Secondary venous ischemic injury ameliorations through medical treatment in rats

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ABSTRACT

Background: Secondary venous ischemia caused by anastomotic failure is one of the major reasons for the lack of success in free flap surgeries. Development of damage can be mediated with single or multiple drugs. This study examined the effects of allopurinol, cyclosporine A, and deferoxamine as monotherapies and combination therapies on inguinal island flap in rats.

Material and Methods: 54 rats were divided into the following nine groups containing six animals each as controls, monotherapies, and combination therapies. Intravenous cyclosporine A (30 mg/kg), allopurinol (100 mg/kg), and deferoxamine (150 mg/kg) were administered after secondary venous ischemia. One hour after termination of the secondary venous ischemia, a biopsy was removed for biochemical (levels of malondialdehyde, the last product of lipid peroxidation; myeloperoxidase, the indicator of neutrophil infiltration; and glutathione, a potent cellular antioxidant) and histopathological (neutrophil count, an indicator of tissue inflammation) assessment. Flap viability was examined on the seventh day following surgery.

Results: The treatment groups had greater flap viability and glutathione levels, and lower levels of malondialdehyde, myeloperoxidase, and neutrophil count. The differences between the control, monotherapy and combination therapy groups were significant (p < 0.05).

Conclusions: The beneficial effect of allopurinol, previously demonstrated in primary ischemia models and arterial ischemia models, was also seen in the secondary venous ischemia model. Dual and triple combinations were more effective than monotherapies in terms of the viability of the flap. These results supported our hypothesis that combination therapies with these agents progressively decrease ischemic damage.

Key words: Flap, free radicals, ischemia, secondary, venous

Introduction

The first tissue damage that develops after free tissue transfer until anastomosis of the vascular pedicle is called primary ischemia and the subsequent arterial or venous obstruction is referred to as secondary ischemia [1].

Based on advancements in microsurgery, prolonged primary ischemia is rarely observed during free tissue transfer. In contrast, secondary anastomosis obstructions remain the major reason for the loss of the flap. In clinical practice, venous thrombosis is the most common cause of anastomosis obstruction. Secondary venous ischemia causes tissue damage, and immedi-
ate renewal of the anastomosis is essential for the viability of the flap. However, a considerable time passes between the realization of the obstruction and the revision of the anastomosis [1,2]. Therefore, this study focused on medical treatment to reduce the destructive effects of secondary venous ischemia.

Deferoxamine (DF) is a potent chelating agent and is often used for the treatment of iron overload [3]. Moreover, DF reduces the formation of free oxygen radicals via an unknown mechanism [4]. Benefits of DF in secondary venous ischemia have been observed previously in a rat model of epigastric island flap [5].

Cyclosporine A (CsA) is a potent immunosuppressant that is commonly employed during organ transplantations [6], and exerts a protective effect on ischemic injury of the liver [7]. CsA inhibits neutrophil chemotaxis [8], thus reducing the formation of free oxygen radicals that are formed during reperfusion [9]. CsA has also been shown to increase flap viability in a rat model of epigastric island flap [10].

Allopurinol (AL) is a xanthine oxidase inhibitor that has an important part in the formation of free radicals during reperfusion damage [11]. Benefits of AL have been studied in global and arterial ischemia models, and it is known to decrease ischemic damage [12]. However, its effects on secondary venous ischemia, to date, have not been studied. Moreover, no investigation has compared the effects of AL, CsA, and DF, which reduces the formation of free oxygen radicals via various mechanisms, as singular or combination therapies (Figure 1).

In the present study, we hypothesized that combination therapy with AL, CsA, and DF may contribute to reducing damage from secondary venous ischemia.

Materials and Methods

This study included 54 Wistar rats (weight, 200–250 g). All the animals were fed and kept in an experimental animal laboratory. The animals were operated on based on the rules put forth by the Ethics Committee of the National Health and Medical Research Council. In addition, permission was granted by the Animal Care and Use Committee of Trakya University. All the surgeries were performed by the same surgeon (ST). Animals were anesthetized by administering ketamine (Ketalar® 50 mg/kg) and xylazine (Rompun® 5 mg/kg) intraperitoneally.

