Effect of cineole, alpha-pinene, and camphor on survivability of skin flaps

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1. Introduction

Random-patterned skin flaps are often created during plastic surgery procedures; however, necrosis can occur as a complication. Improving the survival of random-patterned skin flaps is an important goal to reduce duration of hospitalization, additional surgical procedures, and treatment costs. Although many agents have been used for improving skin flap survival, none that can completely prevent flap necrosis has been identified (1–4).

Rosmarinus officinalis (RO) is one of the agents that have been used systemically and topically in the prevention of flap necrosis (5,6). RO has antimicrobial (7–10), antioxidant (11–18), antiinflammatory (19,20), antifungal (7,9,10), antidiabetic (15), anticarcinogenic (13,19), antiplatelet (21), antimetastatic (13,19), antiproliferative (16), and antimutagenic (22) effects.

In previous studies investigating the effects of RO on the survival of skin flaps, the viable surface areas of RO-treated flaps were reported to be significantly greater in all treated groups than that in control groups (5,6). The authors claimed that, in addition to antiinflammatory and antioxidant effects, RO had vasodilatory effects that contributed to increased flap survival. It was determined that application of this oil increased skin flap survival; however, they did not determine the specific component responsible for the improvement. In addition, the specific components that have a dominant effect in improving flap survival are still unknown.

The aim of the present study was to determine the specific component of RO that were responsible for increased flap survival. The most effective feature of RO was the antiinflammatory effects.

1. Materials and methods

Female Wistar albino rats, aged 12–14 weeks and weighing 243–310 g, were divided into 8 groups comprising 7 rats each. The backs of all rats were shaved. Ketamine hydrochloride (50 mg/kg) (Ketalar vial, Pfizer, USA) and 5 mg/kg xylazine hydrochloride (Rompun amp, Germany) were administered intramuscularly. The back surface of rats was dissected to create a rectangular random-patterned flap. Group I was the control group. In group II 0.1 mL of cineole, in group III 0.1 mL of alpha-pinene, in group IV 0.1 mL of camphor, in group V 0.1 mL each of alpha-pinene and cineole, in group VI 0.1 mL each of alpha-pinene and camphor, in group VII 0.1 mL each of cineole and camphor, and in group VIII, 0.1 mL each of alpha-pinene, cineole, and camphor was orally administered once a day before surgery. The luminal area of the largest blood vessel in the proximal flap was measured. Interleukin-1, tumor necrosis factor alpha, thiobarbituric acid reactive substances, and vascular endothelial growth factor values were measured.

Results: The mean percentage of the viable surface area was significantly greater in groups VIII, III, and V. The mean percentage of vessel diameter was significantly greater in groups V, VIII, and VII.

Conclusion: We suggest that alpha-pinene and cineole were the components of RO that were responsible for increased flap survival. The most effective feature of RO was the antiinflammatory effects.

Key words: Rosmarinus officinalis, flap survivability, tissue defect, rosemary

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Bayer, Germany) were used for anesthesia and were intraperitoneally administered before surgery. No dressing was applied after surgery. The study was approved by the ethics committee and conformed to the Helsinki Declaration. The rats were followed under standard laboratory conditions in the experimental medical research and application center. The feed and water needs of the animals were regularly met.

Group I was designated as the control group. Rectangular random-patterned flaps measuring 8 × 2 cm were elevated from the back of each rat. Electrosurgery or hemostatic agents were not used to control bleeding. The flaps were primer sutured with monofilament absorbable suture (Biosyn, Covidien, USA) to the location from where they were elevated, and 0.1 mL of physiological serum was orally administered with a repeater pipette (Eppendorf, Hamburg, Germany) once daily for 1 week.

Cineole, alpha-pinene, and camphor solutions were obtained with distillation from RO extract. The cineole solution contained 73.6% cineole, 8.67% camphor, 5.07% P-Cymene, 3.71% alpha pinene, 2.31% beta pinine, 1.93% camphene, 1.19% terpinolene, 1.5% dipropylene glycol, 0.7% gamma terpinene, 0.55% delta 3 carene, 0.43% alpha terpineol, and 0.33% isoborneole. The alpha-pinene solution contained 62.19% alpha pinene, 15.85% camphene, 8.17% cineole, 6.17% beta pinene, 4.38% limonene, 1.19% P-Cymene, 0.59% camphor, 0.55% alpha phellandrene, 0.42% delta 3 carene, 0.28% sabinene, and 0.22% myrcene. The camphor solution contained 85.8% camphor, 6.14% dipropylene glycol, 3.44% alpha terpineole, 3.19% isoborneol, and 0.92% cineole. The solutions were diluted with physiological saline to 60% purity.

