doi: 10.48095/ccachp202460

Systemic allopurinol administration reduces malondialdehyde, interleukin 6, tumor necrosis factor α, and increases vascular endothelial growth factor in random flap Wistar rats exposed to nicotine

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Summary

Introduction: Smoking poses a risk to flap viability, with nicotine being a major contributor to the formation of free radicals. Allopurinol, known for its antioxidant properties, has been shown to enhance tissue survival in ischemic conditions by reducing the production of reactive oxygen species (ROS). This study aims to assess the impact of allopurinol on the viability and success of skin flaps in Wistar rats exposed to nicotine. **Methods:** This study examined skin flap survival in nicotine-exposed rats treated with allopurinol. Twenty-eight rats were separated into two groups. During 1 month of nicotine exposure, the treatment group received systemic allopurinol 7 days before and 2 days after the flap procedure, while the control group received no allopurinol. Pro-angiogenic factors, proinflammatory factors, anti-inflammatory factors, and oxidative markers were assessed on the 7th day after the flap procedure using enzyme-linked immunosorbent assay method. Macroscopic flap viability was evaluated on the 7th day using Image J photos. **Results:** As an oxidative marker, malondialdehyde levels were significantly lower in rats given allopurinol than in controls ($P < 0.001$). The levels of interleukin 6 and tumor necrosis factor α, as markers of inflammatory factors, were significantly lower in the group of rats given allopurinol compared to controls ($P < 0.001$). The level of angiogenesis in rats given allopurinol, measured by vascular endothelial growth factor levels, was also higher in the treatment group compared to controls ($P < 0.001$). Macroscopically, the percentage of distal flap necrosis in Wistar rats given allopurinol was lower and statistically significant compared to controls ($P < 0.001$). **Conclusions:** Xanthine oxidoreductase is part of a group of enzymes involved in reactions that produce ROS. Allopurinol, as an effective inhibitor of the xanthine oxidase enzyme, can reduce oxidative stress by decreasing the formation of ROS. This reduction in oxidative stress mitigates the risk of ischemic-reperfusion injury effects and significantly increases the viability of Wistar rat flaps exposed to nicotine.

Key words
allopurinol – angiogenesis – flap – flap necrosis – nicotine

Gitaswari I, Hamid AR, Sanjaya IG et al. Systemic allopurinol administration reduces malondialdehyde, interleukin 6, tumor necrosis factor α, and increases vascular endothelial growth factor in random flap Wistar rats exposed to nicotine. Acta Chir Plast 2024; 66(2): 60–66.

Introduction

Flap reconstruction is a commonly used modality for addressing skin defects resulting from trauma, post-tumor excision, and chronic wounds. However, a primary complication of flap procedures is tissue necrosis, often observed in the distal part of the flap, primarily due to ischemia-reperfusion (I/R) injury, leading to partial destruction of the flap. Flap necrosis not only compromises the success of the reconstruction but also increases patient's morbidity. Ischemia refers to inadequate blood flow to specific tissue areas. Prolonged periods of

ischemia beyond tissue tolerance result in inflammation and necrosis. Additionally, ischemic injury triggers reperfusion to restore the connection between tissues and blood vessels. However, following ischemia, the reperfusion period commences a series of actions, including restoring blood flow and oxygen influx into the ischemic tissue. This can cause alterations in cellular, inflammatory, and metabolic processes in live cells. These alterations, driven by free radicals, disrupt cell structure and function, eventually terminating in tissue necrosis [1].

