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Ascorbic acid and calcitriol reduce TNF-α to improve the viability of modified McFarlane random skin flaps in Wistar rats

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ABSTRACT

Introduction: Distal flap necrosis is one of the most common complications in flap procedure. Ascorbic acid (vitamin C) is one of the most widespread antioxidants available, while calcitriol, a metabolite of vitamin D, has anti-inflammatory and antioxidant properties. The purpose of this study was to determine the effect of ascorbic acid and calcitriol on the viability of Modified McFarlane random skin flaps in Wistar rats and its mechanism through suppression of proinflammatory cytokine Tumor Necrosis Factor-α (TNF-α).

Methods: An experimental study used 28 Wistar rats, by creating a caudally based 3 cm x 9 cm Modified McFarlane random skin flap and divided into four groups. The first group was injected with 200 mg/kg/day ascorbic acid, the second group with 2μg/kg/day calcitriol, and the third group with 200 mg/kg/day ascorbic acid combined with 2μg/kg/day calcitriol was injected two hours after flap elevation for seven days, respectively. The control group was not given any intraperitoneal injection. After seven days, the percentage of flap viability rate was measured and tissue sampling was performed to measure the amount of capillary density and TNF-α in all groups.

Results: The higher percentage of viable flap area was found in all treatment groups, the highest in the ascorbic acid group 80.63% ($p<0.001$). The highest capillary density was found in ascorbic acid combined with the calcitriol group (27/visual field, $p<0.001$) compared to all groups. It was also found that the amount of TNF-α in the tissue was lowest in the group administered by ascorbic acid combined with calcitriol compared to all groups 57.38 ng/L and 0.62 nmol/mL respectively compared to all groups ($p<0.001$). The higher percentage of viable flap area was correlated with a lower amount of TNF-α ($p<0.001$), but not correlated with the amount of capillary density ($p>0.05$).

Conclusion: Ascorbic acid in combination with calcitriol improves the viability of Modified McFarlane random skin flaps by lowering TNF-α levels.

Keywords: Modified McFarlane random skin flap, flap viability, TNF- α, Calcitriol, Ascorbic Acid.
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INTRODUCTION

Random skin flaps are commonly used procedures in plastic and reconstructive surgery to provide coverage to soft tissue defects due to surgery, trauma, infection, or any other etiologies.[1] However, flap necrosis represents one of the most common complications after defect closure using flap procedures, which may require further interventions, generating morbidity to patients and socioeconomic burden to patients and healthcare providers.[1,2] The necrosis rate of flaps is 10–15%.[2,3] The factors influencing flap survival include adequate blood supply, metabolic factors, and tissue tolerance to hypoxia and ischemia.[4] Therefore, improving the local blood circulation, inhibiting the production of inflammatory mediators, alleviating ischemia–reperfusion (I/R) injury, inhibiting oxidative stress of skin flap tissue cells, and promoting angiogenesis are key factors in improving the success rate of a random flap operation.[5,6]

Ascorbic acid is a hydrophilic vitamin that is commonly used worldwide and has various beneficial effects especially in reducing oxidative stress and accelerating the wound healing process.[7] While calcitriol, a lipophilic vitamin, is known to attenuate remodelling via antioxidant, anti-inflammatory and immunomodulatory effects.[7,8] There are studies suggesting that a combination of antioxidants, as a result of additive or synergistic effects, may be more effective for various diseases.[7] However, there has been no study that has assessed the impact of a combination of high doses of vitamin C and calcitriol as an antioxidant on the extent of necrosis of the distal flap on the random dorsal skin flap in rats as the animal model.

This study aims to determine the effect of high-dose ascorbic acid and calcitriol

in improving the viability of Modified McFarlane random skin flaps of Wistar rats through their effects in increasing viability of through TNF-α levels on random skin flap tissue.

METHODS

Preparation of Animals

This study is an experimental, in vivo study using a randomized post-test control group design that aimed to investigate the survival of random dorsal skin flaps in Wistar rats.

