

Febuxostat Reduces Muscle Wasting in Tumor-bearing Mice via Inhibition of Reactive Oxygen Species Generation

Tadashi Tsukamoto

Department of Orthopaedic Surgery, Mie university Graduate school of Medicine <https://orcid.org/0000-0002-3929-8225>

Masaya Tsujii (✉ m-t727@clin.medic.mie-u.ac.jp)

Mie university Graduate school of Medicine <https://orcid.org/0000-0002-0167-2824>

Kazuya Odake

Department of Orthopaedic surgery, Mie university Graduated school of Medicine

Takahiro Iino

Department of Orthopaedic Surgery, Mie university Graduate school of Medicine

Tomoki Nakamura

Department of Orthopaedic Surgery, Mie university Graduate school of Medicine

Akihiko Matsumine

Department of Orthopaedic Surgery, Fukui university Faculty of Medical science

Akihiro Sudo

Department of Orthopedic Surgery, Mie University Graduate school of Medicine

Research

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Abstract

Background

To analyze the effects of the administration of febuxostat, a selective xanthine oxidase (XO) inhibitor, on both *in vitro* and *in vivo* models of the waste of skeletal muscles, due to LM8 osteosarcoma cells.

Methods

C2C12 myotubes were incubated in the conditioned medium of LM8 osteosarcoma cells. At the same time, febuxostat was added at a concentration of 3 μM and 30 μM , and reactive oxygen species (ROS) was measured using 2',7'-dichlorofluorescein diacetate (DCF-DA) at 2 h and 24 h after exposure. Furthermore, an *in vivo* study was performed on male C3H mice (5 weeks old) that were subcutaneously injected with LM8 osteosarcoma cells on their backs. Febuxostat was administered in the drinking water at a concentration of 5 $\mu\text{g}/\text{ml}$ (group F5), and 25 $\mu\text{g}/\text{ml}$ (group F25). In addition, tumor-bearing mice without febuxostat (group TB) and control mice (group C) were established. At 4 weeks after the inoculation of tumor cells, body weight, wet weights of the gastrocnemius muscles, XO activity, 8-hydroxy-2'-deoxyguanosine (8-OHdG), and expression of TNF- α and IL-6 were evaluated and compared among the 4 groups.

Results

ROS generation was clearly observed in C2C12 myotubes following incubation in the conditioned medium of LM8 osteosarcoma cells. ROS generation was significantly inhibited by febuxostat administration. Furthermore, LM8-bearing mice showed significant loss of body weight and wet weight of the gastrocnemius muscles, in which XO activity, 8-OHdG, and expression of IL-6 were significantly increased compared to those in mice without injections of the tumor cells. Further, febuxostat administration not only significantly improved the body weight and wet weight of the skeletal muscles, but also reduced markers of oxidative stress and expression of pro-inflammatory cytokines. Febuxostat did not show anti-tumor effects, including inhibition of lung metastasis and improved overall survival.

Conclusion

Febuxostat, which is clinically used for treatment of hyperuricemia, is effective against the waste of the skeletal muscles induced by LM8 osteosarcoma cells.

Background

Cachexia, due to malignant tumors, is a multifactorial syndrome characterized by an ongoing loss of skeletal muscle mass (with or without the loss of fat mass), that cannot be fully reversed by conventional

nutritional support and leads to progressive functional impairment (1). This condition has been a challenging problem for orthopedists and oncologists, as cachexia has been shown to be associated with increased risk of postsurgical complications, functional impairment, and impaired responses to chemotherapy and anti-neoplastic treatments (2).

Although the pathophysiology of this disorder is unknown, we currently know that malignant tumors alter their surrounding environments via tumor-derived and host-derived paracrine factors(3). As one of the factors, reactive oxygen species (ROS) have been well known to contribute to various steps of tumor genesis, including transformation, growth promotion, and malignant progression. A large number of studies have been undertaken to study the role of ROS in the pathophysiology of malignant tumors (4–7). The role of ROS in the important signaling events of skeletal muscle atrophy due to denervation, aging, and disuse (8, 9), has also been implicated. It is reasonable that ROS could also be used as targets against skeletal muscle atrophy caused by malignant tumors as well as other disorders.

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 also catalyzes the reduction of oxygen to superoxide. This drug is clinically used for treatment of patients with gout, and has been reported to be useful for treatment of various conditions related to ROS as well as hyperuricemia (10–12). However, no reports are available confirming its effectiveness against the deleterious effects of ROS in skeletal muscles under unfavorable circumstances such as malignant tumors.