In group II, 0.1 mL of cineole (60% purity) (Aksu Vital, Turkey) was orally administered once daily for 1 week before surgery. At the end of the week, 0.1 mL of cineole was orally re-administered 2 h prior to surgery. Skin flaps were elevated and replaced as described for group I. Cineole was orally administered once daily for 1 week.

In group III, 0.1 mL of alpha-pinene (60% purity) was orally administered once daily for 1 week before surgery. At the end of the week, 0.1 mL of alpha-pinene was orally re-administered 2 h prior to surgery. Skin flaps were elevated and replaced as described for group I. Alpha-pinene was orally administered once daily for 1 week.

In group IV, 0.1 mL of camphor (60% purity) was orally administered once daily for 1 week before surgery. At the end of the week, 0.1 mL of camphor was orally re-administered 2 h prior to surgery. Skin flaps were elevated and replaced as described for group I. Camphor was orally administered once daily for 1 week.

In group V, 0.1 mL each of alpha-pinene and cineole was orally administered once daily for 1 week prior to surgery. At the end of the week, 0.1 mL each of alpha-pinene and cineole was orally re-administered 2 h prior to surgery. Skin flaps were elevated and replaced as described for group I. Alpha-pinene and cineole were orally administered once daily for 1 week.

In group VI, 0.1 mL each of alpha-pinene and camphor was orally administered once daily for 1 week before surgery. At the end of the week, 0.1 mL each of alpha-pinene and camphor was orally re-administered 2 h prior to surgery. Skin flaps were elevated and replaced as described for group I. Alpha-pinene and camphor were orally administered once daily for 1 week.

In group VII, 0.1 mL each of cineole and camphor was orally administered once daily for 1 week before surgery. At the end of the week, 0.1 mL each of cineole and camphor was orally re-administered 2 h prior to surgery. Skin flaps were elevated and replaced as described for group I. Cineole and camphor were orally administered once daily for 1 week.

In group VIII, 0.1 mL each of alpha-pinene, cineole, and camphor was orally administered once a day for 1 week before surgery. At the end of the week, 0.1 mL each of alpha-pinene, cineole, and camphor was orally re-administered 2 h prior to surgery. Skin flaps were elevated and replaced as described for group I. Alpha-pinene, cineole, and camphor were orally administered once daily for 1 week.

The flaps were evaluated 1 week after elevation. All images were acquired using the same digital camera, distance, position, and the viable and necrotic regions were outlined on transparent paper. The surface area of viable and necrotic regions was calculated. To eliminate false-positive results caused by necrosis-induced contraction, only the viable surface area was measured (cm²) on transparent paper using a digital planimeter. The surface area of the necrotic region was calculated by subtracting the viable surface area from the total flap area (16 cm²). A 2 × 2 cm excision biopsy specimen was taken from the most proximal viable area of the flap. Specimens were stained with hematoxylin and eosin and examined under a light microscope. In each group, the luminal area of the largest blood vessel and number of capillary vessels in the papillary dermis of the proximal flap was measured in 400× magnification area (1 high power field). In addition, number of neovascularized vessels and number of inflammatory cells (neutrophil, macrophage, lymphocyte) in the reticular dermis of the proximal flap were measured. The rats were sacrificed with taking 10 mL of blood intracardially. Interleukin-1 (IL-1), tumor necrosis factor alpha (TNF-α), vascular endothelial growth factor (VEGF), and thiobarbituric acid reactive substances (TBARS) (Oxford Biomedical Research, Missouri, USA) values were measured spectrophotometrically to investigate anti-inflammatory, antioxidant, and angiogenic properties in the blood.
The statistical significance of mean values was analyzed using SPSS. One-way analysis of variance and Tukey’s post hoc test were used to compare the viable surface area and vessel diameter among the groups. P-values of <0.05 were considered statistically significant.

