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Febuxostat treatment attenuates oxidative stress and inflammation due to ischemia-reperfusion injury through the necrotic pathway in skin flap of animal model

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ABSTRACT

Background: Ischemia-reperfusion (I/R) injury is a major contributor to skin flap necrosis, which is a serious complication of reconstructive surgery. The purpose of this study was to evaluate the protective effect of treatment with febuxostat, a selective xanthine oxidase inhibitor, on I/R injury in the skin flap of an animal (rat) model.

Methods: Superficial epigastric flaps were raised in Sprague-Dawley rats and subjected to ischemia for 3 h. Febuxostat at a dose of 10 mg/kg/day was administered to rats in drinking water from 1 week before the surgery (Feb group). Control animals received no drugs (Con group). The mean ratio of flap survival and contraction was evaluated and compared between animals with and without administration of febuxostat on day 5 after the surgery. In addition, infiltration by polymorphonuclear leukocytes and muscles of the panniculus carnosus in the flap were histologically evaluated using hematoxylin-eosin staining. Furthermore, xanthine oxidase activity, ATP levels, superoxide dismutase activity, and expression of 8-hydroxy-2′-deoxyguanosine (8-OHdG), tumor necrosis factor-α, and interleukin-1β were quantitatively assessed in the skin flap 24 h after the surgery.

Results: In the Feb group, the survival and contraction rates at the 5 d timepoint post-surgery were significantly higher and lower than those in the Con group, respectively. Histological analysis showed significant reduction in polymorphonuclear leukocyte infiltration and muscle injury scores due to I/R injury in the Feb group. The expression of 8-OHdG was also significantly inhibited in animals administered febuxostat. Biochemical analysis showed a significant reduction in xanthine oxidase activity and significant increases in ATP levels and superoxide dismutase activity in the Feb group. Furthermore, the expression of interleukin-1β was significantly lower in the Feb group than in the Con group.

Conclusion: Febuxostat, which is clinically used for the treatment of hyperuricemia, was effective against necrosis of the skin flap via inhibition of oxidative stress and inflammation caused by I/R injury.

1. Introduction

Flap surgery using a microsurgical technique is essential for reconstruction of skin and soft tissue defects caused by severe trauma and wide resection of malignant tumors. The success rate has increased to 95% or higher owing to accumulated knowledge of the physiology of flap necrosis as well as advancements in surgical techniques [1,2]. However, complete necrosis of the skin flap is still a challenge. Partial necrosis of the skin and soft tissues is also an important problem in flap surgery because additional treatment is needed, including debridement and re-exploration of the anastomosis. As a result, aesthetic problems at the reconstructive site can occur, including texture depression and poor contour of the skin; these issues need to be resolved to improve the quality of life of patients.

During free flap surgery, ischemia of the elevated flap cannot be avoided until revascularization of the anastomosis because nutrient vessels are cut off from blood circulation. Following ischemia, reperfusion to ischemic tissue may result in paradoxical additional tissue injury,

Abbreviations: I/R, ischemia-reperfusion; RIRR, ROS induced ROS-release; PMN, polymorphonuclear leukocytes; XO, xanthine oxidase; 8-OHdG, 8-hydroxy-2′-deoxyguanosine; SOD, superoxide dismutase; ATP, adenosine triphosphate; TNF-α, tumor necrosis factor-α; IL-1β, interleukin 1β; NADPH, nicotinamide adenine dinucleotide phosphate; NLRP3, nucleotide-binding oligomerization domain-like receptor family pyrin domain-containing 3.

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a phenomenon known as ischemia-reperfusion (I/R) injury, which plays crucial roles in necrosis of the flap [3,4]. Although the pathogenesis of I/R injury is not completely understood, the generation of reactive oxygen species (ROS) is a major contributor to I/R injury in various tissues, including the skeletal muscle, heart, and kidneys [5–7] ROS contributes to multiple processes related to cellular injury, such as energy depletion, apoptosis, necrosis, and inflammation, and is primarily generated in the mitochondria [8]. ROS induced ROS-release (RIRR) [9,10] is a novel mechanism that was recently shown to be triggered in neighboring mitochondria following the release of ROS. This process could result in increased mitochondrial dysfunction and cell death. Consequently, many pharmacological interventions to prevent ROS production and attenuate tissue damage due to I/R injury have been evaluated [4,5,11,12].

Febuxostat (2-(3-cyano-4-isobutoxy-phenyl)-4-methyl-1,3-thiazole-5-carboxylic acid is a selective inhibitor of xanthine oxidase (XO), which produces ROS via uric acid and catalyzes the reduction of oxygen to superoxide. This drug is clinically used for the treatment of patients with gout and has been reported to be useful for the treatment of I/R injury in various tissues related to ROS as well as hyperuricemia [13–15]. Additionally, several studies have shown that febuxostat inhibits inflammatory responses by reducing the levels of pro-inflammatory mediators [16,17]. Inflammation is deeply involved in flap necrosis [4,18]. Thus, febuxostat may have applications in reconstructive flap surgery via inhibition of oxidative stress and inflammation.

In this study, we analyzed whether administration of febuxostat alleviated necrosis of the skin flap in an animal model and evaluated the chemical factors associated with I/R injury, including oxidative stress markers and pro-inflammatory cytokines.