Twenty-eight male Wistar rats weighing 200-250 grams were used in this study. Using Federer's sample size calculation, it was estimated that a total of seven samples were required for each study arm (total n = 28). Samples in this study were grouped into four groups, the first group was given high-dose ascorbic acid, the second group was given calcitriol, the third group was given a combination of ascorbic acid and calcitriol and the fourth group was the control group using a random sampling method. This study was double-blinded.

Clinical investigations

The clinical investigation in this study:

1. The percentage of flap viable area was measured on the seventh day by using ImageJ® (National Institutes of Health, Bethesda, MD) software on digital photos taken using a digital camera
2. The capillary amount was measured on the seventh day by counting the number of capillary vessels on the injured tissue area with hematoxylin-eosin (HE) staining. The capillary amount was expressed as count per mm.
3. Tumor Necrosis Factor-α (TNF-α) level was measured on the seventh day with the ELISA method using a Biotechnology kit.

Preparation of Calcitriol and Ascorbic acid

Ascorbic acid (Extrace®) was prepared at the dose of 200mg/kg/bodyweight in a one ml syringe. Calcitriol (Laretol®) was prepared at the dose of 2mcg/kg/bodyweight in one ml syringe. A combination of Ascorbic acid and Calcitriol was prepared in the same one ml syringe. Solutions were injected intraperitoneally (i.p.) within two hours after elevating the flap.

Elevation of random dorsal distal skin flap

All procedures and injections in each animal were performed far from the others to protect them against stressful situations. This study was approved by the Ethics in Medical Research Committee. The combination of ketamine 10% (30 mg/kg) and xylazine 2% (10mg/kg) was injected intramuscularly (i.m) into each rat to induce general anaesthesia before elevating the flap and tissue sampling. The rats were placed in the prone position and dorsal hair was shaved. Modified McFarland random dorsal skin flap was designed with a size of 3 cm × 9 cm with the pelvic joints as the base part of the flap (Figure 1).

The skin marks were incised, and the flap was elevated by dissecting areolar tissue as high as the panniculus carnosus and fascia. The flaps were repositioned to the initial position and sutured using 4-0 nylon with an interval of 0.5 cm.

Data analysis

The data was analyzed using *Statistical Package for the Social Sciences*. The difference between the means of the study group was Based on analysis of variance (One-way NOVA), subsequently followed by post hoc Tukey test, with $p < 0.05$ considered statistically significant.

Table 1. The Effect of treatment on the viability of skin flap

Viability of skin flap	Mean ± SD	p-value
The first group (ascorbic acid)	80,63 ± 3,83	<0,001
The second group (calcitriol)	78,68 ± 4,37	
The third group (ascorbic acid + calcitriol)	80,01 ± 4,50	
Control	63,64 ± 0,82	