3. Results
No rats died during the experiments. In addition, no infection was observed.

3.1. Measurements for group I
One week after flap elevation, the mean ratio of the viable surface area to the total flap area was 28.63 ± 0.412% (Figure 1). The mean value of the luminal area of the largest blood vessel in the proximal flap was 82.84 ± 4 µm (Table). The mean number of capillary vessels in the papillary dermis of the proximal flap was 5 ± 1 and the mean number of neovascularized vessels was 14.75 ± 2.25. The mean number of inflammatory cells in the reticular dermis of the proximal flap was 58.75 ± 11.25. The mean value of IL-1 was 48.38, TNF-α 33.28, VEGF 12.16, and TBARS 8.52 nmol/mL (Figure 2).

3.2. Measurements for group II
One week after flap elevation, the mean ratio of the viable surface area to the total flap area was 46.06 ± 1.298% (Figure 3). The mean value of the luminal area of the largest blood vessel in the proximal flap was 64.02 ± 4 µm (Table). The mean number of capillary vessels in the papillary dermis of the proximal flap was 5.2 ± 1.8 and the mean number of neovascularized vessels was 13.8 ± 1.2. The mean number of inflammatory cells in the reticular dermis of the proximal flap was 56 ± 16. The mean value of IL-1 was 23.18, TNF-α 20.58, VEGF 15.65, and TBARS 6.57 nmol/mL (Figure 2).

3.3. Measurements for group III
One week after flap elevation, the mean ratio of the viable surface area to the total flap area was 62.56 ± 2.102% (Figure 4). The mean value of the luminal area of the largest blood vessel in the proximal flap was 66.63 ± 4 µm (Table). The mean number of capillary vessels in the papillary dermis of the proximal flap was 5.4 ± 1.6 and the mean number of neovascularized vessels was 14.5 ± 1.5. The mean number of inflammatory cells in the reticular dermis of the proximal flap was 48 ± 26. The mean value of IL-1 was 18.88, TNF-α 19.46, VEGF 15.43, and TBARS 7.37 nmol/mL (Figure 2).

3.4. Measurements for group IV
One week after flap elevation, the mean ratio of the viable surface area to the total flap area was 38.63 ± 1.299% (Figure 5). The mean value of the luminal area of the largest blood vessel in the proximal flap was 99.52 ± 4 µm (Table). The mean number of capillary vessels in the papillary dermis of the proximal flap was 5.1 ± 1.1 and the mean number of neovascularized vessels was 14.8 ± 2.2. The mean number of inflammatory cells in the reticular dermis of the proximal flap was 44 ± 12. The mean value of IL-1 was 17.51, TNF-α 28.31, VEGF 14.35, and TBARS 7.98 nmol/mL (Figure 2).

3.5. Measurements for group V
One week after flap elevation, the mean ratio of the viable surface area to the total flap area was 58.06 ± 1.027% (Figure 6). The mean value of the luminal area of the largest blood vessel in the proximal flap was 146.95 ± 4 µm (Table). The mean number of capillary vessels in the papillary dermis of the proximal flap was 5.3 ± 1.7 and the mean number of neovascularized vessels was 14.9 ± 2.1. The mean number of inflammatory cells in the reticular dermis of the proximal flap was 50 ± 15. The mean value of IL-1 was 18.23, TNF-α 15.76, VEGF 25.15, and TBARS 6.72 nmol/mL (Figure 2).

3.6. Measurements for group VI
One week after flap elevation, the mean ratio of the viable surface area to the total flap area was 47.43 ± 1.027% (Figure 7). The mean value of the luminal area of the largest blood vessel in the proximal flap was 97.05 ± 4 µm (Table). The mean number of capillary vessels in the papillary dermis of the proximal flap was 5.1 ± 1.1 and the mean number of neovascularized vessels was 13.6 ± 2.4. The mean number of inflammatory cells in the reticular dermis of the proximal flap was 42 ± 18. The mean value of IL-1 was 16.81, TNF-α 18.2, VEGF 15.41, and TBARS 7.16 nmol/mL (Figure 2).

3.7. Measurements for group VII
One week after flap elevation, the mean ratio of the viable surface area to the total flap area was 51.87 ± 1.027% (Figure 8). The mean value of the luminal area of the largest blood vessel in the proximal flap was 113.44 ± 4 µm (Table). The mean number of capillary vessels in the papillary dermis of the proximal flap was 5.4 ± 1.6 and the mean number of neovascularized vessels was 14.81 ± 2.19.