2. Materials and methods

2.1. Animals

Experiments were performed on 38 male Sprague-Dawley rats (12 weeks old) weighing 350–400 g (SLC, Hamamatsu, Japan). The animals were housed in a temperature-controlled environment and maintained on a 12/12 h light-dark cycle with food and water available *ad libitum*. The study protocol was approved by the Committee of Animal Research of Mie University (approval number: 28–13). The sample size was determined using a power analysis for an α of 0.05 and a power of 0.80 using G*Power 3.1 [19].

2.2. Administration of febuxostat

Febuxostat (kindly provided by Teijin Pharma Limited, Tokyo, Japan) was administered at a dose of 10 mg/kg/day in drinking water, as reported in previous studies [16,20]. First, we examined whether oral treatment with distilled water was appropriate for XO reduction in the skin of the abdominal wall. Ten randomly selected animals were administered drinking water containing febuxostat or no drug (n = 5 each) for 1 week. Thereafter, harvested skin was immediately frozen in liquid nitrogen for analysis of XO activity. Second, 28 animals were randomly divided into two groups as follows: the Feb group was administered febuxostat beginning 1 week before surgery, and the Con group received no drug.

2.3. Evaluation of XO activity

XO activity was measured using a commercially available kit (Sigma-Aldrich, Darmstadt, Germany). Harvested tissues were homogenized in 50 mM phosphate buffer (pH 7.4) containing 1 mM ethylenediaminetetraacetic acid (EDTA) and protease inhibitor cocktail. The homogenate was centrifuged at 15,000 rpm for 15 min, and the supernatant was used for the assay. After the addition of reaction buffer, initial ($T_{initial}$) and final (T_{final}) measurements were performed at 570 nm on a microplate reader (FluoStar Galaxy; BMG LABTECH GmbH, Ortenberg, Germany), and XO activity was calculated according to the change in measurements from $T_{initial}$ to T_{final}.

2.4. Surgical procedures

All rats were anesthetized with an intramuscular injection of a combination of ketamine (50 mg/kg) and xylazine (2.5 mg/kg). Following deep anesthesia, the abdomen and groin were shaved, and the animals were placed in a supine position. An extended epigastric island flap, measuring 9 × 6 cm^2, was raised with the base of the left superficial epigastric artery and vein (Fig. 1) [4]. A microvascular clamp with 147–245 mN (KEISEI, Tokyo, Japan) was placed on the pedicle during the ischemic period. After flap elevation and induction of ischemia, each flap was sutured back to its original location. Thereafter, ischemia was induced for 3 h by clamping the nutrient vessels. Following the I/R injury to the flap, seven pieces of full-thickness specimens were collected from the viable area of the flap using an 8-mm punch for biopsy after 24 h of reperfusion. Five pieces were immediately frozen in liquid nitrogen and preserved at −80 °C until biochemical analysis, and two pieces were immediately fixed in 4% paraformaldehyde for histopathological analysis. All rats received postoperative analgesia by subcutaneous injection of buprenorphine (0.01 mg/kg) every 12 h for 2 days after the surgery.

2.5. Assessment of the flap

The skin flaps from the Feb and Con groups (n = 6 in each group) were evaluated on day 5 after surgery. Zones of dark color were defined as necrotic, and the remaining areas were defined as viable. Flaps were stored as digital images, and the survival area was measured using image analysis software (Lumina Vision ver. 1.11; Minami Shoji Co., Fukui, Japan). Survival rates were expressed as a percentage of the total flap area (survival rate = viable area/total area × 100%) [21]. Contraction rates were also measured and calculated as a percentage of the original flap area, as follows: contraction rate = survival area on day 5/original flap area × 100) [22].

2.6. Histological analysis

The biopsied specimens were fixed for 72 h and embedded in paraffin after dehydration. The specimens were transversely cut to a thickness of 5 μm on a microtome and stained with hematoxylin and eosin (HE) to determine the infiltration of polymorphonuclear leukocytes (PMNs) and the presence of panniculus carnosus muscle injury. Quantification of injured muscle fibers in the panniculus carnosus muscle was performed using a microscope (BX50; Olympus, Japan) at 200× magnification for four sections on each slide. Then, the severity score associated with I/R injury of the skeletal muscle was determined and expressed as a percentage, obtained by dividing the number of injured myofibers by the total number of myofibers scored within all the fields [23]. The intra-observer and interobserver reliability correlation coefficients of muscle injuries at two times were excellent (κ = 0.95 and 0.88, respectively), as determined by the Cohen kappa correlation coefficient.

2.7. Immunohistochemical analysis

Serial sections from histological analysis were dewaxed in xylene and rehydrated in graded ethanol (99%–70% [v/v]) in distilled water. Endogenous peroxidase activity was quenched by incubation for 30 min in 0.3% (v/v) hydrogen peroxide in 99% methanol. Heat-induced antigen retrieval was performed in 10 mM citrate buffer (pH 6.0) using a pressure cooker (Delicio 6 L; T-FAL, Rumily, France). The sections were then left to cool at room temperature in citrate buffer. Nonspecific staining was blocked by incubating the sections in a solution of 1% bovine serum albumin for 20 min at room temperature. The bovine serum albumin was then drained off, and the specimens were incubated

