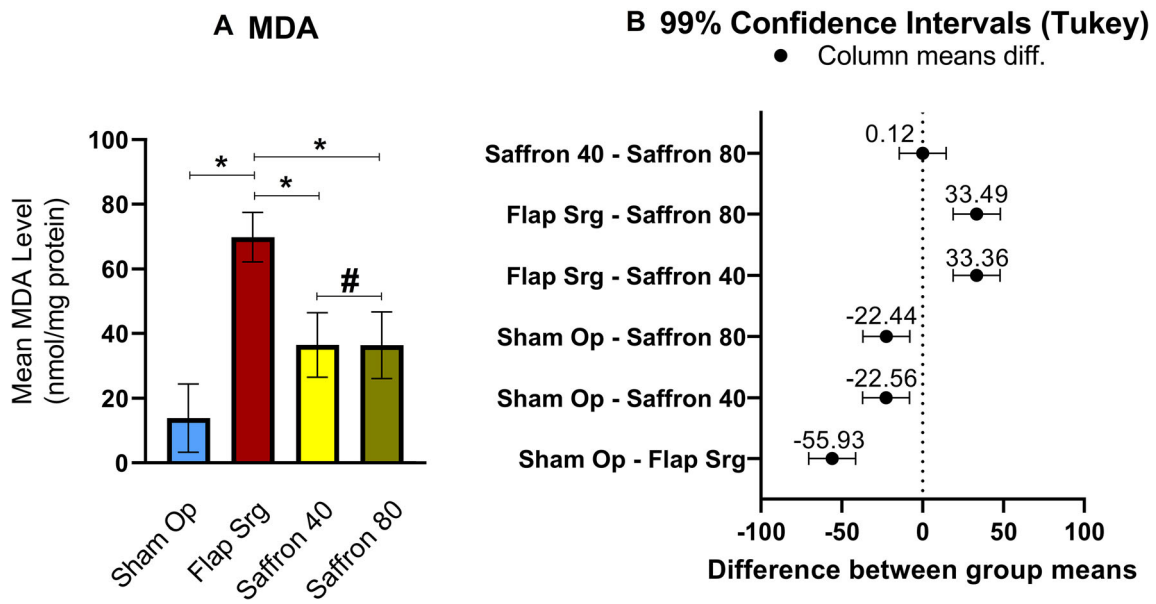

Fig. 5 **A** MDA level seven days after surgery. Data are represented as Mean ± SD. (*) $P < 0.0001$ and significant. (#) $P = 0.999$ and non-significant. **B** Difference between group means

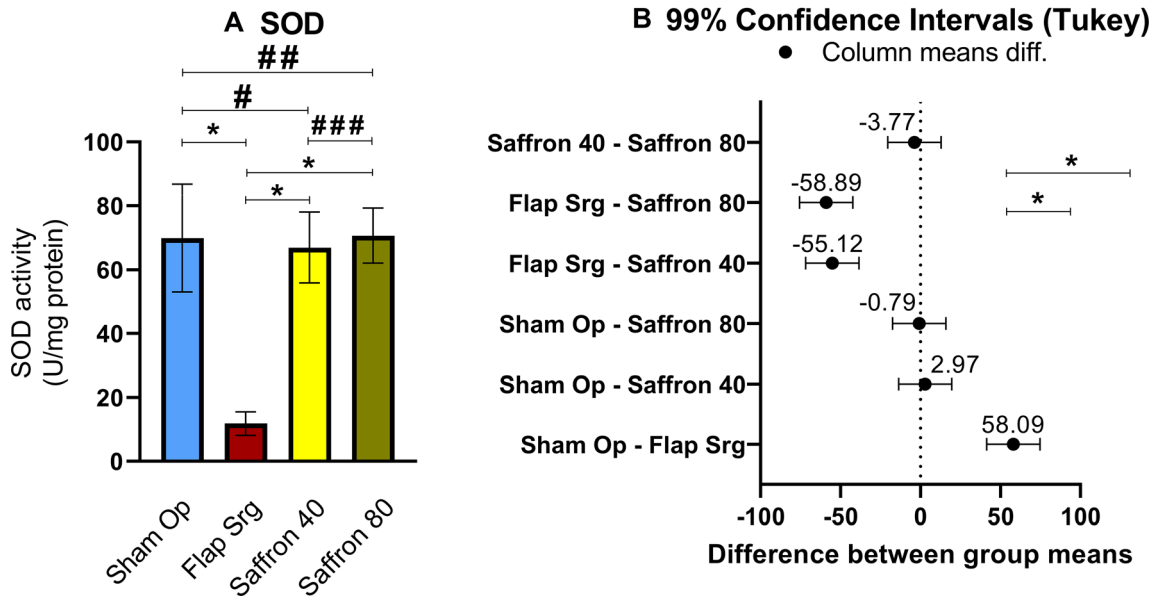

Fig. 6 **A** SOD activity seven days after surgery. Data are represented as Mean ± SD. *$P < 0.0001$ and significant. (#) $P = 0.931$, (##) $P = 0.998$, (###) $P = 0.872$ and non-significant. **B** Difference between group means

