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Original research article

Combination therapy profoundly improved skin flap survival by modulating KATP channels and nitric oxide

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ABSTRACT

Purpose: A potential therapeutic approach on skin flap necrosis is to target parallel pathways involved in necrosis. Azelaic Acid, Minoxidil and Caffeine combination was tried on skin flap survival by their possible interaction with ATP sensitive potassium (KATP) channels and nitric oxide pathway.

Material and methods: Sprauge-Dawley rats were divided into 8 groups for skin flap surgery. Azelaic acid, minoxidil, caffeine, or their combination were applied topically in different groups. Two additional groups were treated with L-NAME or glibenclamide in addition to the combination therapy. Percentage of flap necrosis was calculated and flap samples were removed to measure tissue malondialdehyde (MDA) and nitric oxide (NO) and expression of inducible nitric oxide synthase (iNOS), Bcl-2 and Bax proteins.

Results: Combination therapy profoundly decreased skin flap necrosis, tissue MDA contents, and expression of the pro-apoptotic protein Bax ($p < 0.05$ *vs.* single treatments). These effects were reversed by L-NAME and glibenclamide pre-treatments. Further evaluations showed combination therapy increases flap tissue NO content and iNOS expression ($p < 0.05$ *vs.* single treatments).

Conclusion: Beneficial effect of the combination therapy with azelaic acid, minoxidil and caffeine therapy on rescuing the flap from necrosis by targeting parallel signaling pathways suggested potential applications in clinical practice.

1. Introduction

Skin flap grafting is often applied to repair critical skin damages and underlying soft tissue injuries. Ischemia reperfusion (IR) injury of the grafted tissue remains an important factor contributing to the grafts failure [1–3]. IR injury is characterized by oxidative stress and excessive release of the reactive oxygen species (ROS) and inflammatory cytokines, necrosis and apoptotic cell death [4–11]. Different methods have been applied to improve blood flow to the ischemic flaps, decrease ROS production or scavenge ROS and attenuate reactions [12–16].

Among the pharmacological approaches to limit the IR injury there are dihydrotestosterone (DHT) inhibitors with protective effects in rat models of skin flap [17] or other models of skin injury [18,19]. DHT has been recognized for its detrimental effects on ischemic events [20]. Inhibitors of DHT synthesis, azelaic acid and finasteride, preserve skin flap survival through reducing the apoptotic cell death [21] and increasing the expression of inducible nitric oxide synthase (iNOS) [17]. Vasoactive properties of DHT partially result from its effect on inducible nitric oxide synthase (iNOS). Activation of iNOS induces local vasodilation and improves the blood supply of the affected tissue [22,23].

Minoxidil, which is prescribed to patients with androgenetic alopecia (AGA), acts by activation of the ATP-sensitive potassium (KATP) channels to relax vascular smooth muscle and decrease blood pressure [24]. KATP channels activation plays a significant role in reducing ischemic injury and protecting different tissues from oxidative damage [25–27]. Previous studies confirmed interplay of KATP channels

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opening with other signaling pathways to attenuate skin flap necrosis [28]. According to the previous studies minoxidil topical treatment preserve skin flap viability by its vasodilatory effects [29].

Caffeine is another agent widely used in cosmetic industry due to its strong antioxidant properties [30]. Caffeine has regulatory effects on KATP channels function. Its effect on activation of the KATP channels is sulfonylurea receptor (SUR)-dependent and requires cGMPPKG and intracellular Ca(2+)-dependent signaling [31]. Here, the effect of caffeine or its combination with azelaic acid and minoxidil were studied on skin flap survival. We further examined the interplay of the parallel signaling pathways on protecting flap against ischemic injury.

2. Materials and methods

Forty eight Sprauge-Dawley male rats weighing between 200 and 250 g underwent surgical operation for random pattern cranial based skin flap elevation [32]. Rats were kept in separate cages with free access to food and water in standard dark-light cycle [33].