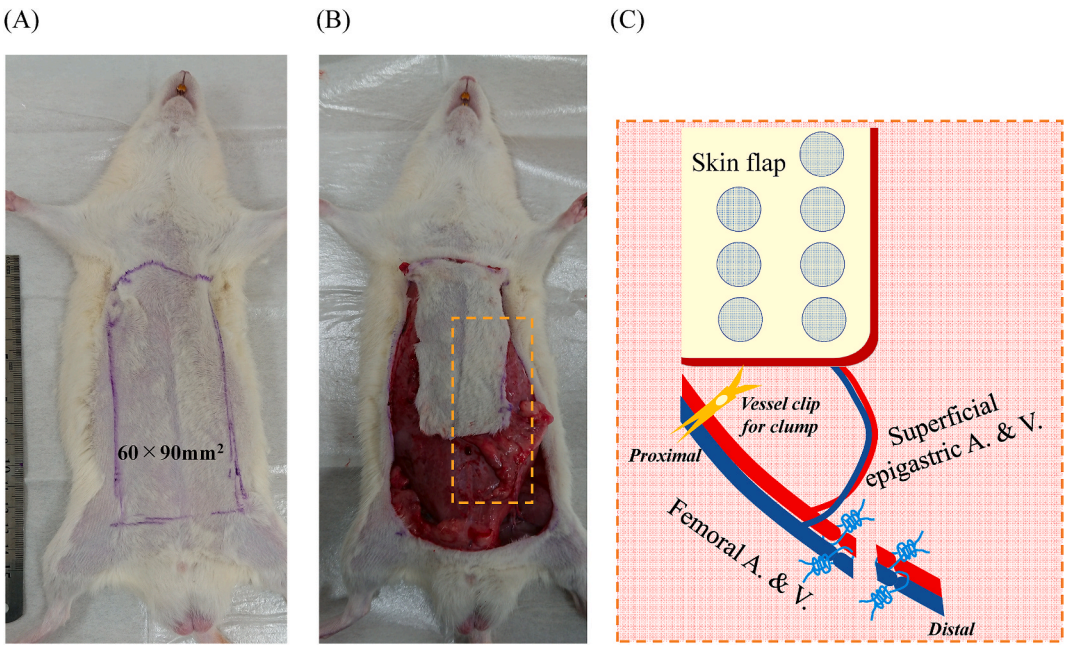

Fig. 1. The skin flap was designed to be 60 × 90 mm^2 at the abdominal wall (A). The flap was elevated with the pedicle of the superficial epigastric artery (B). The illustration (C) is an enlargement of the boxed region (orange dotted line) in (B). The femoral artery was clamped with vessel clips at the proximal part and ligated at the distal part for ischemia-reperfusion injury to the flap. In addition, seven pieces were biopsied from the pedicle side of the skin flap for histological, immunohistochemical, and biochemical analyses.

at room temperature overnight with goat monoclonal anti-8-OHdG antibodies (1:50 dilution; Japan Institute for Control of Aging, Tokyo, Japan). Bound primary antibodies were detected using secondary anti-goat IgG antibodies and anti-rabbit IgG antibodies conjugated to horseradish peroxidase (1:100; Dako Japan, Kyoto, Japan) for 1 h at room temperature. Bound antibodies were visualized by reaction with 3,3′-diaminobenzidine. Between the incubation steps, the sections were washed with phosphate-buffered saline (PBS; 3 × 5 min) to eliminate excess unbound antibodies or reagents. The sections were counterstained with hematoxylin. We counted the number of 8-OHdG-labeled nuclei and calculated the positive rate of 8-OHdG expression by dividing this number by the total number of nuclei.

2.8. Superoxide dismutase (SOD) activity

SOD activity was also determined in homogenized biopsy tissues using a commercially available assay kit (Northwest Life Science Specialties LLC, Vancouver, WA, USA), according to the manufacturer's instructions. The content was measured at 560 nm using a microplate reader.

2.9. Assessment of adenosine triphosphate (ATP) levels

An ATP assay kit (TA100; Toyo B Net, Japan) was used to measure ATP levels. Briefly, biopsied specimens were washed twice with PBS, resuspended in ATP extraction reagent after homogenization, and then centrifuged at 1000 × *g* for 10 min. The supernatants were used for the assays. ATP levels were quantified using luciferin and luciferase.

2.10. Enzyme-linked immunosorbent assay (ELISA)

The expression of tumor necrosis factor-α (TNF-α) and interleukin 1β (IL-1β) was measured using ELISA kits (R&D Systems, Minneapolis, MN, USA). For protein extraction, the tissues were homogenized and centrifuged at 12,000 × *g* at 4 °C for 20 min, and the supernatant was assayed according to the manufacturer's instructions.

2.11. Western blot analysis

The harvested tissues were frozen in liquid nitrogen, homogenized using a Cryopress (Microtech, Chiba, Japan) and mixed in RIPA buffer (10 mM Tris-HCl [pH 7.4], 1% NP40, 0.1% sodium deoxycolate, 0.1% sodium dodecyl sulfate [SDS], 0.15 M NaCl, 1 mM EDTA, 10 μg/mL aprotinin) for 1 h. The supernatants were separated by SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose membranes, and immunoblotted with primary antibodies, including polyclonal rabbit anti-TNF-α (Abcam, Cambridge, UK), polyclonal goat anti-IL-1β (Santa Cruz Biotechnology, Santa Cruz, CA, USA), and anti-β-actin antibodies (Abcam). The bands were visualized using an enhanced chemiluminescence western blotting detection system (GE Healthcare United Kingdom, Ltd., Buckinghamshire, UK) and were detected using an LAS-4000 imager (Fujifilm, Tokyo, Japan). Subsequently, the bands were analyzed using ImageJ software (National Institutes of Health, Bethesda, MD, USA) to quantify the expression levels of TNF-α and IL-1β.