Surgical Technique

On the skin of the abdomen, a fasciocutaneous island flap based on inferior epigastric artery and vein with a size of 3x6 cm was elevated as described by Weinberg et al. [13]. At the distal part of the pedicle, the femoral artery and vein (superficial) were dissected and ligated using 5/0 silk. After elevating the flap, a microvascular clamp (20–25 g pressure, Vascu-Statt®) was placed on both the artery and vein of the pedicle for 2 hours. After 24 hours, venous obstruction was applied by placing a microvascular clamp on just the vein for 4 hours [14-16].

Experimental Groups

The animals were divided into the following nine groups containing six animals each: (1) group 1 (sham group): animals in this group did not develop ischemia and were not provided with any medication (this group was created to standardize biochemical and histopathological values; results of this group were excluded during statistical analysis); (2) group 2 (control group): animals in this group received 1-cc serum physiologic; (3) group 3: animals in this group received only CsA (A8003-25G; Sigma Aldrich, Saint Louis, MO, USA); (4) group 4: animals in this group received only AL (Sandimmune®, 50-mg/ml ampule; Novartis, Istanbul,
Turkey); (5) group 5: animals in this group received only DF (Desferal®, 0.5 g/animal; Novartis, Istanbul, Turkey); (6) group 6: animals in this group received CsA + AL; (7) group 7: animals in this group received CsA + DF; (8) group 8: animals in this group received AL + DF; and (9) group 9: animals in this group received CsA + AL + DF. The drugs were injected via the dorsal tail vein 4 hours after inducing secondary venous ischemia. Various publications in the literature was taken as examples for the dosage and administration of the medicine, and accordingly, the doses were determined to be 100 mg/kg for AL, 150 mg/kg for DF, 30 mg/kg for CsA [5,10,12]. Secondary venous ischemia developed in all animals, except those in group 1.

At 24 hours after termination of secondary venous ischemia, the tissue at the distal part of the flap was removed through biopsy (3 × 1 cm² in size and complete thickness). The defect was repaired primarily by using 5/0 polyvinyl difluoride (Trofile®).

Biopsied tissue (3 × 1 cm²) was further divided into two parts: 1 × 1 cm², which was used for histological examination, and 2 × 1 cm², which was utilized for biochemical examination. For histopathological examination, the tissue specimens were numbered and fixed with 10% formaldehyde. For biochemical examination, tissue specimens were frozen in liquid nitrogen, numbered, and wrapped in gelatin cases containing dry ice (-80°C). The animals were then examined on the seventh day for flap viability and were sacrificed by cervical dislocation.

Evaluation Methods

**Flap Viability:** Viable and necrotic parts of the flap were examined on the seventh day in a blind manner with a method described by Sasaki and Pang [17]. Black tissues showing dehydration and eschar formation were considered necrotic based on their typical signs. The viable and necrotic tissues were drawn on a transparent acetate paper. The drawn areas were counted on unit squares, and viable areas were divided into total area of the flap to calculate the viability percent (viable area/total flap area).

**Measurement of Myeloperoxidase:** Frozen tissue samples were gradually defrosted on the day of analysis and were homogenized at a ratio of 1/20 (w/v) in lysis buffer (200 mM NaCl, 10 mM PMSF, 10% glycerol, 1 μg/mL leupeptin, and 28 μg/mL aprotinin). The homogenate was centrifuged twice at 1500 × g and 4°C for 15 min, and the clear supernatant was used to measure myeloperoxidase (MPO) levels [18]. MPO levels were evaluated using double-sandwich enzyme-linked immunosorbent assay with a mercantile kit (Hycult Biotechnology, Uden, Netherlands). A calibration curve was prepared with 0, 3.9, 7.8, 15.6, 31, 63, 125, and 250 ng/mL standard solutions. Regression analysis determined the following equation of the calibration curve: $y = 0.41 + 0.015x$. This equation was used to calculate MPO levels in the tissue homogenate. The evaluated MPO levels were then proportioned to tissue protein values, and the results were expressed as μg/mg of the protein.