mg/kg/day and 80 mg/kg/day treated groups (Sham vs. Saffron 40 group: $P = 0.931$, Sham vs. Saffron 80 group: $P = 0.998$, Saffron 40 group vs. Saffron 80 group: $P = 0.872$). Difference between group means is illustrated in Fig. 6B. As shown in this figure, difference of the mean between the Sham group and Flap Surgery group is the most positive one, which indicates that flap surgery had a reducing effect on the SOD activity. In the other hand, the most negative differences of the means are related to the difference between Flap Surgery group and the groups treated with Saffron, which indicates that Saffron treatment had an enhancing effect on the SOD activity. Difference between Flap Surgery group mean, with Saffron 80 and Saffron 40 is − 58.89 U/mg protein and − 55.12 U/mg protein, respectively. Also, the difference between the two groups of Saffron 40 and Saffron 80 is almost close to the zero line, which shows that the results of these two groups are not significantly different.

MPO Activity

Leucocyte infiltration to the flap tissue was measured by determining MPO activity levels in the flap tissue. MPO activity of each group has been reported in Table 1, Supplementary Table 4, and illustrated in Fig. 7A. Sham group had the least MPO activity (0.87 ± 0.15 U/mg protein). After flap elevation we observed a significant increase in the flap tissue MPO activity in Flap Surgery group ($P <$ 0.0001 vs. Sham group) and MPO activity reached 1.96 ± 0.33 U/mg protein in this group. Saffron administration in 40 mg/kg/day dosage group, significantly decreased the MPO activity of the flap tissue to 1.20 ± 0.18 U/mg protein ($P < 0.0001$ vs. Flap Surgery group). Also, in Saffron 80 mg/kg/day dosage group, MPO activity significantly decreased to 1.25 ± 0.33 U/mg protein ($P < 0.0001$ vs. Flap Surgery group). We did not observe a significant difference in the MPO activity between Saffron 40 mg/kg/day and 80 mg/kg/day treated groups ($P = 0.964$). Difference between group means is illustrated in Fig. 7B. As shown in this figure, difference of the mean between the Sham group and Flap Surgery group is the most negative one, which indicates that flap surgery had an increasing effect on the MPO activity. In the other hand, the most positive differences of the means are related to the difference between Flap Surgery group and groups treated with Saffron, which indicates that Saffron treatment had a reducing effect on the MPO activity. Difference between Flap Surgery group mean, with Saffron 80 and Saffron 40 is 0.70 U/mg protein and 0.76 U/mg protein, respectively. Also, the difference between the two groups of Saffron 40 and Saffron 80 is almost close to the zero line, which shows that the results of these two groups are not significantly different.

Bax and Bcl-2 Expression

Expression of the Bax and Bcl-2 proteins were measured by western blotting. Bax is as a pro-apoptotic indicator and Bcl-2 is as an anti-apoptotic indicator. On each sample the expression of the Bax or Bcl-2 was normalized to the expression of the control protein beta-actin. Bax expression of each group has been reported in Table 1, Supplementary Table 5, and illustrated in Figs. 8A, 10A and 11. Sham group had the least Bax expression (0.51 ± 0.17). After flap elevation we observed a significant increase in the flap tissue Bax expression in Flap Surgery group ($P < 0.0001$ vs. Sham group) and Bax expression reached 1.64 ± 0.40 in this group. Saffron administration in 40 mg/kg/day dosage group, significantly decreased the Bax expression of the flap tissue to 0.75 ± 0.25 ($P < 0.0001$ vs. Flap Surgery group). Also, in Saffron 80 mg/kg/day dosage group, Bax expression significantly decreased to 0.62 ± 0.23 ($P < 0.0001$ vs. Flap Surgery group). We did not observe a significant difference in the Bax expression between Saffron 40 mg/kg/day and 80 mg/kg/day treated groups ($P = 0.540$). Difference between group means is illustrated in Fig. 8B. As shown in this figure, difference of the mean between the Sham group and Flap Surgery group is the most negative one, which indicates that flap surgery had an increasing effect on the Bax expression. In the other hand, the most positive differences of the means are related