2.12. Statistical analysis

Analyses were performed using EZR version 1.52 (Saitama Medical Center, Jichi Medical University, Saitama, Japan) [24]. Comparative analysis was performed using Mann-Whitney U tests. Results with *P* values less than 0.05 were considered statistically significant.

3. Results

3.1. XO activity in the skin at the abdominal wall was significantly reduced by oral application of febuxostat

The mean XO activity of normal skin at the abdominal wall was 21.1 ± 7.1 mU/mL, and the value was significantly decreased to 8.5 ± 3.8 mU/mL by oral administration of febuxostat for 1 week.

3.2. Administration of febuxostat significantly inhibited both necrosis and contraction of the extended epigastric flap due to I/R injury

Five days after surgery, partial necrosis of the skin flap was observed in all animals with or without administration of febuxostat. The mean flap survival rates were 79.2% ± 8.6% in the Feb group and 48.6% ± 16.6% in the Con group; a significant inhibitory effect of febuxostat administration was observed against necrosis induced by I/R injury in the extended epigastric flap (Fig. 2A–B). In addition, the mean rate of flap contraction in the Feb group was 38.0% ± 4.2%, which was significantly lower than that in the Con group (50.1% ± 5.7%; Fig. 2A–C). Histological analysis showed that the mean severity score due to I/R injury was 4.8% ± 3.1% on the panniculus carnosus muscle in the skin flap of the Feb group at 24 h after surgery. By contrast, the mean severity score in the Con group was 28.7% ± 12.7%; this was significantly higher than that in the Feb group (Fig. 3D–F).

3.3. Oxidative stress marker levels were significantly decreased, and antioxidant marker levels were significantly increased in skin flaps subjected to I/R injury following pretreatment with febuxostat

At 24 h after I/R injury, the XO activity in the skin flap of rats in the Con group was 240.7 ± 130.3 mU/mL. Pretreatment with febuxostat significantly inhibited XO activity to 6.5 ± 1.8 mU/mL (Fig. 4A). In addition, the ratio of 8-OHdG-expressing cells was significantly lower in the skin flap of rats in the Feb group than in that of rats in the Con group (Fig. 4B). By contrast, SOD activity was significantly higher in the Feb group than in the Con group (Fig. 4C). Furthermore, ATP levels were significantly higher in the Feb group than in the Con group (Fig. 4D).

3.4. Inflammation was reduced by pretreatment of the skin flap with febuxostat following I/R injury

Histological analysis showed a significant reduction in the number of PMNs in the Feb group compared with that in the Con group at 24 h after I/R injury (Fig. 3A–C). In ELISA, the mean expression levels of TNF-α and IL-1β were 7.9 ± 10.3 and 95.9 ± 48.8 pg/mL, respectively, in skin flaps subjected to I/R injury. Following pretreatment with febuxostat, the expression levels of these pro-inflammatory cytokines were decreased to 3.2 ± 1.4 and 40.4 ± 27.1 pg/mL, respectively (Fig. 5A and B). The difference in the expression of IL-1β between the Feb and Con groups was statistically significant. Western blot analysis showed that the mean intensities of staining for TNF-α and IL-1β normalized to β-actin expression were 0.32 ± 0.07 and 1.10 ± 0.47, respectively, in the Con group and 0.50 ± 0.19 and 0.34 ± 0.32, respectively, in the Feb group; The normalized intensity of IL-1β was significantly higher in the Con group than in the Feb group (Fig. 5C).

4. Discussion

Various agents that counteract the effects of I/R injury have been investigated for the suppression of skin necrosis in flap surgery [4,18,21,25]. Febuxostat may have applications in the treatment of this condition; indeed, the beneficial effects of this drug have been reported in I/R injury to various tissues [14–16,20,26,27]. In I/R injury, the production of ROS involves the XO, nitric oxide synthase, and NADPH oxidase systems, which show complex crosstalk mechanisms [28]. Activation of one system can lead to activation of the other systems, resulting in further augmentation of ROS levels [29,30]. Moreover, this chain reaction, including the RIRR, must be terminated to prevent unwanted and widespread cell loss [9,10]. In this study, we focused on the XO system because drugs that inhibit XO are used in the clinical setting to treat hyperuricemia. Some reports have described the usefulness of XO inhibition by allopurinol for the management of I/R injury during flap surgery [31,32]. However, allopurinol is a structural isomer of hypoxanthine, and the effects of the drug may be inadequate. Additionally, allopurinol is metabolized to oxypurinol and can cause serious side effects, such as severe dermatitis and aplastic anemia, in clinical practice. By contrast, febuxostat inhibits XO selectively; thus, its effect is remarkable, and there are no concerns related to the potential side effects of its metabolites [33]. In addition, febuxostat, like other drugs that affect XO activity, is clinically used for hyperuricemia treatment by oral administration; this administration route has the advantage of increased adherence compared with intravenous application [34,35]. Despite these benefits, no studies have evaluated the usefulness of orally administered febuxostat for treating damaged skin tissues. In this study, we showed that oral administration of febuxostat had inhibitory effects on XO activity in the abdominal skin, which was used as a donor site in