Smoking represents a major health issue in Indonesia and worldwide, prevalent as a lifestyle choice, particularly in developing countries. Cigarette smoke comprises approximately 4,000 compounds, including nicotine, tar, 3,4-benzopyrene, carbon monoxide (CO), carbon dioxide, nitrogen oxide, ammonia, and sulfur, with over 200 toxic substances and more than 40 carcinogenic substances. These compounds interact with cells in the body, leading to the formation of free radical species or reactive oxygen species (ROS). Empirical data extensively elucidate the adverse effects of smoking on various types of skin flaps. A 2009 study revealed that among patients who experienced tissue slough following a facelift, 80% were active smokers, consuming more than one pack of cigarettes each day at the time of operation. This resulted in a relative risk of 12.46 for smokers compared to non-smokers [2]. Smoking exacerbates the conversion of xanthine dehydrogenase to xanthine oxidase (XO), a process known to be crucial in the pathogenesis of I/R injury. During skin flap ischemia, there is significant upregulation of the XO system. XO serves as the primary source of ROS, which causes the release of some inflammatory mediators [3]. Cigarette smoke, known to generate ROS, necessitates the presence of antioxidants to mitigate ROS production within the body.

Various methods and techniques have been used to safeguard flaps from the harmful effects of I/R damage or to minimize stress throughout and after ischemic events. Among these approaches, the impact of antioxidants on flap failure has been extensively studied, with allopurinol being one such antioxidant of interest [3]. Given that exposure to nicotine from cigarettes is a significant contributor to flap failure, this study aims to enhance flap viability in smokers by administering allopurinol, an antioxidant known to mitigate I/R injury, a primary mechanism underlying skin flap failure.

Material and methods

The management of animals

Twenty-eight male Wistar rats were allocated randomly into two groups of 14 each. The rats ranged in age from 8 to 12 weeks and weighed between 250 and 300 grams. Individual rats were housed in separate cages with adequate airflow before and after surgery. Standard living circumstances were maintained, with humidity levels ranging from 50 to 70% and room temperatures ranging from 25 to 30 °C based on a 12-hour day/night cycle.

Animal groups

The Wistar rats in this study were separated into two groups. Group 1 received oral allopurinol for 7 days before the McFarlane flap procedure, which was performed on the dorsal area, and continued for 2 days after the procedure. Group 2 did not receive oral allopurinol and underwent the McFarlane flap procedure after nicotine exposure. All groups were subjected to nicotine exposure for 30 days before the flap procedure. Additionally, consistent postoperative interventions were administered to all animals across the study groups.

Smoking chamber preparation

The room designated for nicotine exposure contained homemade main cages made of plastic, each measuring 35 × 25 × 15 cm. Each cage housed one rat during the exposure period. The roof of each cage was equipped with an exhaust system consisting of a cooling fan on the outside and a smoking pipe on the inside. During the exposure, one cigarette was placed inside each cage. The exhaust system was connected to an electrical line. Once the exposure to cigarette smoke was completed, the exhaust system was activated, and the cigarette smoke was drawn out of the cage through the exhaust. Control over the burning cigarette was achieved through a vacuum or a smoke valve.

Nicotine exposure

Rats were exposed to nicotine through cigarette smoke, with each rat exposed to smoke from 3 cigarettes per day. Each exposure session lasted 20 min, and the rats underwent three daily exposure sessions. The cigarettes used were commercial filter cigarettes containing 0.8 mg nicotine, 10 mg tar, and CO. Nicotine exposure was conducted according to the treatment category, spanning 30 days for all groups. Following the 30-day exposure period, rats underwent a nicotine-free period lasting 7 days before the flap procedure.

Allopurinol intervention

Group 1 rats comprised the control group without the intervention of oral allopurinol. These rats were exposed to nicotine for 30 days before undergoing the McFarlane flap procedure under healthy conditions. Group 2 rats, on the other hand, constituted the intervention group. These rats received systemic allopurinol treatment for 7 days after 30 days of nicotine exposure, leading up to the McFarlane flap procedure. Allopurinol administration continued for 2 days following the procedure. The dosage of allopurinol administered was 100 mg/kg body weight per day, delivered via an oral feeding cannula steel tube for rats.