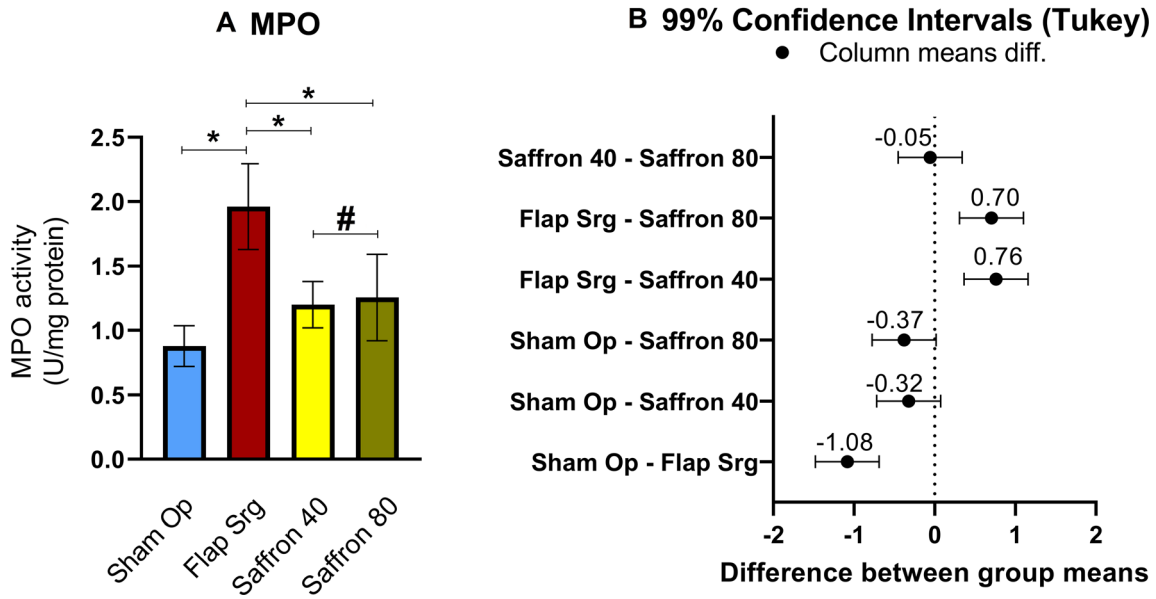

Fig. 7 **A** MPO activity seven days after surgery. Data are represented as Mean ± SD. (*) $P < 0.0001$ and significant. (#) $P = 0.964$ and non-significant. **B** Difference between group means