Osteosarcoma is the most common primary malignant tumor of the bone. Due to the development of combination chemotherapy regimens, the curative rate has increased to 60–70% for 5-year survival, even though 5–20% patients survive using surgical resection alone (13). A therapeutic strategy is needed for the maintenance and improvement of daily and social activities in patients with osteosarcoma. In addition, previous research has reported 5-year survival rate of 35%, even in patients with lung metastasis, which occurs in approximately half of patients with osteosarcoma, of which 30–50% of the patients die due to lung metastasis (14–16). Chemotherapy is an essential component of osteosarcoma treatment. In various types of cancers, the waste of the skeletal muscles has been reported to be associated with the quality of the skeletal muscles (17–19). Further, inhibition of the muscle wasting can be also advantageous for osteosarcoma treatment.

Furthermore, increased inflammation is a characteristic feature of the cachexic condition in numerous diseases, including malignant tumors (20). Several cytokines, including TNF- α and IL-6, were shown to be implicated as mediators of cachexic progression in both human and animal models (21, 22). The cytokines can regulate intracellular signaling, which results in muscle wasting and disrupted metabolic homeostasis in the skeletal muscles during the cachexic condition. Accordingly, the inhibition of inflammatory cytokines may be important in the control of cachexia.

The aim of this study is to analyze the effectiveness of febuxostat against cachexia, *in vitro* and *in vivo*.

Methods

Subjects

C2C12 cell lines (ATCC, Manassas, VA, USA) were used for *In vitro* study. C2C12 murine myoblasts were cultured in a growth medium consisting of low-glucose Dulbecco's Modified Eagle's Medium (DMEM) containing 10% fetal bovine serum (FBS) in a 5% CO₂ incubator at a temperature of 37 °C. After culturing until 70–80% confluence, C2C12 cells were used for the following analysis.

An *in vivo* study was performed on 68 male C3H mice aged at 5 weeks (SLC, Hamamatsu, Japan). The animals were housed in a temperature-controlled environment and maintained on a 12-hour light–dark cycle with the standard chow diet for mice and rats (Rodent Diet CA-1, CLEA Japan Inc., Tokyo, Japan); water was available *ad libitum*. The experimental protocol was approved by the Animal Ethics Research Committee at Mie University.

Collection of conditioned medium

LM8 osteosarcoma cells, which were derived from the murine osteosarcoma cell line Dunn osteosarcoma through a repetition of eight cycles of the procedure, were grown in 10% FBS at 37 °C in 5% CO₂ (23). When the confluence reached 80–90%, the cells were washed with phosphate-buffered saline (PBS) and the medium was replaced with DMEM without phenol red or serum. After 48 h, the conditioned medium was collected by centrifugation at 12,000 rpm at 4 °C for 10 min and filtration with a 0.22 µm filter to remove debris (24). The conditioned medium was preserved at -80 °C for further experiments.

Antioxidant effect of myoblasts incubated in conditioned medium

C2C12 cells were seeded at 1.0×10^4 cells/well on a 96 well-plate in DMEM with 10% FBS. After 24 h, the medium was replaced with DMEM containing 2% FBS for differentiation into the myotubes. At the same time, febuxostat was added at a concentration of either 3 µM or 30 µM for 24 h as premedication. Febuxostat was gratuitously provided by Teijin Pharma Limited (Tokyo, Japan). Following incubation in the conditioned medium for 2 and 24 h, levels of ROS were examined using 2',7'-dichlorofluorescein diacetate (DCF-DA), according to the manufacturer's instructions (Abcam, Tokyo, Japan). Myotubes were washed with PBS and fresh DMEM without phenol red, and incubated with 10 µM DCF-DA for 30 min in darkness at room temperature. The cells were immediately analyzed, and ROS levels were measured by an increase in the DCF fluorescence. DCF fluorescence was measured at an excitation wavelength of 488 nm and an emission wavelength of 519 nm.

Animal models and febuxostat administration: Male C3H mice (5 weeks old; SLC, Hamamatsu, Japan) were inoculated with 1.0×10^7 LM8 osteosarcoma cells in 0.2 ml of PBS by subcutaneous injection into the backs of the mice. Control mice were injected with 0.2 ml of PBS. Febuxostat was administered in the drinking water, from the day of inoculation with the tumor cells. Four different groups were established: group F5, tumor-bearing mice with febuxostat 5 µg/ml; group F25, tumor-bearing mice with febuxostat

25 µg/ml; group TB, tumor-bearing mice without febuxostat; and group C, control mice. At 4 weeks after tumor inoculation, mice were deeply anesthetized with an intraperitoneal injection of sodium pentobarbital (0.05 mg/g body weight), followed by body weight measurement. Then, muscles of gastrocnemius were fully excised from the attachment site of the bone, and wet weights were measured. Furthermore, lung metastasis was macroscopically observed.