Flap surgical procedure

The flap technique was perfomed under general anesthesia in both groups. For group 1, the procedure took place

the day after 30 days of nicotine exposure, while for group 2, it was performed 7 days after the administration of oral allopurinol. An adjustment of the McFarlane flap was conducted, involving a random skin flap vascularized by its subdermal plexus on the dorsal side of the rats. After administering general anesthesia and shaving the dorsal hair, each rat's dorsum was cleaned, and a 4 × 10 cm rectangular section was marked. Anatomical landmarks were discovered, including the superior iliac crest edge, spinous process, and lower scapular angle. The flap was then raised, confirming the inclusion of the panniculus carnosus, and the major caudal artery supplying the flap was meticulously identified. After elevation, the flap was sewed back into position using 4/0 nylon sutures. The data was collected on the 7th day post-surgery. Photographs of the necrotic area were taken for all rats in Groups 1 and 2 using a digital camera and measured digitally using Image J software (Media Cybernetics, Silver Spring, MD, USA). The Image J software version 1.52 was utilized to compute the necrotic area of each flap, expressed as a percentage of the total flap area – flap viability rate area (%) = (area of viable skin flap/area of whole flap) × 100.

Blood serum samples were collected from the subconjunctival of the eye on the 7th day after the flap procedure in the control group and the 7th day after the last administration of allopurinol in the intervention group. These samples were used to measure the levels of carboxyhemoglobin (HbCO), malondialdehyde (MDA), vascular endothelial growth factor (VEGF), tumor necrosis factor α (TNF-α), and interleukin 6 (IL-6) using enzyme-linked immunosorbent assay (ELISA). The specimens were finely chopped, homogenized, and centrifuged for 15 min at 12,000 RPM at 4 °C to gain the supernatant. Final concentrations were reported as nmol/l after adjustment for protein content. Upon completion of the research procedure, the rats were euthanized with ketamine (30 mg/head) and high doses of xylazine (6 mg/head) administered intracardially. Subsequently, the experimental animals were disposed of in an incinerator.

Tab. 1. Univariate analysis.

Variable	Treatment group (N = 14)	Control group (N = 14)
	mean (SD)	
HbCO	176.47 (5.235)	245.5 (3.007)
IL-6	2.16 (0.136)	5.43 (0.299)
	median (IQR)	
MDA	0.61 (0.09)	1.81 (0.07)
TNF-α	126.38 (38.38)	70.95 (8.24)
VEGF	513.41 (4.85)	317.48 (225.10)
skin flap	5.50 (5.3)	29.0 (13.5)

SD – standard deviation, IQR – interquartile range, HbCO – carboxyhaemoglobin, IL-6 – interleukin-6, MDA – malondialdehyde, TNF-α – tumor necrosis factor α, VEGF – vascular endothelial growth factor

Data analysis

The data analysis was statistically done using Statistical Package for the Social Sciences (SPSS) software. The quantitative data gained from the research underwent the Shapiro-Wilk test to assess normality. The data that were distributed normally were analyzed using the independent t-test, while non-normally distributed data were analyzed using the Mann-Whitney test. The homogeneity of variance was assessed using the Levene's test. Statistical significance was determined at a $P < 0.05$. IL-6 and HbCO levels were distributed normally and reported as mean and standard deviation (SD). However, levels of MDA, TNF-α, VEGF, and the percentage of flap necrosis were not distributed normally and are displayed as the median and interquartile range (IQR).

Results

The univariate analysis result showed that the mean of HbCO and IL-6 levels in the control group is higher than in the treatment group. This result also showed that the median of MDA levels and skin flap necrosis area in the control group is higher than in the treatment group, and the median of TNF-α and VEGF levels in the control group is lower than in the treatment group (Tab. 1).

HbCO levels

To ensure that rats were adequately exposed to cigarette smoke and to evaluate the effectiveness of allopurinol as an antioxidant in reducing HbCO levels, this study employed the ELISA method to quantify HbCO levels. The mean HbCO levels in the control group's skin flap blood serum were 245.5 (SD 3.007). A significant reduction in HbCO levels was observed in the blood serum of subjects who received oral allopurinol after administering nicotine in all groups. The mean HbCO level was 176.47 (SD 5.235), indicating a mean difference of 69.1, with a 95% CI 56.54–81.68. The difference was determined to be statistically significant ($P = 0.000$) (Tab. 2).