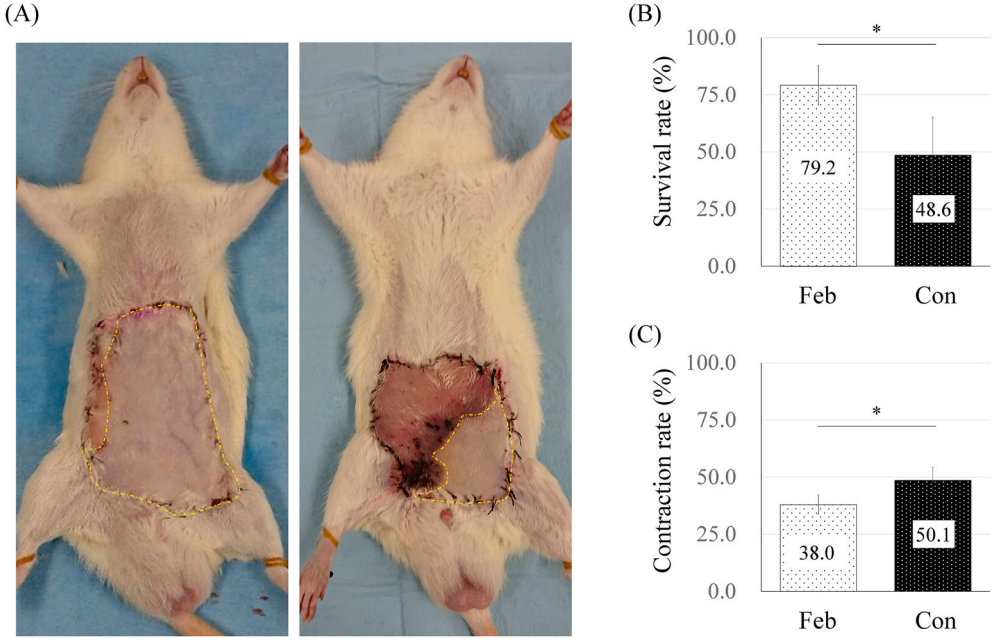

Fig. 2. (A) Appearance of skin flaps in rats administered febuxostat (left) and control rats (right). Survival rates (B) and contraction rates (C) in the skin flap of rats administered febuxostat (Feb) and control rats (Con). Values are the means ± standard deviations of observations from six rats. *$p < 0.05$.

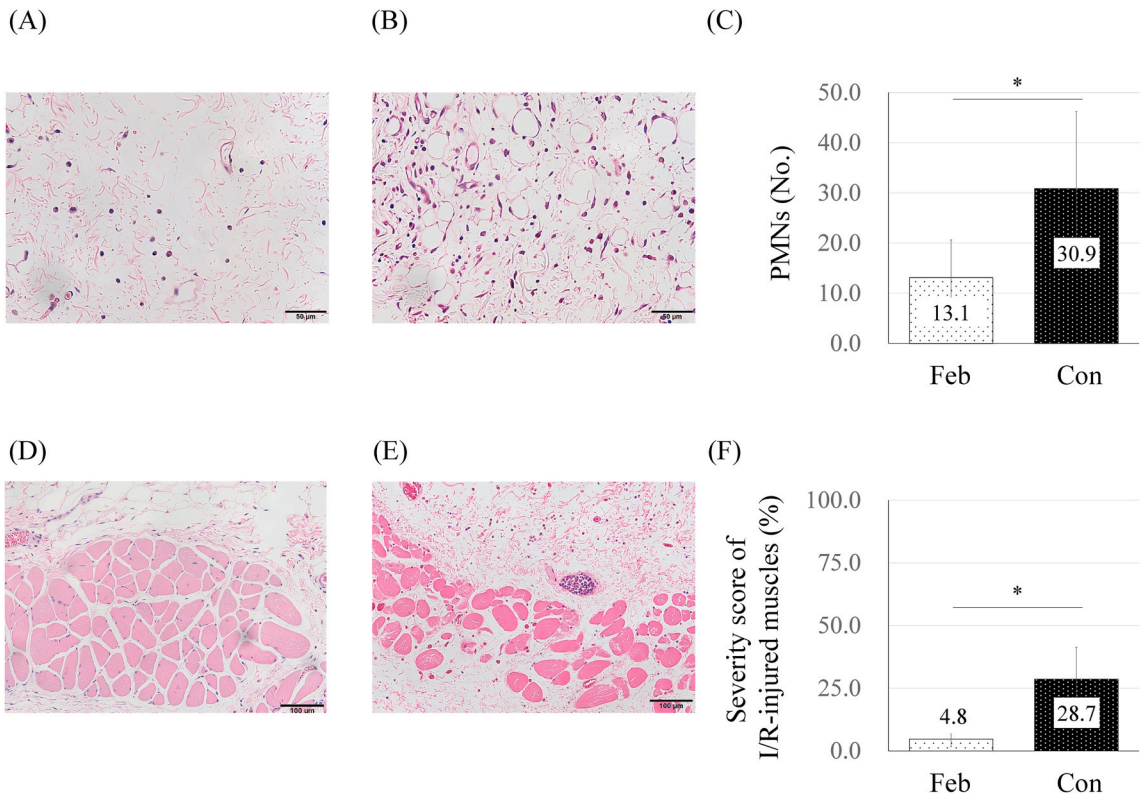

Fig. 3. Histological findings in skin flaps subjected to ischemia. Infiltration of polymorphonuclear leukocytes (A and B; original magnification, 400×; and bar size, 50 μm) in the skin flaps of rats administered febuxostat (Feb) and in controls (Con) (C). Injured muscle fibers following the ischemia-reperfusion injury (D and E; original magnification, 200×; and bar size, 100 μm) in the Feb and Con groups (F). Values are the means ± standard deviations of measurements from five rats. *$p < 0.05$.