2.1. Experimental design

Animals were allocated into 8 experimental groups (n = 6 per group) including: (1) control (normal saline topical application), (2) vehicle (solvent topical application), (3) azelaic acid (1.5%; 100 mg/flap), (4) minoxidil 5%, (5) caffeine 1%, (6) combination of azelaic acid 1.5%, minoxidil 5% and caffeine 1% (AMC), (7) L-N^G-Nitroarginine methyl ester (L-NAME; iNOS inhibitor, 20 mg/kg) + AMC, (8) and glibenclamide (non-selective KATP channel blocker, 0.3 mg/kg) + AMC. In the last two groups, L-NAME and glibenclamide were injected intraperitoneally 30 min before azelaic acid, minoxidil, caffeine or AMC topical application. Drugs were dissolved in 10% (v/v) ethanol in distilled water.

2.2. Skin flap surgery and calculation of necrotic area

Surgical approach for modeling skin flap in rats and necrotic area calculation were performed according to our previous work with brief differences [32]. Before surgery all animals were shaved using clippers, scrubbed with povidone-iodine and isopropyl alcohol, and artificial tear was administered to their eyes. After inducing general anesthesia by intraperitoneal injections of ketamine (50 mg/kg) and xylazine (10 mg/kg) mixture, two 8 cm incisions with 3 cm distance were made caudal to cephalad starting from scapula. Then the distal parts were connected and the flap was elevated to leave a sterile impermeable plastic barrier between the flap and its donor bed (Fig. 1). Incisions were sutured and animals were returned into their cages for one week topical treatments. To calculate necrotic areas photographs were taken of the flaps, and necrotic areas were measured by superimposition of photographs on graph paper. The length of the necrotic area was multiplied on width of the flap to calculate the necrotic surface of the flap. Percentage of the necrotic area in each flap was calculated as follows:

$$PF = \frac{NF}{TF} \times 100$$

PF is the percentage of the flap necrosis, NF is equal to the surface of the necrotic area, and TF is the total surface of the flap.

2.3. Flap MDA and NO metabolites contents

The skin tissue homogenized in ice-cold 50 mM Tris–ethylenediaminetetraacetic acid (EDTA) buffer (pH 7.0, 4 °C). The homogenate was centrifuged at 14,000 × *g* for 15 min (4 °C). Acquired supernatant was used to determine the levels of MDA and NO. Tissue malondialdehyde (MDA) level, as an index of lipid peroxidation, was determined in homogenized solutions using the Malondialdehyde Assay Kit (Northwest Life Science Specialties, Vancouver, Canada) according to the manufacturer's guide.

Nitric oxide metabolites were measured by assaying nitrite ($NO2^-$) plus nitrate ($NO3^-$) using a Nitric Oxide Colorimetric Assay Kit (Roche Applied Science, Indianapolis, IN 46250-0414 USA). In the first step the nitrate was reduced to nitrite by reduced nicotinamide adenine dinucleotide phosphate (NADPH) in the presence of nitrate reductase and then nitrite reacts with Griess reagents (sulfanilamide and N-1(-naphthyl)-ethylene diamine dihydrochloride) to give a red-violet diazo dye. Absorbance of this diazo dye was measured at 540 nm and determined the nitrite concentration. The value was expressed as the total nitrite plus nitrate (μmol per gram of tissue homogenate).

2.4. iNOS, Bcl-2 and Bax protein expression in flap tissue

The expression of proteins was examined using Western blotting [17,21]. Skin patches from the flap area were removed and placed immediately into liquid nitrogen. Frozen samples were homogenized in lysis buffer (containing 0.1% protease inhibitor cocktail). Acquired product was resolved and centrifuged at 22,000*g* for 20 min (4 °C) and the resulting lysate was kept at −80 °C. Protein concentration was determined by Bradford Protein Assay Kit. Extracted protein (20 μg) was mixed with sample buffer and boiled for 5 min. Samples were subjected to electrophoresis and transferred to nitrocellulose membranes for immunolabeling. Membranes were blocked in 5% skim milk in TBS plus 100 μl of Tween 20 for 1 h at room temperature and then incubated with the rabbit anti-iNOS, anti-Bcl-2, anti-Bax or mouse anti-β-actin antibodies (all from Abcam) overnight at 4 °C, followed by incubation with horseradish peroxidase conjugated secondary antibody

