Assessing the effects of melatonin and N-acetylcysteine on the McFarlane flap using a rat model

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OBJECTIVE: To determine the effects of N-acetylcysteine (NAC) and melatonin, alone and in combination, on McFarlane flap viability in a rat model.

METHODS: Forty Wistar rats were divided into four groups and received daily intraperitoneal injections for one week before surgery: control (sham [n=10]); melatonin (n=10); NAC (n=10); and NAC+melatonin (n=10). One week after surgery, the experiment was terminated and photographs were taken for topographic studies. A transillumination study was performed to observe vascularization in the flaps and biopsies were obtained for histopathological studies.

RESULTS: Flap viability was significantly greater in the antioxidant- (ie, NAC and melatonin) treated groups compared with the control group; however, there were no significant differences among the groups that received antioxidants.

CONCLUSIONS: Melatonin and NAC are important antioxidants that can be used alone or in combination to increase flap viability and prevent distal necrosis in rats.

Key Words: *Antioxidant; McFarlane flap; Melatonin; NAC; N-acetylcysteine*

Évaluer les effets de la mélatonine et de la N-acétylcystéine sur un lambeau de type McFarlane dans un modèle de rat

OBJECTIF : Déterminer les effets de la N-acétylcystéine (NAC) et de la mélatonine, seules ou ensemble, sur la viabilité d'un lambeau de type McFarlane dans un modèle de rat.

MÉTHODOLOGIE : Les chercheurs ont réparti 40 rats Wistar en quatre groupes à qui ils ont administré des injections intrapéritonéales quotidiennes pendant une semaine avant l'opération : sujets témoins (n=10), mélatonine (n=10), NAC (n=10) et NAC+mélatonine (n=10). Une semaine après l'opération, ils ont mis fin à l'expérience et pris des photos en vue d'études topographiques. Ils ont réalisé une étude de transillumination pour observer la vascularisation des lambeaux et effectué des biopsies pour mener des études histologiques.

RÉSULTATS : La viabilité des lambeaux était beaucoup plus importante dans les groupes traités aux antioxydants (NAC et mélatonine) que dans le groupe témoin. Cependant, les différences n'étaient pas significatives entre les divers groupes qui avaient reçu des antioxydants.

CONCLUSIONS : La mélatonine et la NAC sont d'importants antioxydants qu'on peut utiliser seuls ou ensemble pour accroître la viabilité des lambeaux et prévenir la nécrose distale chez les rats.

Skin flaps are frequently used in plastic and reconstructive surgery. However, distal skin ischemic necrosis is a common complication that may require secondary surgical intervention(s) and delay future treatments (1). To overcome this problem, researchers have worked intensively to increase flap viability, particularly in high-risk patients (2). Necrosis is triggered by severe ischemia resulting from impaired arterial inflow, particularly in the distal part of the flap. Pharmacological solutions, including sympatholytics, vasodilators, calcium channel blockers, antihemorrhagics, antioxidants, prostaglandin inhibitors, anticoagulants and glucocorticoids, have been used (3).

N-acetylcysteine (NAC) and melatonin are two effective antioxidant agents that are believed to increase flap viability (4,5). Several studies support the hypothesis that these agents can improve flap viability (6,7). NAC has been shown to protect from ischemia/reperfusion injury in a variety of organs and tissues (8-10). The elevation of flaps with randomly perfused areas does not cause ischemia/reperfusion injury but may be associated with critical ischemia, eventually compromising the survival of flap tissue. The exact mechanism of action of NAC, however, has not been elucidated. Melatonin, a primary secretion of the pineal gland, is a strong free-radical scavenger and an antioxidant. Melatonin may decrease oxidative stress by stimulating antioxidant enzymes (ie, superoxide dismutase and catalase) (11).

Limitations associated with flap surgery have prompted surgeons to investigate new methods of enhancing flap viability, including the use of antioxidant agents. In the present study, we used a rat model to investigate the effects of NAC and melatonin – alone and in combination – on McFarlane flap viability.

METHODS

Study design
The present experimental study was performed in the Medical Sciences Experimental Research and Application Centre and the pathology laboratories of the Çukurova University (Adana, Turkey) with the approval of the Institutional Animal Care and Use Committee. Animals were housed under standard conditions of temperature (20°C to 22°C) and humidity (50% to 60%), in a quiet and well-aerated room with day/night cycles of 12 h/12 h and ad libitum access to water and food (21% raw protein).