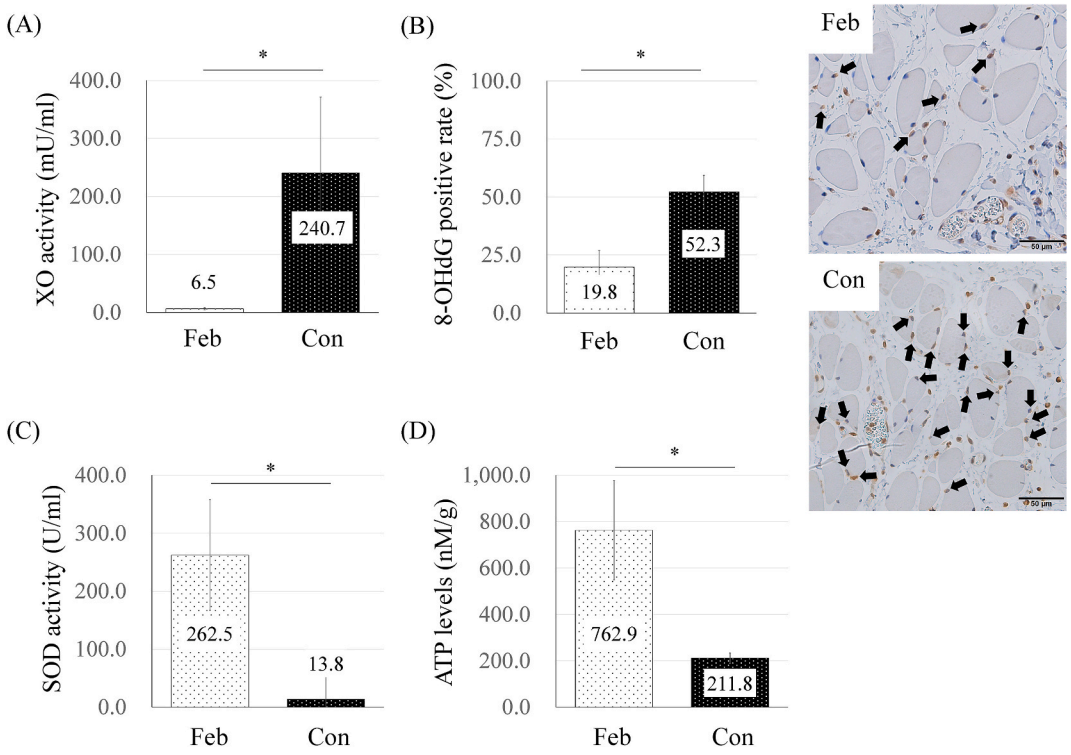

Fig. 4. Biochemical analyses. Xanthine oxidase (XO) activity (A) and 8-OHdG expression (B) in skin flaps from rats administered febuxostat (Feb) and control rats (Con). Photographs of the immunohistochemical analysis were observed under the original magnification of 400× (bar size, 50 μm). 8-OHdG-positive nuclei are marked with black arrows. Superoxide dismutase activity (C) and adenosine triphosphatase (ATP) levels (D) in the Feb and Con groups. Values are means ± standard deviations of measurements from five rats. *$p < 0.05$.