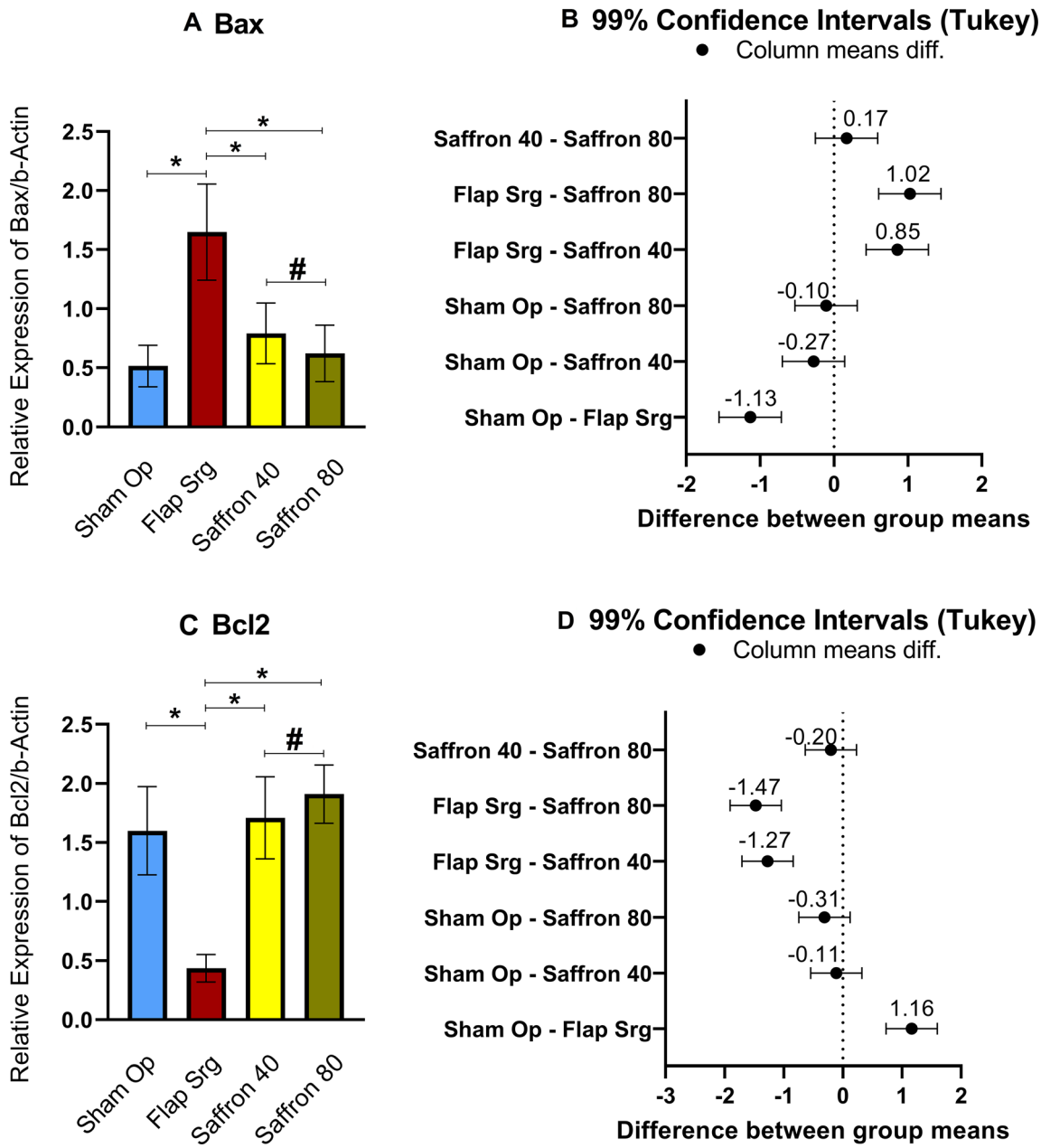

Fig. 8 Relative expression of protein/b-actin 7 days after surgery. Data are represented as Mean ± SD. **A** Bax expression. **C** Bcl-2 expression. (*) $P < 0.0001$ and significant. # in diagram **A** $P = 0.540$ and non-significant. # in diagram **C** $P = 0.418$ and non-significant. **B** and **D** Difference between group means

to the difference between Flap Surgery group and groups treated with Saffron, which indicates that Saffron treatment had a reducing effect on the Bax expression. Difference between Flap Surgery group mean, with Saffron 80 and Saffron 40 is 1.02 and 0.85, respectively. Also, the difference between the two groups of Saffron 40 and Saffron 80 is almost close to the zero line, which shows that the results of these two groups are not significantly different.

Bcl-2 expression of each group has been reported in Table 1, Supplementary Table 6, and illustrated in Figs. 8C, 10A and 11. Flap Surgery group had the least Bcl-2 expression (0.43 ± 0.11) and we observed a significant decrease in the flap tissue Bcl-2 expression in this group ($p < 0.0001$ vs. Sham group). Saffron administration in 40 mg/kg/day dosage group, significantly increased Bcl-2 expression of the flap tissue to 1.70 ± 0.34 ($P < 0.0001$ vs. Flap Surgery group). Also, in Saffron 80 mg/kg/day dosage group, Bcl-2 expression significantly increased to 1.91 ± 0.21 ($P < 0.0001$ vs. Flap Surgery group). We did not observe a significant difference in the Bcl-2 expression

between Sham, Saffron 40 mg/kg/day and 80 mg/kg/day treated groups (Sham vs. Saffron 40 group: $P = 0.828$, Sham vs. Saffron 80 group: $P = 0.094$, Saffron 40 group vs. Saffron 80 group: $P = 0.418$). Difference between group means is illustrated in Fig. 8D. As shown in this figure, difference of the mean between the Sham group and Flap Surgery group is the most positive one, which indicates that flap surgery had a reducing effect on the Bcl-2 expression. In the other hand, the most negative differences of the means are related to the difference between Flap Surgery group and the groups treated with Saffron, which indicates that Saffron treatment had an enhancing effect on the Bcl-2 expression. Difference between Flap Surgery group mean, with Saffron 80 and Saffron 40 is − 1.47 and − 1.27, respectively. Also, the difference between the two groups of Saffron 40 and Saffron 80 is almost close to the zero line, which shows that the results of these two groups are not significantly different.

mTOR and p-mTOR Expression

mTOR and p-mTOR expression were evaluated in flap tissues to determine whether Saffron affects via modifying this pathway or not. The results are prepared in Table 1, Supplementary Table 7 and 8, and illustrated in Figs. 9, 10B and 11. There were no significant differences between any of the experimental groups for both mTOR and p-mTOR expression. It is obvious that according to heat map of protein expression (Fig. 11), there is no significant change between expression of mTOR and p-mTOR in experimental groups.

Histopathologic Changes

Histologic sections of flap tissue are prepared in Table 2 and illustrated in Fig. 12. Neovascularization as an important effect of treatment has been investigated in samples. Flap Surgery group sections was fully necrotized. Saffron 40 group showed moderate epithelialization, fibroblast migration, and neovascularization. Saffron 80 group showed severe epithelialization, moderate fibroblast migration and neovascularization. Epithelialization thickness in Saffron 40 group and Saffron 80 group, was + and ++, respectively.