Forty Wistar rats (200 g to 250 g) were divided into four groups as follows:

- Control (n=10): Daily intraperitoneal (ip) administration of physiological saline for one week.
- Melatonin (n=10): 10 mg/kg/day melatonin administered ip for one week.
- NAC (n=10): 40 mg/kg/day NAC administered ip for one week.
- NAC+melatonin (n=10):10 mg/kg/day melatonin plus 40 mg/kg/day NAC administered ip for one week.

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TABLE 1
Flap area, necrotic area and percentage of necrosis in the four groups

Group	Flap area, cm²	Necrotic area, cm²	Necrosis, %
Control	18.4±5.1 (11.8–29.1)	7.8±2.7 (3.2–12.5)	41.5±10.1 (27–58)
Melatonin	23.9±5.4 (16.6–32.6)	5.7±4.0 (1.0–14.2)	22.2±12.5 (4–43)
N-acetylcysteine	21.3±3.9 (16.8–28.1)	5.1±2.4 (1.8–8.9)	22.3±7.8 (10–33)
Melatonin + N-acetylcysteine	18.7±3.7 (12.5–24.2)	4.2±1.9 (1.7–8.5)	22.2±10.0 (9–39)
Total	20.6±4.9 (11.8–32.6)	5.7±3.1 (1.0–14.2)	27.1±12.9 (4–58)
P	0.061	0.051	0.002

Data presented as mean ± SD (minimum–maximum) unless otherwise indicated

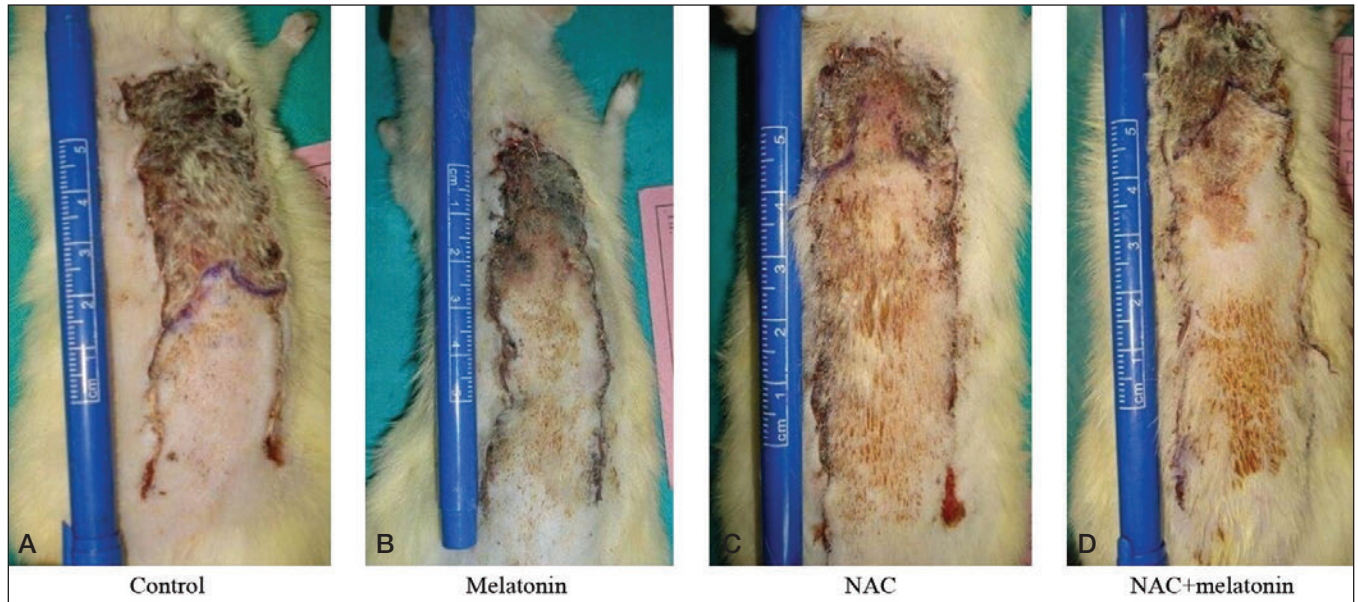**Figure 1)** **A** *to* **D** *Area of necrosis in the four experimental groups. NAC N-acetylcysteine*

The rats were anesthetized with 10% ketamine HCl (50 mg/kg) and xylazine HCl (2.5 mg/kg) before surgery. For flap viability experiments, the McFarlane flap, described by McFarlane etal in 1965, was elevated including the panniculus carnosus and muscle on the dorsal skin of the rat(12).