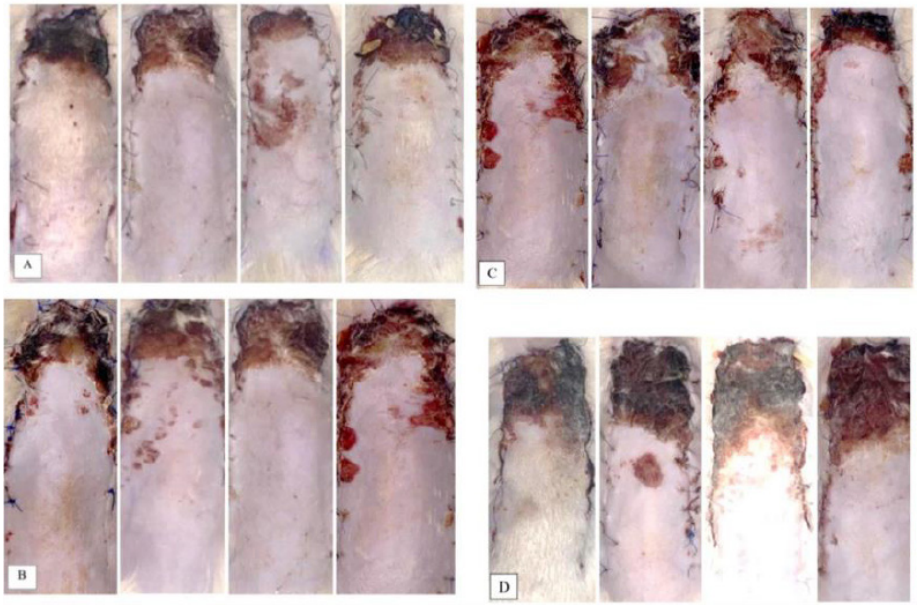

Figure 1. Comparison between groups after seven days of treatment. (a) The first group (ascorbic acid) (b) The second group (Calcitriol), (c) The third group (combination of ascorbic acid and calcitriol), (d) the Control group.

Table 2. Evaluation of capillary density after treatment

Capillary density	Mean ± SD	p-value
The first group (ascorbic acid)	8,1 ± 3,13	<0,001
The second group (calcitriol)	17,3 ± 0,82	
The third group (ascorbic acid + calcitriol)	27,0 ± 6,22	
Control	15,3 ± 8,78	

RESULTS

After the seventh day of treatment, the mean percentage of the viability area in the first group showed the highest viability (80,63 ± 3,83) and showed a remarkable protective effect against necrosis compared to the other group (Table 1). The third group (combination of ascorbic acid + calcitriol) showed the highest capillary density (27,0 ± 6,22) (Table 2), which exerted a remarkable effect on angiogenesis (Figure 2). The third group also showed the lowest TNF-α levels after treatment (Table 3). We performed a Pearson analysis test to find the correlation between variables, showing that flap viability and TNFα levels are correlated, and capillary density and TNFα levels are also correlated (Table 4).

DISCUSSION

One of the most common problems with the skin flap procedure is tissue necrosis due to ischemia, inflammation, and ischemia/reperfusion injury. Therefore, to improve flap viability is to improve blood flow and tissue endurance.[1,2] Inflammation plays an important role in the viability of random skin flaps. The greater the extent of necrosis, the more pronounced the inflammation, which compromises flap success.[1] Induction of moderate inflammation with biomaterials promotes wound healing, suggesting an important role in the balance of proinflammatory signals during regeneration. Tumor necrosis factor (TNF) is one of the pro-inflammatory cytokines involved in skin regeneration.[9] TNF-α is a pleiotropic cytokine produced by a cell types of variety, including keratinocytes, macrophages, and mast cells. TNF-α amplify the inflammatory response by activating more neutrophils and cells such as macrophages, which, although essential for the repair cells activation, can generate deleterious effects when an exacerbated release occurs by prolonging the inflammatory phase and, thus, preventing keratinocyte and fibroblast proliferation. Balanced TNF production in the inflammatory state still appears to be important for the protective functions of the cutaneous wound.[5,9,10]