Discussion

Despite all scientific advances and the use of methods that increase skin flap survival, the maintenance of flap tissue against necrosis and tissue damage is still a major concern for surgeons and researchers [1, 38]. So, for the first time in the present study, we aimed to evaluate the effect of Saffron (*Crocus sativus* L.) on Skin flap survival and investigate whether it affects via mTOR pathway. Our studies were performed at three levels: macroscopic, microscopic and molecular. Our results indicated that the use of Saffron extract significantly reduced tissue necrosis rate. We observed that MDA (as a marker of lipid peroxidation), MPO (as a marker of neutrophil infiltration), and Bax (pro-apoptotic) significantly decreased in Saffron treated groups. In the other hand, SOD (anti-ROS enzyme), Bcl-2 (anti-apoptotic), neovascularization, epithelialization, epithelialization thickness, and fibroblast migration increased consequent to Saffron administration. Saffron had also no effect on mTOR or p-mTOR expression.

Nowadays, increasing of skin flap survival is the challenging issue of research. The survival of skin flaps is threatened by mal-perfusion, mal-oxygenation, low excretion and retention of tissue waste, necrosis and apoptosis, ischemia-induced injury, reperfusion injury, ROS injury, and inflammatory processes. It seems much more useful to avoid the creation of flap threatening processes and factors, rather than confronting them such as prevention of tissue necrosis via reduction of ROS production, elimination of created ROS, increasing of total antioxidant capacity (TAC) of flap tissue, and even use of exogenous antioxidants [10, 15, 36, 39, 40].

Studies have demonstrated the protective effect of Saffron on oxidative stress in different tissues such as abdominal organs, kidney, testis, and endothelial cells [21, 41–46]. Crocin, safranal, carotene and crocetin as the most abundant constituents of Saffron stigmas, represent antioxidant effects. Noticeably, Saffron extract is mentioned as a potent anti-oxidant with high ability to scavenge ROS, especially superoxide anions and hydrogen peroxide (H_2O_2) resulting in inhibitory effect on lipid peroxidation [22, 29, 32, 41]. Also, Saffron is suggested in autoimmune encephalomyelitis, and cerebral ischemia [23, 47, 48]. In these ischemic models, Saffron or its active components, reduced MDA level and lipid peroxidation, increased the activity of SOD, GPx, and antioxidant capacity.

In our study, flap necrosis and MDA levels increased with skin flap surgery. MDA, as a stable metabolite of lipid peroxidation, indicates the amount of oxidative stress. Our results also showed that flap necrosis and the level of MDA in skin flap tissue was significantly reduced by administering Saffron. This directly confirms the antioxidant effect of Saffron on the skin flap tissue. By reducing oxidative stress, peroxidation and degradation of lipids are also reduced and less MDA is produced. By a decrease in lipid degradation, it can be expected that cell membranes remain undamaged which potentially prevents arachidonic acid-dependent inflammation. Considering that we have administrated Saffron orally, it is possible to have a good assurance of the absorption and bioavailability of Saffron,