The independent t-test results established a statistically significant difference in HbCO levels among the treatment and control groups ($P = 0.000$, i.e., < 0.001).

MDA levels

MDA levels were evaluated by collecting blood serum samples from the

Tab. 2. Analysis of carboxyhaemoglobin levels.

Variable	Mean (SD)		Mean difference (CI 95%)	P-value
	treatment group (N = 14)	control group (N = 14)		
HbCO	176.47 (5.235)	245.5 (3.007)	69.1 (56.54–81.68)	0.000*

SD – standard deviation, HbCO – carboxyhaemoglobin, *significant value (P < 0.05)

Tab. 3. Analysis of malondialdehyde levels.

Variable	Median (IQR)		P-value
	treatment group (N = 14)	control group (N = 14)	
MDA	0.61 (0.09)	1.81 (0.07)	0.000*

IQR – interquartile range, MDA – malondialdehyde, *significant value (P < 0.05)

Tab. 4. Analysis of interleukin-6 levels.

Variable	Mean (SD)		Mean difference (CI 95%)	P-value
	treatment group (N = 14)	control group (N = 14)		
IL-6	2.16 (0.136)	5.43 (0.299)	3.26 (2.57–3.95)	0.000*

IL-6 – interleukin-6, *significant value (P < 0.05)

Tab. 5. Analysis of necrosis factor α (TNF-α) levels.

Variable	Median (IQR)		P-value
	treatment group (N = 14)	control group (N = 14)	
TNF-α	126.38 (38.38)	70.95 (8.24)	0.000*

TNF-α – necrosis factor α, IQR – interquartile range, *significant value (P < 0.05)

subconjunctival on the 7th day after the flap procedure to assess the impact of oral allopurinol therapy on MDA levels in the flap. MDA levels were measured using the ELISA method (BT Laboratory, Zhejiang, China). The findings revealed that the median values of VEGF levels in rats administered oral allopurinol (median 0.62, IQR 0.09) were significantly lower compared to rats without oral allopurinol therapy (median 1.81, IQR 0.07) (P = 0.000) (Tab. 3).

The Mann-Whitney test results demonstrated a statistically significant difference in MDA levels between the treatment and control group (P = 0.000, i.e., < 0.001).

IL-6 levels

The mean IL-6 levels in the control group's skin flap blood serum were 5.43 (SD 0.299). Following the administration of oral allopurinol therapy (100 mg/kg) in the intervention group, the IL-6 levels in the blood serum noticeably decreased, with a mean IL-6 level of 2.16 (SD 0.136). The mean difference observed was 3.26, with a 95% CI 2.57–3.95. This difference was statistically significant (P = 0.000) (Tab. 4).

The independent t-test results established a statistically significant difference in IL-6 levels between the treatment group and the control group (P = 0.000, i.e., < 0.001).

TNF-α levels

The highest value of TNF-α on the 7th day was found in the control group, with a median result of 70.95 (IQR 8.24), whereas in the treatment group, the median value was 126.38 (IQR 38.38). The difference was significant in statistics (P = 0.000) (Tab. 5).

The Mann-Whitney test results demonstrated a statistically significant difference in TNF-α levels between the treatment and control group (P = 0.000, i.e., < 0.001).

VEGF levels

On the 7th day, VEGF levels were measured from blood serum samples obtained from Wistar rats using ELISA examination. The highest value of VEGF on the 7th day was found in the treatment group, with a median result of 513.41 (IQR 4.85), while in the control group, the median value was 317.48 (IQR 225.10). Nicotine-exposed rats treated with oral allopurinol showed significantly higher levels of angiogenesis VEGF compared to the control group, similar to IL-6, TNF-α, and MDA (P = 0.000) (Tab. 6).

The Mann-Whitney test results demonstrated a statistically significant difference in VEGF levels between the treatment and control group (P = 0.000, i.e., < 0.001).