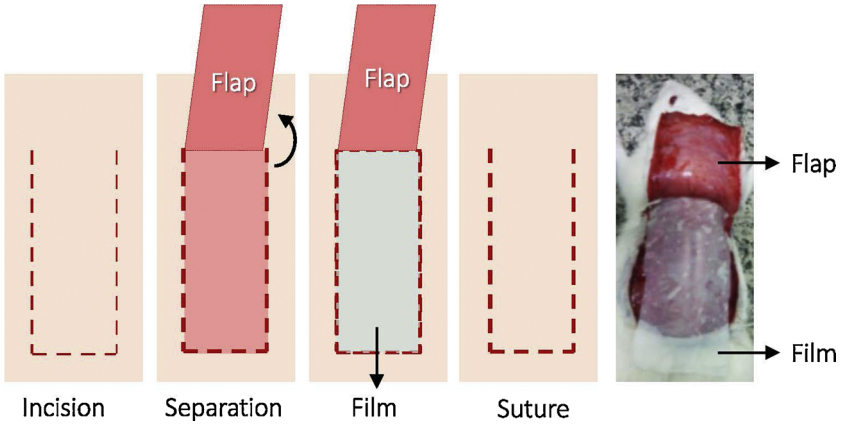

Fig. 1. Flap surgery procedure including the incision (8 × 3 cm), flap elevation, inserting the transparency film of the same dimensions into the flap area, returning the flap to its place and suturing it.

(1:5000) for 1 h at room temperature. Immunoreactive bands were detected using chemiluminescence reagents and developed on light sensitive X-ray film (Pierce, Rockford, IL, USA). Densitometry quantification was performed on digital 16-bit TIFF images using ImageJ software (version 1.45 s), after obtaining these images by CCD camera-based Fuji/GE LAS-4000 system. Relative densities of immunoreactive bands were normalized to the density of corresponding bands for β-actin in each sample and averaged in each group for statistical analysis.

2.5. Ethical issues

The study was performed in compliance with the institutes' guidelines based on the guidelines of National Institute of Health guide for the care and use of laboratory animals for medical research. Ethical approval for this work was issued by Iran University of Medical Sciences, Tehran, Iran (approval number 20926).

2.6. Statistical analysis

To measure the flap necrosis and MDA we performed analysis on 6 rats per group or 6 samples prepared from these rats. For NO, iNOS and Bax, Bcl-2 we prepared 6 samples from each rat (flap), therefore there were 36 samples for each group. SPSS statistical package V. 17 (IBM corporation, US) in 64-bit Microsoft Windows XP Professional (Version 2010) was used for data analysis. The skin flap and MDA was compared between the groups using Kruskal-Wallis test. For the rest of variables, since the data showed normal distribution pattern using Kolmogorov-Smirnov test as well as homogeneity of variance, the results were statistically evaluated by One-Way Analysis of Variance (ANOVA). Tukey's post-hoc test was applied for between group comparisons. P-value less than 0.05 was considered statistically significant.

As a limitation in the present work the percentage of the flap necrosis was calculated non-blindly to make homogenous scoring for all groups by one evaluator.

3. Results

3.1. Flap tissue necrosis

Tissue necrosis in the control group and in the vehicle group were 63.5 ± 5.1% and 59.8 ± 6.3%, respectively. All single-drug treatments decreased percentage of the flap tissue necrosis compared to the control group (Figs. 2 and 6 ; $p < 0.05$). In the group treated with the combination of 3 drugs, tissue necrosis was significantly less than groups treated with any single agents (Figs. 2 and 6; $p < 0.05$). Injection of L-NAME or glibenclamide reversed the beneficial effects of the combination therapy and significantly increased the flap tissue necrosis at distal parts (Figs. 2 and 6; $p < 0.01$).