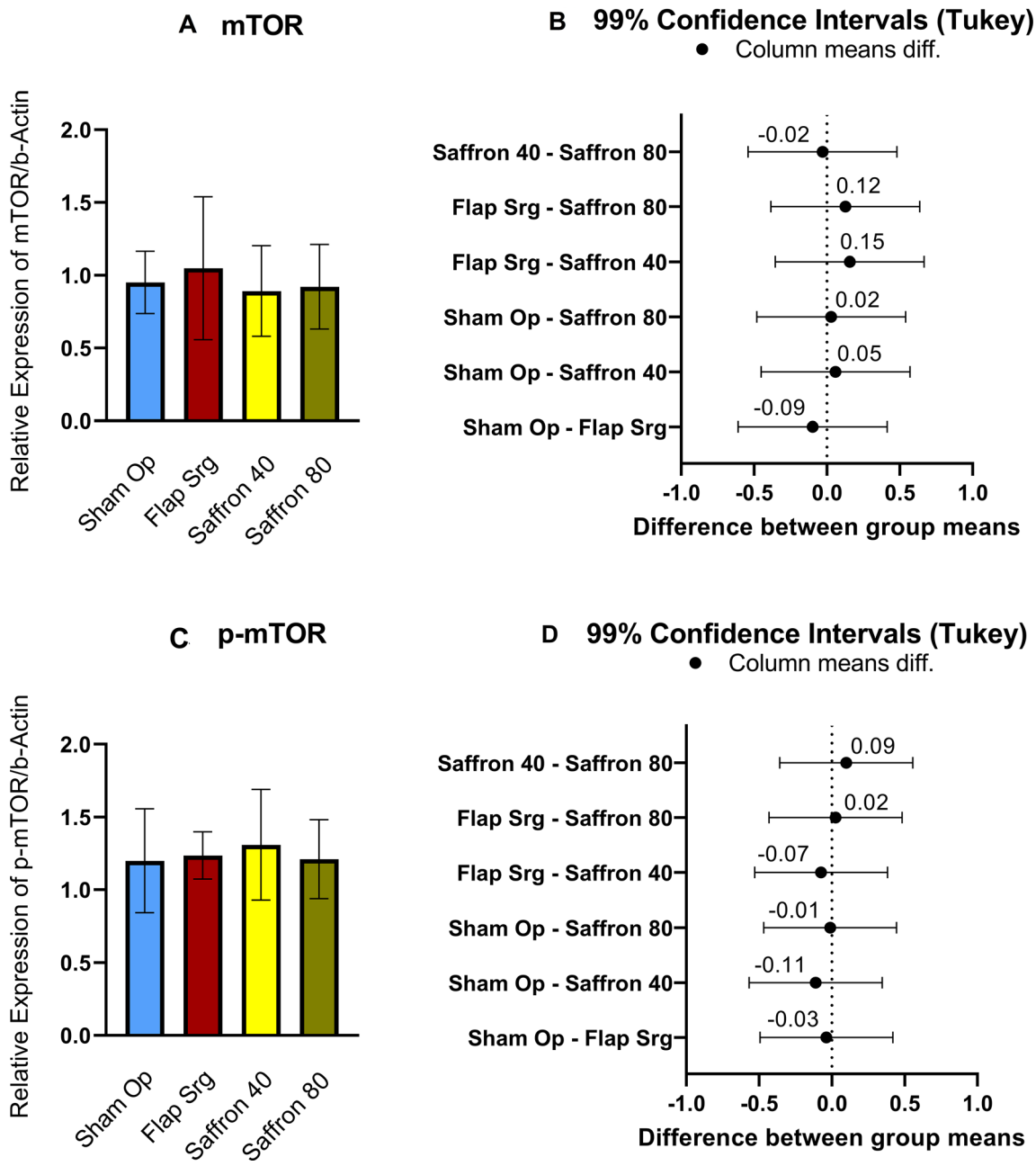

Fig. 9 Relative expression of protein/b-actin seven days after surgery. Data are represented as Mean ± SD. **A** mTOR expression. **C** p-mTOR expression. Comparison of every group with another one was non-significant for both mTOR and p-mTOR expression. **B** and **D** Difference between group means

because its effects have appeared in the skin tissue. Oral administration has numerous benefits, such as easily applicability, availability, and being more physiologic. All of these results are consistent with pervious findings which mentioned above [5, 49]. Another study [30] has evaluated Saffron effect on mitogen-activated protein kinase (MAPK) signaling pathways, in mediation of endothelial cell-survival against oxidative stress. The results of this study showed that Saffron administration has improved viability of cells in response to H_2O_2-induced toxicity and has reduced production of ROS in H_2O_2-treated cells. It is a confirmation to our results.

Superoxide dismutase (SOD) is an enzyme that scavenges superoxide radicals and prevents their destructive effects. In our study, skin flap surgery significantly reduced SOD activity in the flap tissue. This means that flap surgery reduces ability of tissue to cope with oxidative stress. In the groups that treated with Saffron, the SOD activity increased significantly. Therefore, it can be concluded that Saffron has increased tissue ability to deal with reactive

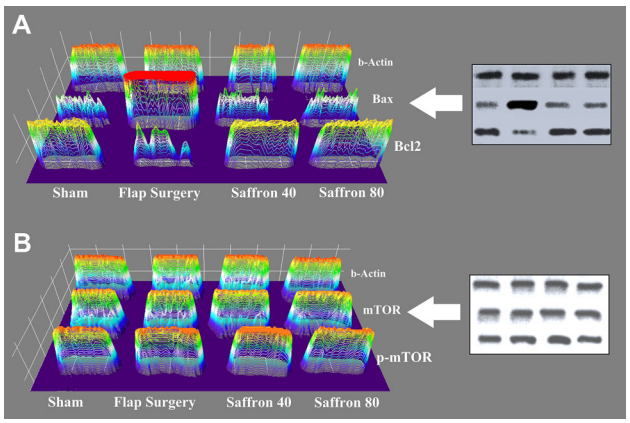

Fig. 10 3-Demential Western blot data virtualization of Bax, Bcl-2, mTOR, p-mTOR and b-actin expression