Figure 1. The mean ratio of the viable surface area to the total flap area was 28.63% ± 0.412% in group I a week after surgery.
The mean number of inflammatory cells in the reticular dermis of the proximal flap was 45 ± 15. The mean value of IL-1 was 17.8, TNF-α 21.66, VEGF 18.68, and TBARS 7.51 nmol/mL (Figure 2).

### Table. The mean viable areas of the flaps and value of the luminal area of the largest blood vessel in the proximal flap in all groups.

<table>
<thead>
<tr>
<th>Group no.</th>
<th>The mean viable areas of the flaps (cm²)</th>
<th>The mean viable areas of the flaps (%)</th>
<th>The mean value of the luminal area (µm)</th>
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</table>

The mean viable areas of the flaps and value of the luminal area of the largest blood vessel in the proximal flap in all groups.

**Figure 2.** The mean value of IL-1, TNF-α, VEGF, and TBARS in all groups.

**3.8. Measurements for group VIII**

One week after flap elevation, the mean ratio of the viable surface area to the total flap area was 74.43 ± 1.027% (Figure 9). The mean value of the luminal area of the largest blood vessel in the proximal flap was 149.74 ± 4
µm (Table). The mean number of capillary vessels in the papillary dermis of the proximal flap was 5.4 ± 1.6 and the mean number of neovascularized vessels was 14.83 ± 2.17. The mean number of inflammatory cells in the reticular dermis of the proximal flap was 41 ± 19. The mean value of IL-1 was 14.4, TNF-α 16.63, VEGF 23.46, and TBARS 6.64 nmol/mL (Figure 2).

Figure 3. The mean ratio of the viable surface area to the total flap area was 46.06 ± 1.298% in group II a week after surgery.

Figure 4. The mean ratio of the viable surface area to the total flap area was 62.56 ± 2.102% in group III a week after surgery.

Figure 5. The mean ratio of the viable surface area to the total flap area was 38.63 ± 1.299% in group IV a week after surgery.

Figure 6. The mean ratio of the viable surface area to the total flap area was 58.06 ± 1.027% in group V a week after surgery.

Figure 7. The mean ratio of the viable surface area to the total flap area was 47.43 ± 1.027% in group VI a week after surgery.

Figure 8. The mean ratio of the viable surface area to the total flap area was 51.87 ± 1.027% in group VII a week after surgery.
The mean percentage of the viable surface area was significantly greater in all treatment groups than that in group I, and was significantly greater in groups VIII, III, and V than in groups II, IV, VI, and VII (P < 0.05). The mean percentage of vessel diameter was significantly greater (P < 0.05) in groups V, VIII, and VII than in group I, and no statistically significant differences were found between groups V and VIII (Figure 10). When the mean number of capillary vessels in the papillary dermis of the proximal flap was investigated, no statistically significant differences were found between the groups. In all groups, of the mean number of inflammatory cells in the reticular dermis of the proximal flap, 5–6 were neutrophils, 5–6 were macrophages, and the rest were lymphocytes (Figure 11). No statistically significant differences were found between the groups.

When the plasma level of VEGF was investigated, there was no effect found in groups I, II, III, IV, and VI. Groups V, VII, and VIII displayed effects. Moreover, no statistically significant differences were found between groups V and VIII.

When the plasma level of IL-1 was investigated, there was no effect found in groups I and II. Groups III, IV, V, VI, VII, and VIII had effects. The minimum level of IL-1 was in group VIII.

When the plasma level of TBARS was investigated, there was no effect shown in groups I, IV, and VII. Groups II, III, V, VI, and VIII had effects. However, no statistically significant differences were found between groups II, III, V, VI, and VIII.

When the plasma level of TNF-α was investigated, there was no effect in group I. Groups II, III, IV, V, VI, VII, and VIII had effects. Group IV displayed the least efficacy. No statistically significant differences were found between groups II, III, V, VI, VII, and VIII.

4. Discussion
Random-patterned skin flaps are widely used in soft tissue reconstruction. If the procedure is not well-planned or circulatory disorders occur, flap necrosis can become a complication. Many studies have been performed to improve the survival of random-patterned skin flaps. As a result of anaerobic metabolism occurring in cells due to ischemia occurring in the flap, free radicals increase within the cell. This increase in free oxygen radicals and
neutrophils can lead to tissue damage, and subsequently flap necrosis. Antiinflammatory drugs and antioxidants have been used to reduce the cellular effects of ischemia (23,24). In addition, RO can greatly enhance flap viability with vasodilatory effects (5).