3.2. MDA contents of the flaps tissues

Tissue MDA content, lipid peroxidation level, was reduced by azelaic acid (4.2 ± 0.82 μg/ml), minoxidil (5.1 ± 0.93 μg/ml) or caffeine (6.0 ± 0.87 μg/ml) treatments compared to the control group (8.3 ± 1.3 μg/ml; $p < 0.05$, Figs. 3 and 7). Treatment with combination of these 3 drugs significantly decreased the amount of tissue MDA (3.0 ± 0.57 μg/ml; $p < 0.05$ *vs.* azelaic acid-, minoxidil- or caffeine-treated groups). Glibenclamide or L-NAME abolished the effect

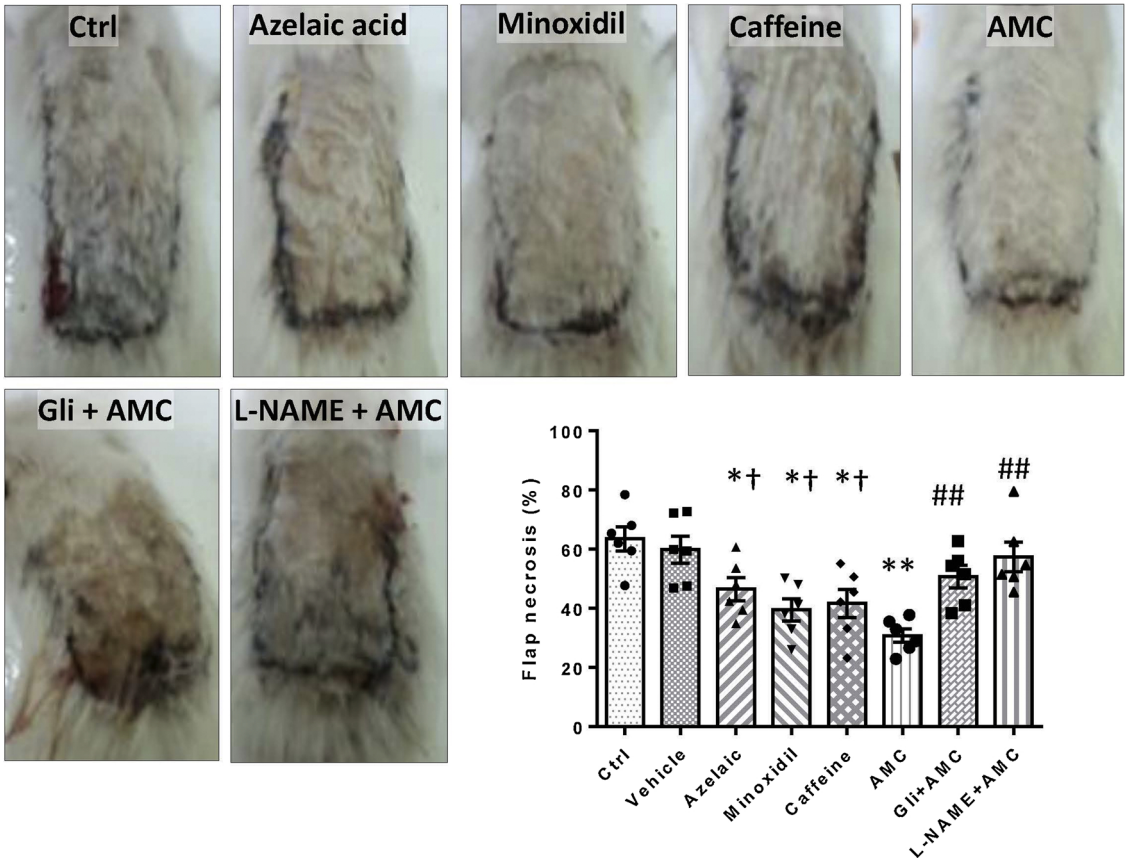

Fig. 2. Skin flap necrosis seven days after surgery in different groups. AMC: Azelaic + Minoxidil + Caffeine. The graph represents Mean ± Standard error of mean (SEM) percentage of the necrotic area in each group. Ctrl: Control group without treatment, Vehicle: Sham topical treatment, Azelaic: Azelaic acid, AMC: Azelaic + Minoxidil + Caffeine. *$p < 0.05$ and **$p < 0.01$ *vs.* control group. †$p < 0.05$: Azelaic, Minoxidil and Caffeine treated groups *vs.* AMC group. ## $p < 0.01$: Gli + AMC and L-NAME + AMC treated groups *vs.* AMC group.