The rats were euthanized seven days after surgery, and viable tissue and necrotic areas were analyzed using planimetric methods. Using transillumination photography, the extent of tissue vascularity in the flaps was analyzed (13,14). General appearance and percentage of survivingareas of the flaps were recorded as described by Acarturk et al (14). Photographs were obtained utilizing standardized parameters (lighting in 45° in relation to the object, column height of 40 cm and distance from the object of 40 cm). Aplanimetric evaluation with image analysis software (Photoshop®, Adobe, USA) was used to assess and measure the percentageof the surviving area. The area of survival of the skin flapswere expressed as a percentage of the total flap area (survival rate = surviving area/total area × 100). The measurements were performed by a researcher who was unaware of the specific groups and treatment. In addition, a necropsy was performed to evaluate the status of the arterial system within and at the base of the flaps.

Biopsy specimens from necrotic regions of the flaps were stained with hematoxylin and eosin and examined under a light microscope (Olympus BX-51, Japan) for vessel proliferation, erythrocyte extravasation and evidence of inflammation based on a modified scoring system, including three different categories, ie, vessel proliferation, erythrocyte extravasation and evidence of inflammation (15). Each category was scored from 0 to 4.

Statistical analysis
Data were analyzed using SPSS version 17.0 (IBM Corporation, USA) for Windows (Microsoft Corporation, USA). A parametric test (independent-samples *t* test) was applied to normally distributed data, while a nonparametric test (Mann-Whiney U test) was applied to non-normally distributed data. Continuous data are presented as mean ± SD or median (minimum–maximum), as appropriate; P≤0.05 was considered to be statistically significant.

RESULTS
Flap area, necrotic area and percentage of necrosis in the four groups are summarized in Table 1. The area of the flap and the area of necrosis did not show significant difference among groups (P=0.061 and P=0.061, respectively) (Figures 1A to 1D).When percentages of necrosis in the control group were compared with the treatment groups, a significant difference was observed (P=0.002) (Table 1, Figure 2).

On histopathological examination, necrotic areas in the control group were generally full thickness (Figure 3A), while those in the melatonin group were superficial and did not involve the dermis (Figure 3B). Only focal necrotic areas were observed in the NAC and NAC+melatonin groups (Figure 3C). Although the necrosis in the melatonin group was superficial, fewer animals in the NAC group developed necrosis compared with the melatonin and NAC+melatonin groups. Moreover, the rate of necrosis in the NAC+melatonin group was notlower compared with the melatonin and NAC groups.

Full-thickness samples were obtained from flap sections and were histopathologically examined for edema, inflammation and vessel proliferation; rates of necrosis and locations of necrotic areas were also assessed. Macroscopic examination revealed evidence of edema and infection in four rats in the control group, and in one rat each in the melatonin and NAC+melatonin groups. There was no evidence of edema or infection in animals that were treated with NAC only. The control group showed significantly increased levels of inflammation and edema compared with the other three groups (P≤0.05) (Figure 4A).

Vessel proliferation rates in histopathological sections were considerably higher in the NAC, melatonin and NAC+melatonin groups compared with the control group (P=0.035) (Figure 4B). Vascular proliferation observed in the NAC+melatonin group was less than in the groups in which these agents were administered alone. In addition, the extent of vascularization was assessed by examining transilluminated flaps (Figures 5A to 5D). The rate of vascularization in the control group was less than that observed in the treatment groups and was noted on only proximal portions of control group flaps compared with the other groups. In contrast, the treatment groups exhibited increased vascularization extending to distal parts of the flaps. No difference between the melatonin and NAC+melatonin groups was found; the most extensive vascularization structures were observed in the NAC group.

DISCUSSION

In the present experimental study, we attempted to determine the effects of NAC and melatonin, alone and in combination, on McFarlane flap viability in a rat model. The present study showed that melatonin and NAC are important antioxidant agents that can improve skin flap viability. Although they had similar effects when used alone, they did not work synergistically to further improve skin flap outcomes.