In this study, the group who were treated with high-dose ascorbic acid

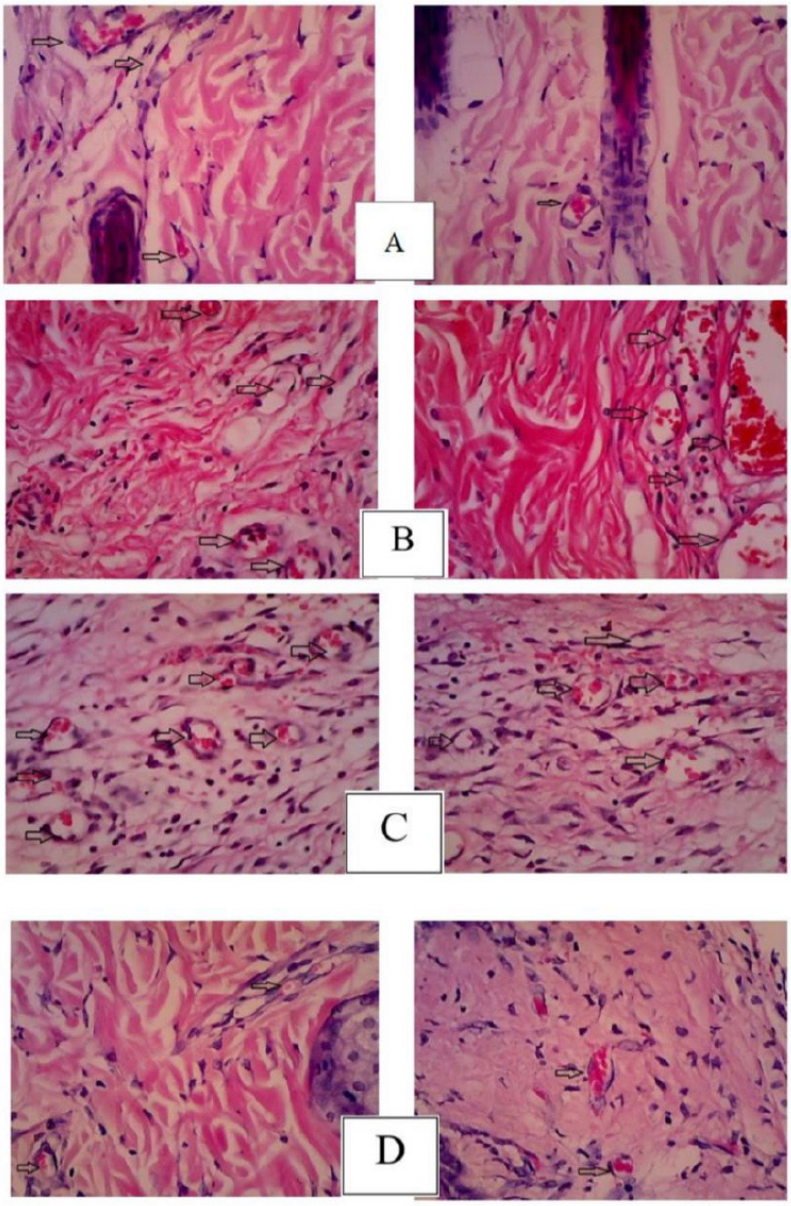

Figure 2. Capillary microscopic view between groups, samples were taken on the seventh day after treatment. (a) The first group (ascorbic acid) (b) The second group (Calcitriol), (c) The third group (combination of ascorbic acid and calcitriol), (d) Control group.

Table 3. TNF-α levels after treatment

TNF-α Levels	Mean ± SD	p-value
The first group (ascorbic acid)	78,59 ± 3,34	<0,001
The second group (calcitriol)	64,26 ± 3,01	
The third group (ascorbic acid + calcitriol)	57,38 ± 2,74	
Control	94,76 ± 1,63	

Table 4. Pearson Correlation Analysis between Variables.

Variables	r	T-test	p-value	Correlation
Flap & Capillary Density	.32	1.70	0.0508	10.36
Flap & TNF-α levels	-.66	-4.40	<0.001**	43.59
Capillary density & TNF-α levels	-.74	-5.58	<0.001**	55.47

Note. *p*-value significant,*) p < 0.05; **) p<0.01.

showed the highest flap viability rate (80,63 ± 3,83), p = <0,05). This is consistent with previous studies by Kianian et al. and Wijaya et al., which that showed the administration of ascorbic acid reduced flap necrosis due to the strong antioxidant effect, collagen production, and fibroblast proliferation.[11,12] Vitamin C downregulates the proinflammatory cytokines tumor necrosis factor-α (TNF-α), and interleukin (IL)-6. Vitamin C has been shown to increase neutrophil migration in response to chemoattractant (chemotaxis), enhance microbial phagocytosis, stimulate the formation of reactive oxygen species (ROS) and support caspase-dependent apoptosis, increase uptake and clearance by macrophages and inhibit necrosis, thereby supporting resolution of the inflammatory response and reduced tissue damage.[9,10]