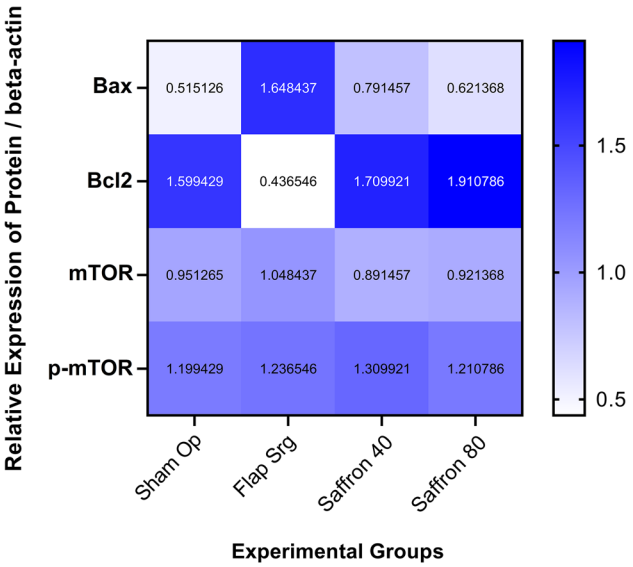

Fig. 11 Heat map of Bax, Bcl-2, mTOR, and p-mTOR expression in skin flap tissue

oxygen species. In recent research [50] about crocin effect on the viability of random pattern skin flaps, the authors have reported SOD increases after crocin application. This research and aforementioned ones [6, 11, 18, 28, 38, 42] is in line with our results and have reported that SOD increasing would result in tissue protection against ROS damage [16, 51].

Recent articles have also focused on anti-inflammatory properties of Saffron [52] and even declared that Saffron

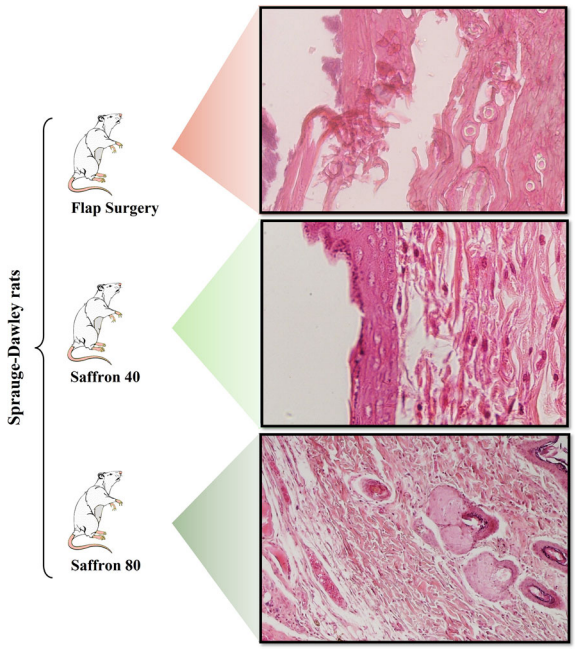

Fig. 12 Histopathologic study: H&E staining (× 100 magnificence) of Flap Surgery group, Saffron 40 group, and Saffron 80 group. In Saffron treated groups, healing level increased and necrosis decreased

represents a promising substance for toning down cytokine storm during COVID-19, as well as a potential preventive treatment for COVID-19 [26]. Scholars also mentioned that crocin anti-inflammatory properties results from effecting on cyclooxygenase pathway and inhibition of both cyclooxygenase1 (COX1), and cyclooxygenase 2 (COX2) enzymes, and inhibition of prostaglandin E2 (PGE -2) production, in a dose dependent manner [53]. Crocin has presented an inhibitory role on the high levels of bone joint exo-glycosidases, cathepsin-D and tartarate resistant acid phosphatases. Saffron and crocin are used to show relieving effects on arthritis by modifying cartilage deteriorating enzymes, inflammatory mediators, and matrix metalloproteinases MMPs [28]. The Myeloperoxidase (MPO) enzyme is active in tissue-infiltrated neutrophils, producing oxygen-reactive species so that neutrophils can fight their targets with these ROS. Therefore, the activity of MPO enzyme is a manifestation of inflammation and chemotaxis of neutrophils. In addition to other effects of inflammation, these ROS also cause damage to the skin flap tissue [1, 36, 49]. In our study, skin flap surgery increased MPO

Table 2 Results of histopathologic study

	Epithelialization	Epithelialization thickness	Fibroblast migration	Neovascularization
Grading	0–3	+/++	0–3	0–3
Flap surgery	Necrosis	Necrosis	Necrosis	Necrosis
Saffron 40	2	+	2	2
Saffron 80	3	++	2	2

activity, but in the groups that received Saffron, the enzyme activity level decreased significantly and resulted in an increase of skin flap survival. However, this effect did not change much with increasing the dose of Saffron in our experiment, and in both groups of Saffron 40 and Saffron 80, the rate of MPO activity decrease, was the same. Other studies [1, 49, 54] confirm our results about increasing the survival of skin flaps following a decrease in MPO activity.