Skin flap necrosis area

On the 7th day after surgery and administration of systemic allopurinol orally, the viability of the rat dorsal skin flap was assessed to determine the extent of

necrosis. The median percentage of necrosis flap area was lower in rats treated with oral allopurinol compared to rats not receiving oral allopurinol, measuring a median value of 5.50 (IQR 5.3) and 29.0 (IQR 13.5), respectively. The difference was significant in statistics (P = 0.000) (Tab. 7, Fig. 1).

The Mann-Whitney test results demonstrated a statistically significant difference in skin flap necrosis area between the treatment and control group (P = 0.000, i.e., < 0.001).

Tab. 6. Analysis of vascular endothelial growth factor (VEGF) levels.

Variable	Median (IQR)		P-value
	treatment group (N = 14)	control group (N = 14)	
VEGF	513.41 (4.85)	317.48 (225.10)	0.000*

VEGF – vascular endothelial growth factor, IQR – interquartile range, *significant value (P < 0.05)

Tab. 7. Analysis of Wistar rat McFarlane flap necrosis extent.

Variable	Median (IQR)		P-value
	treatment group (N = 14)	control group (N = 14)	
skin flap	5.50 (5.3)	29.0 (13.5)	0.000*

IQR – interquartile range, *significant value (P < 0.05)

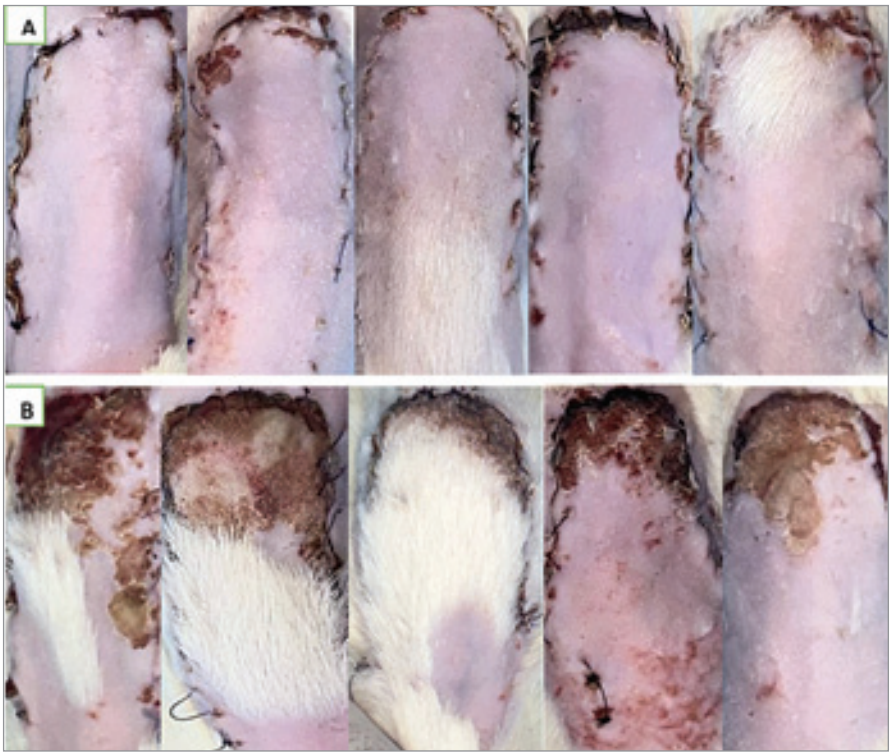

Fig. 1. Skin flap necrosis area in Wistar rats. A) allopurinol intervention group; B) control group.

Discussion

Allopurinol, a XO inhibitor, is known to reduce ROS production. Studies conducted by Manson et al., Ashoori F et al., Schein O et al., and Angel M et al. have demonstrated that allopurinol can enhance skin flap viability. Its mechanism of action involves both direct effects and indirect vasodilation, which increases flap perfusion. Allopurinol administration in rats before flap procedures aims to bolster skin resistance and mitigate the risk of edema, contributing to improved flap outcomes [4]. XO serves as the primary source of ROS, which can trigger the release of inflammatory mediators. Allopurinol, functioning as an antioxidant drug, inhibits the activity of XO, thereby preventing the release of ROS. Systemic administration of allopurinol effectively reduces oxidative stress, thereby mitigating I/R injury and ultimately increasing flap viability [3].