Increased blood circulation in the flap can prevent necrosis. In a previous study, the authors claimed that RO treatment is useful in patients with circulatory problems and can be used as an alternative therapy to increase blood circulation in flaps (5).

When these studies are considered together, the bioavailability of RO is the highest when administered orally (5,6). Thus, oral administration was used in our study.

The proportions of the basic components of RO vary among genotypes. These components and their proportions are as follows: alpha-pinene (7.3%–37.8%), camphene (0%–12.1%), myrcene (0%–6.6%), cymol (0%–4.5%), limonene (0%–7.2%), 1.8-cineole (13.6%–67.3%), camphor (2.2%–48.3%), and borneol (1.3%–12.7%) (25). In a previous study, the authors used Kozan-genotype RO (5). This genotype contains alpha-pinene (13.5%), camphene (4.1%), myrcene (2.2%), cymol (2.8%), limonene (2.4%), camphor (2.2%), borneol (5.3%), and 1.8-cineole (67.3%) (25). In our study, most of the 3 components studied, which were from the Kozan genotype, were obtained with 60% purity when the RO components were separated.

Cineole was evaluated by being administered singly in group II. Cineole has antiinflammatory and antioxidant properties. Alpha-pinene was evaluated by being administered singly in group III. Alpha-pinene also has antiinflammatory and antioxidant properties. Camphor has only antiinflammatory properties.

There were no statistically significant differences found in VEGF compared to the groups in which components of RO were given singly. However, VEGF was significantly higher in groups V, VII, and VIII, in which RO components were given in combination. We hypothesized that although no RO components had an angiogenesis feature, the components may be mutually induced. Three components of RO were given in group VIII. The antiinflammatory effect was highest in this group; in addition, group VIII had antioxidant properties and the VEGF plasma level was high.

There were antiinflammatory and antioxidant properties and the VEGF plasma level was high in group V, in which alpha-pinene and cineole were given together.

We performed flap elevation after 1 week of treatment because the average ratio of the viable surface area to the total flap area was found to be the highest in previous studies investigating the effects of RO on flap viability (5,6). Flap elevation after 1 week of treatment can increase flap survival in clinical practice. In addition, it was suggested that the bioavailability of RO is the greatest when orally administered (5,6). Thus, oral administration was used in our study.

Although the average ratio of the viable surface area in the control groups was similar in previous studies and in our study, it was different in the experimental groups. The average ratio of viable surface area was 67.68% and 96% in previous studies (5,6). However, in our study, the highest average ratio of viable surface area was 74% in group VIII, in which all 3 components were given. The reason for this difference could be that we used only 3 components of RO in the experimental groups. Other components of RO such as camphene, myrcene, cymol, limonene, and borneol may also increase flap survival. On the other hand, in group
VIII, unlike the Kozan genotype of RO, more alpha-pinene and camphor were given to the rats. Although alpha-pinene and camphor have antiinflammatory and antioxidant properties, the flap viability was poorer than that in a previous study. Giving a substance that has higher purity or a more concentrated amount does not mean that it will be more effective. Another probability may be that other components of RO induce the effects of alpha-pinene, cineole, and camphor.

The amount of cineole, which is 67.3% in the Kozan genotype (5), had 60% purity in our study. This difference created a significant difference in flap viability. Cineole and alpha-pinene both displayed antioxidant properties, and the flap viable area rate was the third highest in group V, in which these two components were administered together. However, the second highest viable area was in group III, in which alpha-pinene was given singly.

When flap viable areas were compared, the most viable areas were in groups VIII, III, V, and VII. The common feature of these groups was the antiinflammatory effect. The common component of RO in groups VIII, III, and V was alpha-pinene. In addition, the common component of RO in groups VIII, V, and VII was cineole. We suggest that alpha-pinene and cineole were the components of RO that were responsible for increased flap survival. As a result, although RO was effective in flap viability with antiinflammatory, antioxidant, and vasodilatory properties and in increasing VEGF, the most effective of these features were the antiinflammatory effects.

This study demonstrated that cineole, alpha-pinene, and camphor can help to prevent flap necrosis; however, investigation of other components of RO for their effects on the viability of flaps is also required in further studies.

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