In addition to tissue necrosis, apoptosis is mentioned as a noticeable process in tissue loss. Numerous mediators could co/regulate apoptosis pathways. It would be a challenging issue that except decrease in ROS, necrosis, and tissue inflammation, we could increase anti-apoptotics and decrease pro-apoptotics, which would directly result in enhancement of skin flap survival [16, 55]. Our study showed skin flap surgery results in increasing of Bax (pro-apoptotic) and decreasing of Bcl-2 (anti-apoptotic), meaning apoptosis promotion and consequent skin flap tissue loss. Saffron administration reversed this issue and resulted in decreasing of flap tissue loss and also expression of Bax, and increasing of tissue viability and Bcl-2. So, it might have concluded that Saffron moderates tissue loss via effecting on the expression intracellular apoptosis regulators. Also, a study [30] demonstrated that apoptosis has reduced significantly by Saffron administration in endothelial cells, which is similar to our results. Other studies on skin flap survival have confirmed that skin flap viability enhances with Bcl-2 increasing and Bax decreasing [16, 56, 57].

New research in recent years have suggested the role of autophagy in apoptosis, and declared that autophagy could decrease apoptosis, oxidative damage, and skin flap tissue loss. Moreover, the mTOR signaling pathway has been shown to act as an inhibitor of autophagy. We aimed to study the possible involvement of the mTOR pathway in the protective effects of Saffron. Studies have been performed on the effect of Crocin in liver cancer [58] and Crocetin in breast cancer [59] and their effects on autophagy have been reported, but the results of our study showed that Saffron has no effect on mTOR or p-mTOR expression in skin flap tissue. We did not observe significant mTOR and p-mTOR changes between groups. This indicates that Saffron effect on flap survival might be independent of mTOR and p-mTOR expression.

Histologically, neovascularization causes better perfusion and blood supply, so it prevents necrosis. Fibroblast migration helps tissue repair and epithelialization is as an indicator of healing process. In our study, Saffron treatment increased all of these items. Our results are consistent with previous studies [1, 6, 18, 50, 60–62].

Finally, it seems that Saffron has protective effect on skin tissue and could prevent skin flap tissue loss. Our study was an experimental study and all limitations of experimental studies apply to ours. More research is needed to determine all the positive effects or toxicities of Saffron. To determine the greater extent of the impact of Saffron on the survival of the skin flap, it is suggested that researchers who will work on this issue in the future consider (1) measurement of skin flap survival while using mTOR inhibitors such as rapamycin to determine the contribution of this pathway to the rate of tissue loss, and (2) immunohistochemistry (IHC) examination of flap tissue for vascular growth factors (3) investigation of other molecular markers that are related to autophagy (4) measure markers at RNA level and protein level (by western blotting and IHC).

Conclusion

Firstly, according to the results, it could be concluded that Saffron has a great potential to be applied for increasing survival of skin flap tissue due to its ability to decrease apoptosis, oxidative stress and inflammatory-derived oxidative injury, and also enhancing flap survival via more effective neo-vascularization and epithelialization. Finally, we declare that all of aforementioned effects are independent of mTOR expression.

Secondly, this study gave an overall view to researchers and aesthetic surgeons about the positive effects of Saffron on random skin flap survival. Moreover, our study was experimental and it is not possible to use our method in skin flap surgeries immediately. Therefore, this is an opportunity for clinical researchers and aesthetic surgeons to design a clinical trial to approve our study and find a proper dose of Saffron for clinical use. All efforts are worthy to achieve enhanced life quality of patients.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s00266-022-03048-6.

Acknowledgement The authors would like to thank the Iran University of Medical Sciences, Tehran, Iran.

Author Contributions Study conception and design: Z.H., M.H., H.P., M.A., G.H. Acquisition of data: Z.H., H.P., M.B. Analysis and Interpretation of data: M.B., Z.H. Drafting of Manuscript: Z.H., M.B., H.P., M.H., G.H. Critical Revision: All authors

Funding The authors have no financial relationships relevant to this article to disclose. No funding source

Declarations

Conflict of interest The authors declare that they have no conflicts of interest to disclose.

Ethical Approval The study protocol was approved by Research Ethics Committees of School of Medicine - Tehran University of

Medical Sciences (Tehran, Iran), Approval ID: IR.TUMS.MEDICINE.REC.1400.1013. All applicable institutional and/or national guidelines for the care and use of animals were followed.

Informed Consent For this type of study informed consent is not required.

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