The nicotine content in cigarettes enhances the activity and strength of CO in binding to hemoglobin, leading to a decrease in hemoglobin levels in the body. Haemoglobin bound with CO is referred to as HbCO. In smokers, HbCO levels typically range between 3 a 5%. Exposure to nicotine also results in elevated exhaled CO levels, both in active smokers and passive smokers [5]. Assessing the levels of HbCO could be valuable in evaluating the efficacy of oral allopurinol therapy in rats exposed to nicotine. Clinical diagnosis of acute CO poisoning typically involves measuring HbCO levels. The toxicity of CO extends beyond hypoxia resulting from HbCO, involving direct cellular changes and inflammatory damage through various mechanisms. Our study found a significant decrease in HbCO levels in the intervention oral allopurinol group (P < 0.001). This result aligns with the previous research by Susanti et al., which demonstrated the impact of administering antioxidant melon extract (Cucumis melo) on the

HbCO levels of Wistar rats exposed to cigarette smoke. The study revealed that the group solely bare to cigarette smoke exhibited an average HbCO level of 9.77 ± 0.81%. In contrast, the treatment group receiving melon extract demonstrated the lowest mean HbCO levels at 9 IU/day (7.55 ± 0.97%) ($P < 0.05$) [6]. This research suggests that allopurinol can function as an antioxidant, reducing HbCO levels in the blood. By decreasing HbCO levels, allopurinol may help to prevent the development of further ROS, thereby lowering oxidative stress and its associated cellular damage.

The formation of MDA in smokers is attributed to the generation of oxidative radicals, which damage cellular lipids and promote increased production of lipid peroxidase, namely MDA. Based on the examinations conducted in this study, it was observed that the systemic administration of oral allopurinol led to lower levels of MDA compared to controls ($P < 0.001$). This finding aligns with previous research, highlighting allopurinol's potential as an antioxidant. Previous studies have demonstrated that allopurinol significantly reduces serum concentrations of MDA by 0.403 nmol/ml (95% CI –0.618, –0.189; $P < 0.001$) [7].

Nicotine has been shown to stimulate the expression of IL-6 by activating the transcription factor activator protein 1. Understanding the involvement of these molecules in the inflammatory process is crucial for preventing inflammation-induced cell death, particularly in cases of nicotine exposure. Several studies have reported that smoking behavior increases serum IL-6 levels. This aligns with the study by Musfufatun et al., which highlights a relationship between elevated serum IL-6 levels in smokers and non-smokers. Cigarette smoke-induced oxidative stress increases the release of proinflammatory cytokines such as C-reactive protein and IL-6 [8]. In our study, it was observed that IL-6 levels in Wistar rats exposed to nicotine and treated with systemic allopurinol for 7 days before and 2 days after the flap procedure were lower compared to Wistar rats exposed to nicotine without allopurinol treatment ($P < 0.001$).

In addition to IL-6, the activation of TNF-α in active smokers can lead to a prolonged inflammatory phase, inhibiting wound healing. Our study revealed that the levels of TNF-α in Wistar rats exposed to nicotine and treated with systemic allopurinol were statistically significantly lower than the control group ($P < 0.001$). This suggests that allopurinol effectively inhibits oxidative stress and inflammation. Furthermore, the use of TNF-α as an indicator in reducing inflammatory reactions and increasing flap viability is clarified by the results. Li et al. conducted a therapy aimed at preventing necrosis in random skin flaps by increasing angiogenesis and minimizing oxidative stress and inflammation. Their study demonstrated a significant inhibition of TNF-α ($P \leq 0.01$), a proinflammatory cytokine expressed in vascular endothelium and stromal cells [9].