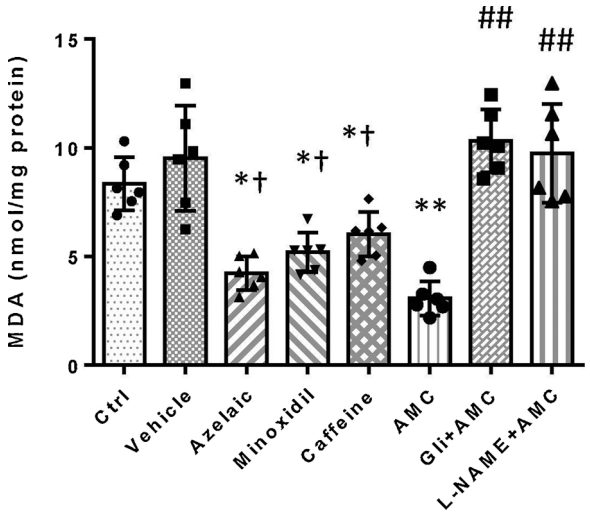

Fig. 3. MDA contents at distal section of the flap seven days after the surgery. Data are shown as Mean ± Standard error of mean (SEM). Ctrl: Control group without treatment, Vehicle: Sham topical treatment, Azelaic: Azelaic acid, AMC: AMC: Azelaic + Minoxidil + Caffeine. *p < 0.05 and **p < 0.01 *vs.* control group. †p < 0.05: Azelaic, Minoxidil and Caffeine treated groups *vs.* AMC group. ## p < 0.01: Gli + AMC and L-NAME + AMC treated groups *vs.* AMC group.

of the combination therapy and increased the MDA content (p < 0.05 *vs.* combination therapy group; Figs. 3 and 7).

3.3. Bcl-2 and Bax protein expression

Expression of pro-apoptotic protein Bax decreased by azelaic acid, minoxidil or caffeine treatments (p < 0.05 *vs.* control group; Fig. 4). Combination of azelaic acid, minoxidil and caffeine had even more significant effect on decreasing Bax expression (p < 0.05 *vs.* single treatments; Fig. 4). Anti-apoptotic protein Bcl-2 expression increased by single treatments (p < 0.05 *vs.* control group). Combination therapy boosted the increased expression of Bcl-2 (p < 0.05 *vs.* single treatments). L-NAME and glibenclamide both abolished the effect of the combination therapy on decreasing Bax and increasing of Bcl-2 protein expression (p < 0.01 and p < 0.05 *vs.* combination therapy group; Fig. 4). The ratio of Bcl-2 to Bax expression was also calculated as an anti-apoptotic state of the tissue and revealed a sharp effect of the combination therapy on preventing apoptosis (p < 0.001 *vs.* control group; Fig. 4). L-NAME and glibenclamide both reversed the effect of the combination therapy on Bcl-2/Bax expression (p < 0.001 *vs.* combination therapy; Fig. 4).

3.4. Nitric oxide content and iNOS expression

The level of the NO metabolites was increased in flap tissues belonging to the groups treated with azelaic acid, minoxidil or caffeine, or their combination (p < 0.05 *vs.* control group; Fig. 5). Even though glibenclamide decreased NO level in the combination therapy group, NO levels in this group were higher than the NO levels in the control group (p < 0.05 *vs.* control group and p < 0.05 *vs.* combination therapy group; Fig. 5) which showed that glibenclamide partially blocked the effect of the combination therapy on NO release. L-NAME completely blocked the effect of the combination therapy on NO production in the flap tissue (p < 0.001 *vs.* combination therapy; Fig. 5).

iNOS expression increased by all of the single treatments and their combination (p < 0.05 *vs.* control group; Fig. 5). Glibenclamide and L-NAME both abolished the effect of the combination therapy on iNOS expression (p < 0.05 and p < 0.01 *vs.* combination therapy, respectively; Fig. 5).