Classic studies have reported that flap necrosis occurs due to separation between the flap and sympathetic nerves and blood vessels following flap elevation. After the flap loses sympathetic activity, blood flow to the distal region diminishes. Soon thereafter, vasoconstrictor mediators cause a further decrease in blood flow. The risk for ischemia-reperfusion injury is increased if ischemia persists for 6 h to 12 h. Ischemia-reperfusion injury is an additional pathogenic event that occurs when circulation is restored after a period of absent blood flow. Microvascular perfusion is interrupted
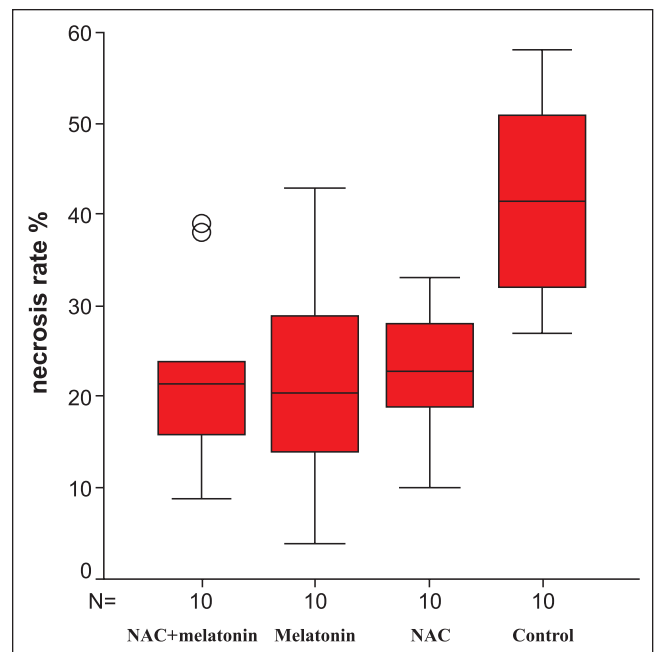

Figure 2) *Percentage of necrosis in the control and treatment groups. NAC N-acetylcysteine*

and necrosis usually develops in the tissues. In ischemic tissues, anaerobic metabolism and glycosylation occur, and the subsequent increase in reactive oxygen species levels leads to lipid peroxidation in the cell membrane, ultimately leading to toxicity. This causes
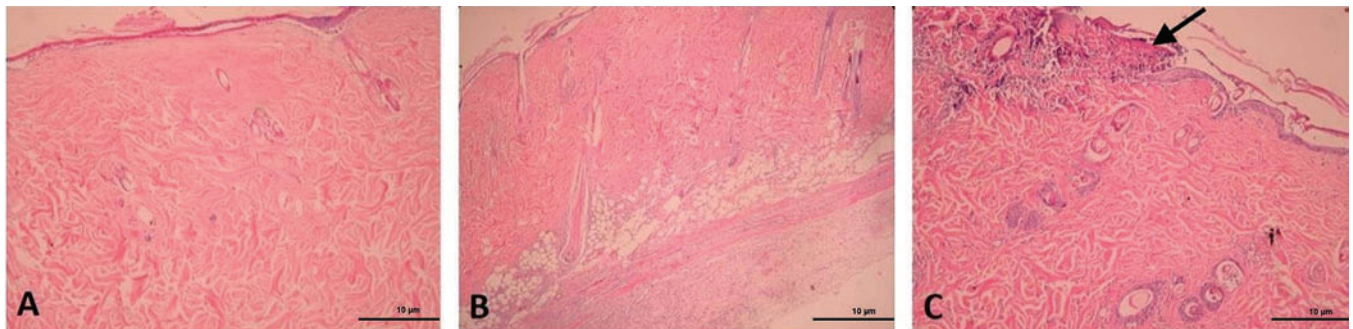