**Evaluation of Malondialdehyde:** Malondialdehyde (MDA) is one of the final products of peroxidation of polyunsaturated fat acids. Tissue MDA levels were established spectrophotometrically at 532 nm by measuring the pink-red compound formed by the reaction of MDA with thiobarbituric acid in a warm environment [19]. Frozen tissue samples were defrosted progressively on the day of analysis and were homogenized at a ratio of 1/5 (w/v) in 0.15 M KCl. The homogenate was centrifuged twice at 2000 × g and 4°C for 10 min, and the clear supernatant was employed to measure MDA levels. All the measurements were performed twice for all samples. MDA concentration in the homogenate was calculated with the molar absorptivity of MDA at 532 nm. The evaluated MDA levels were proportioned to tissue protein values, and the results were expressed as nmol/mg of the protein.

**Analysis of Glutathione and Tissue Proteins:** Sulfhydryl groups in reduced glutathione (GSH) form a yellow compound with 2,2’-dinitro-5,5’-dithiobisbenzoic acid in a basic environment. This compound’s color intensity at a wavelength of 412 nm correlates with GSH quantity [20]. The samples were homogenized at a ratio of 1/5 (w/v) in 0.15 M KCl on the day of their collection. The homogenate was centrifuged twice at 2000 × g and 4°C for 10 min, and the clear supernatant was deproteinized. GSH concentration in the deproteinized supernatant was measured spectrophotometrically at 412 nm after the supernatant was treated.
with Ellman reagent. All the measurements were performed twice for all samples. GSH concentration in the homogenate was calculated using molar absorbptivity of GSH at 412 nm. Evaluated GSH levels were proportioned to tissue protein values, and the results were expressed as nmol/mg of the protein. In an alkali environment, copper ions form compounds with peptide bonds. These copper–peptide complexes react with Folin indicator to form a blue-purple compound [21]. The generated blue color is proportional to the amount of tissue protein. A graph of density versus absorbance was plotted with density values of human albumin (1, 2, 3, 4, and 5 g/L) as the standard. Protein concentration in the homogenate was calculated with the following formula obtained by regression analysis: $y = 0.0384 + 0.01936x$. All the calculations were performed twice.

**Histopathological Analysis:** Tissue specimens were fixed in 10% formaldehyde and placed in paraffin. Cross sections (width, 4 µ) were taken and dyed with hematoxylin and eosin. Neutrophil count in all the sections was assessed using a light microscope at 100× magnification and values from five different areas were averaged.

**Statistical analysis:** All statistical analyses were performed using SPSS 20 (IBM Corp., NY, USA, serial number: 10240642). The relevance of the measured values to the normal range was determined by performing the Kolmogorov–Smirnov test for the values obtained for a single sample. The values were not in the normal range. Therefore, Kruskal–Wallis variance analysis was performed for comparing groups, followed by a Bonferroni-corrected Mann Whitney U test. The acquired values were expressed as average ± standard deviation (Std). P values less than 0.05 were considered statistically significant.

**Results**

No animal was lost during the experiment. No clinically distinctive side effects were observed for the dosages of the used agents. All the obtained values are summarized in Table 1.

**Flap viability:** All the flaps in the non-ischemic group (group 1) survived completely (100%). The mean viability percentage in the treatment groups was significantly higher than that in the control group; moreover, differences among the groups receiving monotherapy, dual combination therapy, and triple combination therapy were significant (p < 0.05; Figure 2).

**Biochemical analysis:** Mean MDA and MPO levels in the treatment groups were significantly lower and mean GSH levels in the treatment groups were significantly higher than those in the control group (p < 0.05); however, other comparisons among the groups were not significant (p > 0.05; Figures 3, 4, and 5).
Table 1. All of the values are summarized here.