4. Discussion

Topical administration of azelaic acid, minoxidil, caffeine or their combination attenuated skin flaps' necrosis and decreased MDA contents. Survival of the skin flap tissue was directly correlated with an increase in anti-apoptotic signaling (Bcl-2 to Bax ratio), iNOS expression and NO release. These protective effects of the combination therapy were reversed by glibenclamide and L-NAME pre-treatments and suggested an iNOS-NO- and KATP channel-dependent mechanism for the above-mentioned combination therapy.

Previous studies confirmed an increase on iNOS expression and NO metabolites in azelaic acid treated skin flaps [17]. iNOS expression and NO production play critical roles in regulating the fate of tissue damage in pharmacological preconditioning of different organs [6,34]. Previous studies reported controversial effects of NO signaling on IR injury. Such controversies mainly result from differences in experimental setups, species and organs and duration of the reperfusion period [35]. For instance iNOS and NO production protect against cardiac and kidney

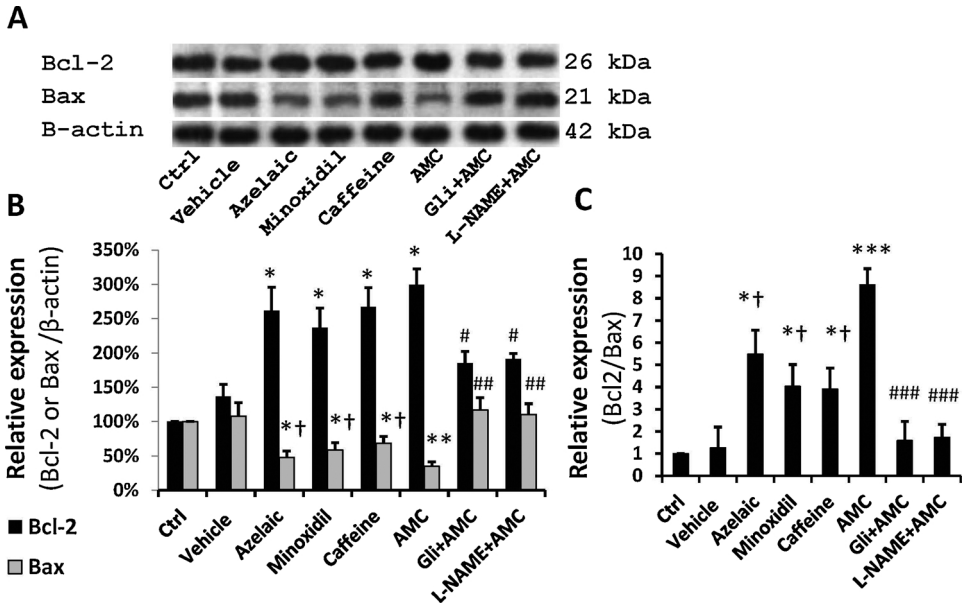

Fig. 4. Bcl-2 and Bax expression in different groups seven days after surgery. A) A representative image of Bcl-2, Bax and β-actin proteins expression in each group. B) In each flap sample expression of Bax or Bcl-2 was normalized in accordance to the expression of β-actin and represented as relative expression of Bax or Bcl-2 to β-actin expression. C) The ratio of Bcl-2 / Bax expression calculated by dividing Bcl-2 expression in each sample to Bax expression. Data are represented as Mean ± Standard error of mean (SEM) for each group. Ctrl: Control group without treatment, Vehicle: Sham topical treatment, Azelaic: Azelaic acid, AMC: AMC: Azelaic + Minoxidil + Caffeine. *p < 0.05, **p < 0.01 and ***p < 0.001 *vs.* control group. †p < 0.05: Azelaic, Minoxidil and Caffeine treated groups *vs.* AMC group. #p < 0.05, ##p < 0.01 and ### p < 0.001: Gli + AMC and L-NAME + AMC treated groups *vs.* AMC group.