Figure 3) *Histopathological examination showing full-thickness necrosis in the control group (A), superficial necrosis in the melatonin group (B), and focal necrotic areas in the N-acetylcysteine (NAC) and NAC+melatonin groups (arrow) (C) (Scale bar: 10 µm)*
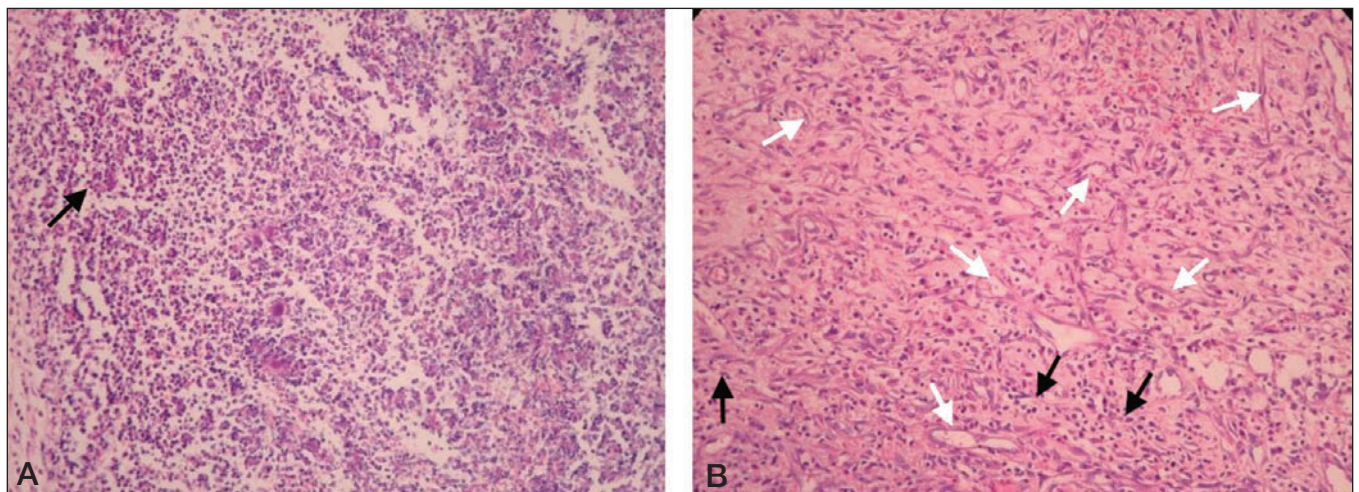

Figure 4) *The control group showed significantly increased levels of inflammation (arrow) and edema (A), while vessel proliferation (white arrows) was significantly decreased compared with the treatment groups (B). Hematoxylin and eosin stain (Scale bar: 10 µm)*
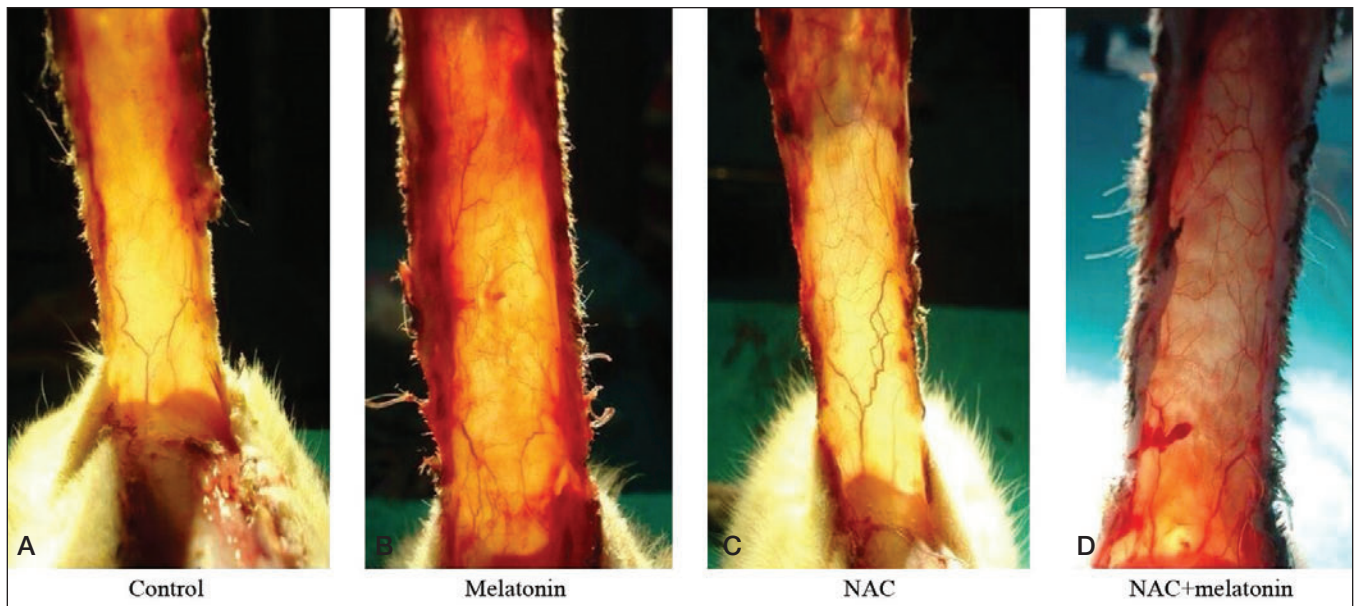
Figure 5) **A** to **D** The extent of vascularization was assessed by examining transilluminated flaps. NAC N-acetylcysteine