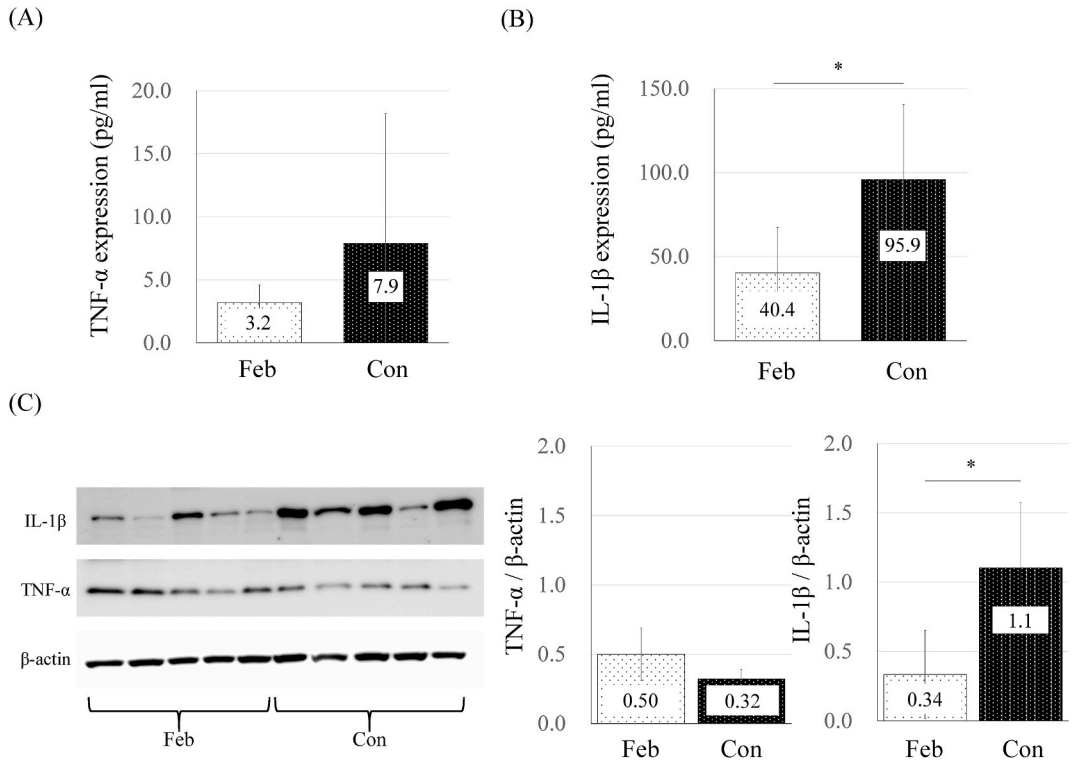

Fig. 5. Concentrations of tumor necrosis factor-α (TNF-α) (A) and interleukin 1β (IL-1β) (B) in the skin flaps of the rats administered with Febuxostat (Feb) compared with those in the control rats (Con). Western blot analysis (C) of TNF-α and IL-1β were examined, and semi-quantitation graphs reflect these staining intensities normalized to β-actin. Each value is the mean ± SD of determinations of five different rats. *$p < 0.05$.

this study and is commonly used for other reconstructive surgeries.

Pretreatment with febuxostat significantly increased survival rates and significantly decreased contraction rates in the skin flap following I/R injury in an animal model. Reconstructive surgery is one of the most effective methods applied during limb salvage following surgically wide resection of malignant tumors [36]. Partial necrosis occurs at the distal end of the skin flap, in which important tissues are frequently covered, including the bone, tendon, and vital organs. Furthermore, excessive contraction of the skin flap can lead to failure of reconstructive surgery, including dehiscence of the wound, delay to adjuvant therapy for the tumor, and aesthetic problems [37]. Additional surgery is needed for wound coverage in response to partial necrosis and for protection against infection. We believe that pretreatment with febuxostat may have therapeutic effects, improving the survival area and quality of the skin flap and thereby allowing patients to return to daily and social activities.

Previous reports have shown that many patients with malignant tumors have hyperuricemia [38–40], which is implicated in many biological processes contributing to tumor progression, including cell proliferation, neoplastic transformation, and inflammation [41]. In addition, serum levels of uric acid are negatively correlated with endothelial function in the conduit artery and microvasculature [42]. Febuxostat can alleviate necrosis of the skin flap by protecting against endothelial dysfunction in the nutrient vessels owing to the presence of uric acid in hyperuricemia generated by primary disorders as well as I/R injury.

Another mechanism involved in suppression of skin flap necrosis was inhibition of oxidative stress. Indeed, administration of febuxostat significantly reduced XO activity, mediating ROS production and oxidative stress marker (8-OHdG) expression in the skin flap and resulting in a significant reduction in myofiber injury following I/R injury. Febuxostat has been shown to protect cardiovascular tissues, the heart, kidneys, and endothelial cells from I/R injury by suppressing oxidative stress [13,14,43]. Similarly, oxidative stress contributes to I/R injury in necrosis of the skin flap [4,44]. Furthermore, in the current study, we showed that administration of febuxostat maintained high activation of SOD, which is as important intrinsic antioxidant [45]. In fact, previous studies reported that SOD administration inhibited flap necrosis after I/R injury in animal models [46–48]. There are three forms of SOD. Mitochondrial manganese-dependent SOD, also known as SOD2, plays key roles in the regulation of ROS production in I/R injury [49]. During oxidative stress, mitochondria employ oxidative stress scavenging systems, such as SOD expression, particularly SOD2 [9,49,50]. Subsequently, inactivation of SOD2 can be caused by tyrosine nitration or dityrosine formation [51,52]. Moreover, elevation of ROS leads to depletion and inactivation of SOD [53,54]. In this study, we showed that febuxostat inhibited the production of ROS by blocking XO activity, thereby increasing SOD activity. Furthermore, previous studies have suggested that febuxostat may directly activate SOD activity [16,54]. Taken together, our findings suggested that increased SOD activity may have been caused by decreased ROS levels within the skin flap after I/R injury or as a direct effect of febuxostat administration.

Increased ATP levels could enhance cellular production of antioxidants. Under ischemia, cellular ATP is gradually converted to adenosine diphosphate, adenosine monophosphate, adenosine, and hypoxanthine. Reperfusion following ischemia promotes conversion of hypoxanthine to uric acid by XO activity, leading to the generation of ROS with high ATP consumption [55]. This production of ROS is a byproduct of ATP metabolism and is independent of mitochondrial energy metabolism [56]. Febuxostat likely increased ATP levels through the salvage pathway, in which the ATP was synthesized from hypoxanthine. Unlike other drugs that inhibit XO, febuxostat was recently shown to increase ATP through the salvage pathway and protect injured organs after I/R injury [57,58]. However, energy metabolism in the mitochondria may also have a role in this mechanism. Indeed, mitochondrial function is involved in I/R injury. In a previous report, febuxostat was shown to protect endothelial cells from hypoxia via the upregulation of gene expression, leading to enhancement of mitochondrial function [59].