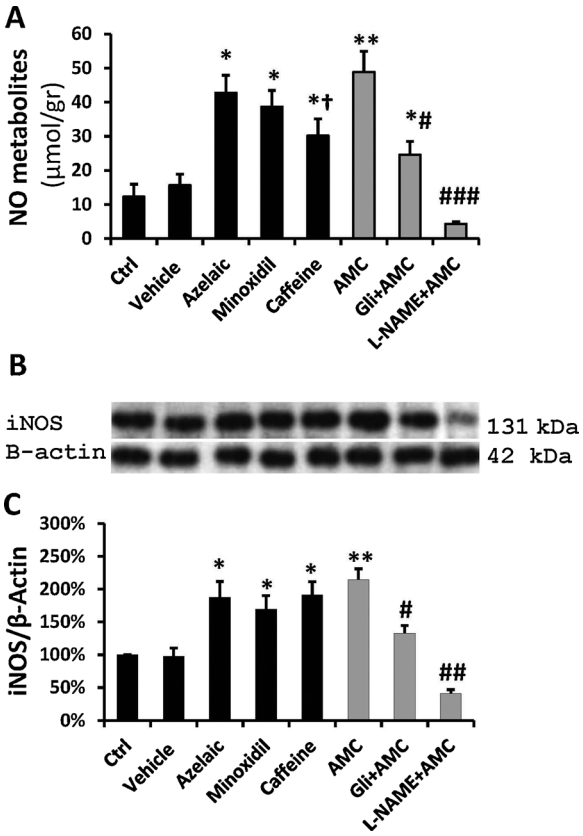

Fig. 5. Nitric oxide contents and iNOS expression in distal flap seven days after surgery. A) NO metabolites concentration were measured in skin tissue homogenates. B) A representative image of iNOS and β-actin proteins expression in each group. C) iNOS protein expression in skin flaps. Relative densities of the bands were normalized to the density of the corresponding bands for β-actin and values were represented as Mean ± Standard error of mean (SEM) of each group. Graph shows the normalized values of iNOS/β-actin expression ratio. Ctrl: Control group without treatment, Vehicle: Sham topical treatment, Azelaic: Azelaic acid, AMC: AMC: Azelaic + Minoxidil + Caffeine. *p < 0.05 *vs.* control group. #p < 0.05, ##p < 0.01 and ### p < 0.001: Gli + AMC and L-NAME + AMC treated groups *vs.* AMC group.

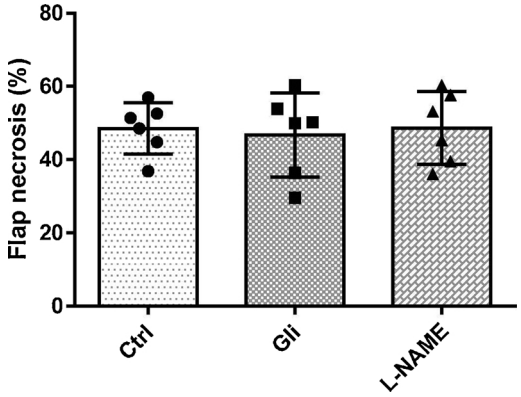

Fig. 6. Skin flap necrosis seven days after surgery in extra control groups (n = 6). Ctrl: Control group without treatment, Gli: Glibenclamide. Analysis was performed by Kruskal-Wallis test and P value = 0.9379.

ischemia in the later phases of the reperfusion [6,36,37]. Whereas higher NO content may be harmful in the acute phases after ischemia [38]. In the present study, increased iNOS expression and NO release on day 7 after flap ischemia was connected to improved flap survival in single or AMC treated groups which were abolished by L-NAME treatment.

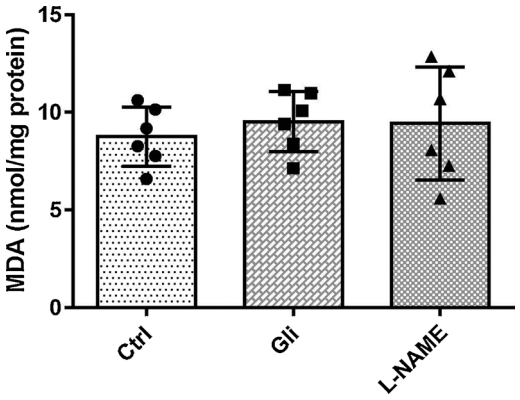

Fig. 7. MDA contents at distal section of the flap seven days after the surgery in extra control groups (n = 6). Data are shown as Mean ± Standard error of mean (SEM). Ctrl: Control group without treatment, Gli: Glibenclamide. Analysis was performed by Kruskal-Wallis test and P value = 0.7150.