local acute inflammation, leukocyte accumulation and adhesion, events that lead to endothelial injury (16). Surgical delay procedures, hyperbaric oxygen and pharmacological agents are used to avoid flap necrosis. Because delay procedures require a staged surgical process and hyperbaric oxygen is not available in every centre, we searched for alternative pharmacological agents.

Several studies have described the antioxidant characteristics of many agents, such as carnitine, desferrioxamine, trimetazidine, coenzyme Q and vitamins E and C, and their positive effects on flap viability (17-19). It has been shown that as an antioxidant, melatonin is at least twice as effective as vitamin E and five times more effective than glutathione (4,20). A few studies have demonstrated melatonin's potential effect on flap viability (4,5,21). Gurlek et al (4,5) performed pinealectomies in rats to prevent circadian melatonin secretion, thus enabling the assessment of antioxidant effects of exogenous melatonin. They observed that skin flap viability increased with ip administration of melatonin. NAC is considered to exert its antioxidant effects both by decreasing free-radical oxygen formation and increasing glutathione synthetase activity (22,23). Other studies have described NAC's ability to ameliorate ischemia-reperfusion injury in certain tissues. Glutathione is a major free-radical scavenger, and NAC (a precursor of glutathione) has been used to block ischemia-reperfusion injury in the kidneys, liver, lungs, myocardium, skin and soft tissue (24). The combination of melatonin and NAC treatment was previously studied in several ischemia-reperfusion models. Sener et al (25) used a hepatic ischemia-reperfusion model to demonstrate the synergistic effect of these two agents. In the present study, we found that the rate of necrosis in the control group was significantly increased compared with the other groups. The necrotic area in the melatonin group was superficial and did not involve the dermis. This is likely the result of melatonin's preventive effect on vasoconstriction due to sympathetic activation during flap elevation and its antioxidant effect in the acute period (26). Although necrosis in the melatonin group was superficial, fewer animals in the NAC group developed necrosis compared with the melatonin and NAC+melatonin groups. Moreover, the rate of necrosis in the NAC+melatonin group was not lower compared with the melatonin and NAC groups. Although NAC and NAC+melatonin decreased the rate of necrosis, co-administration of both antioxidants did not have a synergistic effect.

Other studies have reported that antioxidants can induce angiogenesis and prevent the free radical-mediated decrease in angiogenic growth factors (27-29). As an indicator of neoangiogenesis, significant increases in vascular proliferation were observed in the melatonin, NAC and NAC+melatonin groups compared with the control group in the present study. Vascular proliferation observed in the NAC+melatonin group was less than that observed in the groups in which these agents were administered alone. This result was attributed to competition and decreases in their antioxidant effects against the free oxygen radicals that prevent angiogenesis. We found that NAC alone increased vascular proliferation and neoangiogenesis compared with the other three groups.

Shortly before euthanizing the animals and obtaining samples, we examined the vascular structures of elevated flaps via transillumination. These vascular structures supported the findings of the histopathological examination. That is, the vascularization rate in the control group increased only in the proximal region; however, the other groups exhibited increased vascularization extending to the distal regions of the flaps. The greatest extent of vascularization was observed in the NAC group. Our findings indicate that treatment with antioxidants prevented free radicals from inhibiting the effects of growth factors and attenuating angiogenesis.

CONCLUSIONS

The results of the present animal study indicate that melatonin and NAC may be used by surgeons in the future to increase flap success rates and flap sizes when reconstructing large tissue defects. Additionally, melatonin and NAC may be used to treat chronic skin ulcers due to their ability to induce angiogenesis.

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