VEGF is a crucial growth factor involved in regulating physiological and angiogenic processes in tissues, including chronic inflammation, wound healing, tumors, and diabetic retinopathy. Inflammatory responses and local conditions in wounds can stimulate VEGF production, leading to tissue hypoxia and metabolic decline, ultimately resulting in angiogenesis and enhanced endothelial cell function [10]. The decrease in VEGF levels due to nicotine exposure significantly impacts the wound healing process, thereby reducing flap viability. In our study, systemic administration of allopurinol to Wistar rats exposed to nicotine led to a statistically significant increase in VEGF levels ($P < 0.001$) compared to controls. VEGF levels were assessed on the 7th day after the flap procedure. The mechanism underlying the enhancement in endothelial function with high doses of allopurinol (100 mg/kg/day) is attributed to its ability to decrease vascular oxidative stress rather than its effect on uric acid levels. Abbas et al. investigated the impact of nicotine on mouse transverse rectus abdominis muscle (TRAM) flaps. They demonstrated significant improvements in TRAM flap survival in rats exposed to nicotine by inhibiting Notch signaling as an anti-angiogenic factor [11]. Furthermore, futuristic therapies, such as genetic manipulation in murine animals conducted by Basu et al., have shown promising results. They transferred a plasmid encoding the VEGF isoform to an ischemic flap using electrotransfer, resulting in significantly higher flap viability in the intervention group (98.63 ± 1.1%) compared to the control group (79.8 ± 5.2%) ($P < 0.05$) [12].

Macroscopically, the percentage of necrosis in the distal skin flaps of Wistar rats exposed to nicotine and treated with systemic allopurinol was significantly lower compared to the control group not receiving allopurinol ($P < 0.001$). This is consistent with a study by Ardakani et al., which demonstrated that the mean area of necrotic regions in flaps treated with allopurinol was significantly lower at 7 days of observation ($P < 0.0001$). Furthermore, histopathological analysis in Ardakani's study revealed minimal inflammatory reactions and a higher number of normal tissue structures in skin flaps treated with allopurinol. This suggests that allopurinol administration not only reduces macroscopic necrosis but also contributes to maintaining tissue integrity and reducing inflammatory responses [3].

Numerous studies have demonstrated the beneficial effect of administering allopurinol as a pretreatment, either before flap elevation or before reperfusion. The advantage of administering allopurinol in the pretreatment phase lies in its ability to prevent the generation of free radicals by inhibiting XO rather than directly contributing to the production of free radicals [13]. Allopurinol

administration can result in higher levels of tissue antioxidants, enhancing the defense mechanisms against the necrosis process. In addition to allopurinol, several other antioxidants have been investigated for their potential to increase flap viability. These include the administration of vitamin C with high doses, selenium, zinc, coenzyme Q10, folic acid, and vitamin B12 [14].

The limitations of this study include the use of experimental animals, specifically Wistar rats, which may exhibit differences in biological characteristics and skin flap structure compared to humans. Additionally, the exposure to nicotine in this study was limited to a specific duration, and the content of the cigarettes used was predetermined. It is important to note that variations in nicotine exposure in humans may occur over time, and the composition of cigarette content can vary as well.

Conclusion

Xanthine oxidoreductase is part of a group of enzymes involved in reactions that produce ROS. Allopurinol, as an effective inhibitor of the XO enzyme, can reduce oxidative stress by decreasing the formation of ROS. This reduction in oxidative stress mitigates the risk of ischemic-reperfusion injury effects and significantly increases the viability of Wistar rat flaps exposed to nicotine.

Roles of the authors

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validation – Anak Agung Wiradewi Lestari;
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Disclosure: The authors have no conflict of interest to disclose. There is no external financial support in this study. The Research Ethics Committee Faculty of Medicine, Universitas Udayana, provided ethical clearance for this study to protect the basic rights and welfare of the research subjects and to ensure that the research adheres to the International Conference on Harmonization – Good Clinical Practice (ICH-GCP) guidance and other applicable laws and regulations, with approval number 919/UN 14.2.2.VII.14/LT/2023.

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