Azelaic acid also has a proven effect on reducing lipid peroxidation [17], balancing Bcl-2 and Bax protein expression and suppressing pro-apoptotic signaling pathways [21]. Inducing the expression of the anti-apoptotic protein Bcl-2 or suppressing the expression of the pro-apoptotic protein Bax by pharmacological agents have been shown that improves tissue tolerance against ischemic injury in different organs including brain [39], kidney [40], heart [41], skeletal muscle [25] and skin [21,42,43]. Furthermore, a known mechanism of action for 5α-reductase inhibitors, azelaic acid and finasteride, is induction of the iNOS expression and NO production by blocking the DHT synthesis which have been exploited in the present study to improve the skin flap survival [17,21]. A study by Bae et al. [44] suggested that DHT treatment *of* rat vascular smooth muscle cells decreased the iNOS protein expression. Kolasa et al. [45] applied finasteride to block DHT in the rat testis which led to upregulation of the iNOS protein expression in the tissue.

Minoxidil, the second component of the combination therapy in the present study, was chosen because of its vasorelaxant effects which are acquired mainly by activating the KATP channels. Vasorelaxant agents have been known as potential candidates for reducing IR injury and improving tissue survival after reperfusion injury [46]. These pharmacological agents improve the blood flow reestablishment and microvasculature function, and ameliorate no-reflow phenomenon which is the main source or ROS production in tissue [47]. Besides, KATP channel openers can play an important role in reducing the lipid peroxidation, apoptotic cell death, and tissue damage after IR injury [34,48]. Therefore, KATP channel activation by minoxidil seems to act as an additional contributor to attenuate ischemic flap necrosis.

Caffeine with known antioxidant properties has already been applied to protect other organs against IR injury [49,50]. It has also been reported to improve lung inflammation subsequent to the lower limbs ischemia-reperfusion [51]. In the present work combining caffeine with azelaic acid and minoxidil provided profound protection on the skin flaps. Such combination therapies can affect parallel signaling pathways to overcome oxidative stress, inflammatory responses and apoptotic cell death in ischemic tissue [52,53].

Blocking of the KATP channels or NO release by glibenclamide or L-NAME abolished beneficial effects of combination therapy on flap survival. Even though, glibenclamide was not as effective as L-NAME on abolishing the NO production, it completely suppressed the protective effects of the combination therapy. These data can suggest a more important role for KATP channels in protecting the flap tissue against oxidative damage. Previous evidence suggest KATP channel activation as the final step of the signaling pathways involved in pharmacological protection against IR injury [54]. Furthermore, subtle decrease in iNOS expression and NO production after blocking the KATP channels by

glibenclamide suggested an interplay between the KATP channels and NO signaling. Previous models of the pharmacological protection against IR on other organs demonstrated a molecular link between NO signaling and mitochondrial KATP channels [55,56]. Interplay between the KATP channels and NO signaling requires further evaluation.

5. Conclusions

In conclusion, activation of the several signaling pathways involved in tissue protection may offer great advantages in preserving flap necrosis in clinical practice. This can be achieved by combining different pharmacological agents - each targeting specific molecular pathway [57]. Additionally, activation of the parallel pathways with the combination therapy may decrease the need for single treatments with higher doses and subsequent side effects.

Conflict of interests

The authors declare no conflict of interests.

Financial disclosure

The authors have no funding to disclose.

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The author contribution

Study Design: Rohalah Habibi, Mahmood Hoormand.
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Statistical Analysis: Mehdi Zekriyapanah Gashti.
Data Interpretation: Mehdi Zekriyapanah Gashti, Mahmood Hoormand, Azam Bakhtiarian
Manuscript Preparation: Mahtab Farrokhi, Mehdi Zekriyapanah Gashti, Rohalah Habibi
Literature Search: Mahtab Farrokhi, Mehdi Zekriyapanah Gashti, Azam Bakhtiarian
Funds Collection: n/a.

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