Calcitriol, also known as 1,25-dihydroxy vitamin D3, is the biologically active metabolite of vitamin D and the principal Ca2+-regulatory steroid hormone.[13] Many recent studies have shown that calcitriol has other bioactivities; Zhou et al (2016) have shown that calcitriol promotes skin flap survival in rats by accelerating angiogenesis, having anti-inflammatory effects, reducing oxidative stress, and promoting autophagy.[1] Research shows that calcitriol is a strong immunomodulator that has receptors on all immune cells, and plays a role in T lymphocyte production and B lymphocyte differentiation, optimizing anti-inflammatory function by lowering IL-10 cytokine levels, inducing monocyte and macrophage maturation and differentiation, associated with cytokine production and chemokine via nuclear factor-κB (NF-κB) and induces the secretion of lysosomal enzymes and hydrogen peroxide.[7] Vitamin D also attenuates the inflammatory response by reducing proinflammatory cytokines, tumor necrosis factor (TNF)-α and IL-6. In the skin, vitamin D has the effect of modulating inflammation, angiogenesis and wound healing.[8,14]

There are studies suggesting that a combination of antioxidants, is a result of additive or synergistic effects.[15] Vitamins C and D have been shown to regulate immune response by decreasing the proinflammatory cytokine release from immune cells and inducing the proliferation of other immune cells.[16] Ascorbic acid used in mixtures with other protective compounds, such as α-tocopherol, β-carotene, or 25 -hydroxyvitamin D, significantly downregulates pro-inflammatory molecules, such as interleukin 6 (IL-6) or interferon-γ (IFN-γ), and, to varying degrees, affect levels of the anti-inflammatory interleukin 4 (IL- 4) in human plasma.[17] In this study, the combination of ascorbic acid and calcitriol showed the highest capillary density (27,0 ± 6,22, p = < 0,05) and the lowest TNF-α levels (57,38 ± 2,74, p = <0,05). Combination of ascorbic acid with calcitriol even in ineffective doses reduces oxidative stress and inflammation due to additive effects, able to reduce the infiltration of neutrophils and eosinophils, inflammatory cell infiltration, MDA level and -NF-κB expression.[18] A combination of ascorbic acid and calcitriol is known to increase nitric oxide and superoxide dismutase. In addition to its effects on cellular and humoral immunity, this combination plays an important role in the formation and maintenance of epithelial and endothelial barriers.[19]

A higher percentage of viable flap area was observed in this study in Wistar rats injected with ascorbic acid, calcitriol, and combination groups. Capillary density was higher in the calcitriol and combination group, but not in the ascorbic acid group even though it had similar flap viability. This may be caused by the ascorbic acid effect on NO inhibition limiting angiogenesis but has a positive effect on collagen and fibroblast to support flap viability.[7,11] Reduced tumor necrosis factor-alpha (TNF-α) level was found in all treatment group and the lowest was found in the combination group, correlating with flap viable area, explaining that suppression of inflammation shown by TNF-α plays an important role in promoting flap viability.[2-4]

CONCLUSION

Coadministration of ascorbic acid and calcitriol could improve skin flap survival, probably due to suppression of inflammation and I/R injury subsequently, shown by lower TNF-α. This study finding may repurpose the use of these commonly used vitamins. For the next study, we suggest follow-up research to determine the most optimal dose and timing for the administration.

ETHICAL APPROVAL

This study is approved by The Ethics Committee of Medical Faculty, Universitas Udayana, Prof. dr. I G.N.G. Ngoerah General Hospital, with ethical approval no. 2754/UN14.2.2.VII.14/LT/2022.

CONFLICT OF INTEREST

None declared.

AUTHOR CONTRIBUTIONS

All authors are contributing to the study from the conceptual, data analysis and the results through publication. The authors would like to thank all participants in this study and all the staff of the Department of Surgery, Medical Faculty, Universitas Udayana/ Prof. dr. I G.N.G. Ngoerah General Hospital.

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