| Flap viability percent | MDA         | MPO         | GSH          | Neutrophil count |
|------------------------|-------------|-------------|--------------|------------------|
| Group 1                | 100±0       | 0.901±0.317 | 48,163±16,737| 5,257±0.449      | 22,564±4,471    |
| Group 2                | 10.17±2.858 | 2.873±0.567 | 153,494±30,803| 3,739±0.286      | 37,500±2,141    |
| Group 3                | 40.67±3.266 | 0.731±0.266 | 41,245±12,232| 5,882±0.299      | 24,667±2.728    |
| Group 4                | 40.00±3.347 | 0.743±0.245 | 41,672±10,654| 5,840±0.956      | 32,333±3.062    |
| Group 5                | 41.17±2.714 | 0.754±0.187 | 42,343±11,332| 6,568±0.554      | 33,833±2.750    |
| Group 6                | 53.33±2.160 | 0.684±0.154 | 40,474±10,121| 6,326±0.436      | 21,667±3.313    |
| Group 7                | 53.83±2.994 | 0.720±0.133 | 37,402±9,778 | 6,671±0.514      | 21,000±1,317    |
| Group 8                | 63.50±1.871 | 0.587±0.121 | 33,283±9,436 | 6,718±0.730      | 20,333±1,542    |
| Group 9                | 73.17±1.941 | 0.460±0.099 | 25,136±6,962 | 6,892±0.436      | 18,500±0.885    |

(MDA: Malondialdehyde, MPO: Myeloperoxidase, GSH: Glutathione)

Histopathological analysis: Mean neutrophil counts in the treatment groups, except in groups receiving AL (group 4) and DF (group 5), were significantly lower than those in the control group (p < 0.05). Moreover, the difference between groups receiving dual and triple combination therapies and the control group was statistically significant (p < 0.001). However, other comparisons among the groups were not significant (p > 0.05; Figure 6).

Neutrophil count, inflammation, and edema were evaluated in cross sections that are dyed with hematoxylin and eosin at 100× enlargement. Lower neutrophil counts, inflammation and edema stands out in triple or dual treated groups. CsA group neutrophil count, inflammation and edema was moderate. Neutrophil count, inflammation, and edema in animals receiving AL and DF were lower than those in the control group but higher than those in other groups (Figure 7).

Discussion

Restoration of blood flow in ischemic tissue is essential to prevent irreversible damage during free-flap surgeries. On the other hand, inflammatory response
of reperfusion causes tissue damage (referred to as ischemia-reperfusion damage). Prolonged ischemia leads to loss of tissue viability (referred to as no-reflow phenomenon) even after revascularization [1, 2]. This study examined the effects of CsA, DF, and AL, all which have been shown to possess beneficial effects on different steps of the ischemia-reperfusion mechanism either as monotherapies or combination therapies targeting reduction of ischemia-reperfusion damage.

With advancements in microsurgery, the use of free-flap surgery has increased in clinical practice. Secondary venous failure is the most common side effect associated with this surgery [10, 22]. The flap model chosen in this study was considered the best model to study secondary venous failure because it simulates the condition observed in clinical practice [14-16].

The pathophysiology of secondary ischemia is not well understood. There are multiple potential sources of free radicals in skin flaps [5, 10]. Previous studies on secondary venous ischemic injury have suggested that free radicals, lipid peroxides, and prostanoid metabolism are important factors in tissue necrosis [5, 10, 23-25]. In these studies, the effects of CsA [10], DF [5], 21-aminosteroid U74389F [23], dexamethasone [24], and hyperbaric oxygen [25] were examined in a rat inguinal island flap model of secondary venous ischemia. All these studies demonstrated significant amelioration in the treatment groups but did not elicit complete survival. Therefore, all these studies concluded that other more important factors may result in flap necrosis in addition to free radicals, which should be elucidated in further studies. Although previous investigations have focused on monotherapies, the present study compared the efficacy of monotherapies with that of dual and triple combination therapies because of our hypothesis that combination therapies may play a progressive role.

The results of this study can be summarized in three points:

1. AL, which was beneficial in treating global ischemia, total venous ischemia, and total arterial ischemia in other studies was also effective in treating secondary venous ischemia in our study.

2. Combination of AL with DF or CsA and that of CsA with DF was more beneficial in increasing flap viability than their individual monotherapies.

3. In our study, we focused on the free radical mechanism and tried to prevent the production of free radicals at three different steps by using various agents. The triple combination of AL, DF, and CsA showed the best results for flap viability, thus supporting our hypothesis that combination therapy with these agents progressively decreases ischemic damage. Therefore, unlike the conclusions of previous studies, it is seen that the pathophysiology of secondary ischemia can be explained with the current mechanisms. However, further studies should be performed to examine the effects of the combination of all known pathophysiological mechanisms upon injury by secondary venous ischemia.

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Conflict of interest statement

The authors have no conflicts of interest to declare.

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