Taken together, these findings suggest that febuxostat could be useful for maintenance of ATP levels via both the salvage pathway and regulation of mitochondrial function.

Inhibition of inflammatory reactions, such as increased PMNs and pro-inflammatory cytokine expression, was also observed following administration of febuxostat, which is a selective XO inhibitor but not an anti-inflammatory agent. In the pathophysiology underlying tissue necrosis, inflammation has been implicated in the progression of tissue damage in I/R injury [60]. The suppressive effects of febuxostat on inflammation were likely a result of inhibition of ROS production, which is known to induce the expression of inflammation-related cytokines. In addition, injured cells can induce inflammation, resulting in further tissue damage. Thus, we suggest that administration of febuxostat may protect the skin flap against I/R injury via an indirect mechanism by suppressing inflammatory cytokines, as a result of reduced ROS generation. Moreover, considering crosstalk among other systems, PMNs are rich in phagocytic NADPH oxidase, and a significant reduction in the number of PMNs in the Feb group compared with that in the Con group was observed in histological analyses. Consequently, administration of febuxostat may affect the production of ROS via both the XO system and the NADPH oxidase system.

There were several limitations to this study. First, the outcomes of this study were obtained using 10 mg/kg/day febuxostat, which is equivalent to approximately 110–115 mg/day in the average human [61]. Although this dose is not too high and is within the therapeutic dose used for the treatment of hyperuricemia in the United States of America, only one febuxostat dose was evaluated. Furthermore, differences in XO activity among species may have contributed to the outcomes of this study. XO activity in rats is almost 40 times higher than that in humans and pigs [62]. Thus, XO inhibitors may have reduced effects in humans and pigs compared with that in rats. Indeed, in a pig model, an XO inhibitor was found to have no efficacy in protection against necrosis of the skin flap, and the mechanism of I/R injury without XO activity was discussed [63]. Additionally, in a rat model of skin flap surgery with and without prior elevation, which has been shown to reduce necrosis, the activity of XO did not vary significantly between the two groups, although the group with prior elevation had consistently lower XO activity than the group without elevation. The authors mentioned that large variations in the data may have masked minor differences between groups [64]. Further studies are required to examine the effectiveness of XO inhibitors because of variance. Second, the production of ROS was assessed based on only immunohistochemical measurement, representing an indirect measure of ROS levels. Direct measurement is a more reliable approach for determining the status of oxidative stress; however, direct measurement of ROS with high accuracy and precision is difficult owing to the short lifespan and rapid reactivity of ROS [65]. Therefore, we performed indirect measurement of ROS based on 8-OHdG staining, which is a promising alternative method for assessing damage caused to nucleic acids in cells. Further studies with direct measurement of ROS are needed to confirm our findings. Third, although administration of febuxostat inhibited the inflammatory response, particularly the production of IL-1β, upstream targets of IL-1β, such as nucleotide-binding oligomerization domain-like receptor family, pyrin domain-containing 3 (NLRP3) and caspase-1, were not investigated. A previous report showed that XO-induced ROS triggered IL-1β release and that inhibition of XO impaired IL-1β and caspase-1 secretion. Furthermore, inactivation of the NLRP3 inflammasome has been shown to play important roles in this effect [66]. Thus, analysis of caspase-1 and NLRP3 could provide important insights into the effects of febuxostat on inflammatory reactions. Finally, evaluation of flap survival was performed using only macroscopic findings, and the mechanisms of cell death were not evaluated in this study. Previous studies have reported that both necrosis and apoptosis are involved in cell death caused by I/R injury in various tissues. However, it is difficult to control cell necrosis, whereas apoptosis can be regulated by inhibiting chemical factors, including ROS and inflammatory cytokines. Modulation of apoptosis via these systems is important for tissue necrosis in I/R injury. In addition, mitochondrial function was not assessed, although mitochondria are a major source of ROS and play crucial roles in regulating apoptosis. In our study, febuxostat may have affected mitochondrial function because changes in SOD activation and ATP levels were observed following administration of febuxostat. Further assessment of mitochondrial function may improve our understanding of the mechanisms through which febuxostat mediates skin flap necrosis.

In conclusion, our findings showed that febuxostat had beneficial effects on flap surgery. Indeed, both the survival and contraction rate of the skin flap subjected to I/R injury were significantly improved in rats orally administered febuxostat compared with those in control rats. Moreover, XO activity and 8-OHdG expression were significantly reduced in animals administered febuxostat compared with those in the control rats, suggesting that febuxostat inhibited oxidative stress in the skin flap following I/R injury. By contrast, administration of febuxostat significantly elevated SOD activity and ATP levels, which were involved in regulation of mitochondrial function and the salvage pathway, potentially inhibiting uric acid metabolism. Furthermore, administration of febuxostat blocked inflammation, as demonstrated by the increased numbers of PMNs and the upregulation of pro-inflammatory cytokines. Overall, these findings provide important insights into efficacy of febuxostat in prevention of necrosis of the skin flap via inhibition of oxidative stress and inflammation caused by I/R injury.

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