Immunohistochemical analysis: The harvested gastrocnemius muscles were immediately fixed in 4% paraformaldehyde at 4 °C overnight. Then, the specimens were embedded in paraffin, and were longitudinally cut at 4-mm thickness on a microtome. Specimens were dewaxed in xylene and rehydrated in graded ethanol (99–70% (v/v)) in distilled water. Endogenous peroxidase activity was quenched by 30-min incubation 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 6L; T-FAL, Rumilly, France). The sections were then left to cool at room temperature in the 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 specimens were incubated at room temperature overnight in each antibody (1: 50). Goat monoclonal 80HdG antibodies (Japan Institute for Control of Aging, Tokyo, Japan) were used as primary antibodies. Bound primary antibodies were detected using secondary anti-goat Ig antibodies and anti-rabbit Ig antibodies conjugated to horseradish peroxidase (1:100; Dako Japan, Kyoto, Japan) for 1 h at room temperature. Bound antibodies were visualized by a reaction with 3, 3'-diaminobenzidine. Between the incubation steps, sections were washed with PBS (3 × 5 min) to eliminate excess non-bound antibodies or reagents. Sections were counterstained with hematoxylin. We counted the number of 80HdG labeled nuclei, and calculated the positive rate of 80HdG expression by dividing this number by the total number of nuclei.

XO assay

XO activity was measured using a commercially available kit (Sigma-Aldrich, Darmstadt, Germany). The harvested gastrocnemius muscles were homogenized with an assay buffer. The homogenate was centrifuged at 15,000 rpm for 10 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 by the change in the measurements from T_{initial} to T_{final} .

Measurements of inflammatory cytokine

The harvested gastrocnemius muscles were quickly frozen in liquid nitrogen, homogenized using Cryopress (Microtech, Chiba, Japan), and centrifuged, and the supernatant was preserved at -80 °C. Quantitative analysis of TNF- α and IL-6 was done using enzyme-linked immunosorbent assay (ELISA) kit (BD Bioscience, Franklin Lakes, NJ).

Statistical analyses

SPSS Statistics Software version 23.0 (IBM, Armonk, NY, USA) was used. Results are shown as mean \pm standard deviation (SD). The comparisons among groups were assessed using the Kruskal-Wallis test. Further, overall survival was determined using the Kaplan-Meier survival analysis. Values of $p < 0.05$ were considered statistically significant.

Results

ROS generation and effects of febuxostat administration following exposure of the conditioned medium from LM8 osteosarcoma cells on C2C12 myotubes

The DCF-DA assay ($n = 5$ in each group) showed that ROS were significantly upregulated to 1.9-fold in C2C12 myotubes incubated in the conditioned medium, compared to controls from the early phase (2 h). The upregulation of ROS was predominant at 24 h after incubation in the conditioned medium, where the ROS concentration increased by 3.0-fold compared to controls at 24 h. On the other hand, premedication of the febuxostat significantly inhibited upregulation of the ROS in the C2C12 myoblasts, with a dose of $30 \mu\text{M}$ at 2 h, and both doses of $3 \mu\text{M}$ and $30 \mu\text{M}$ at 24 h after exposure to the conditioned medium (Fig. 1). In addition, no differences in the ROS concentration were observed in group F5 and F25 between 2 and 24 h after incubation in the conditioned medium, even though the ROS significantly increased in the C2C12 myoblasts that lacked any added febuxostat.

Evaluation Of The Mice Inoculated With Lm8 Osteosarcoma Cells

At 4 weeks after inoculation with LM8 osteosarcoma, the body weight was significantly decreased in group TB ($24.9 \pm 1.4 \text{ g}$) compared to group C ($20.8 \pm 1.7 \text{ g}$). The wet weight of the gastrocnemius muscles of group TB mice was significantly lower ($88.3 \pm 10.5 \text{ mg}$) than that of group C mice ($118.3 \pm 10.3 \text{ mg}$). In the skeletal muscles, significant differences were observed in the XO activity as well as the ratio of 8-OHdG positive nuclei in group TB and group C. In addition, expression of pro-inflammatory cytokines in the gastrocnemius muscles of group TB were $12.1 \pm 1.3 \text{ pg/ml}$ in TNF- α and $1.1 \pm 0.3 \text{ pg/ml}$ in IL-6, which are higher than the corresponding $10.0 \pm 1.7 \text{ pg/ml}$ in TNF- α and $0.8 \pm 0.3 \text{ pg/ml}$ in IL-6 of group C.

Inhibitory effects of febuxostat on the general condition and waste of skeletal muscles in tumor-bearing mice with LM8 osteosarcoma cells

Administration of febuxostat significantly inhibited body weight loss in both F5 ($23.2 \pm 1.9 \text{ g}$) and F25 ($23.5 \pm 2.6 \text{ g}$) groups at 4 weeks, although approximately 15% loss of body weight was recorded in the group TB mice, compared to the group C mice (Fig. 2A). Administration of febuxostat in tumor-bearing mice also led to a significant improvement in skeletal muscle weight in both F5 and F25 groups (Fig. 2B). In the skeletal muscles, XO activity was significantly inhibited due to administration of febuxostat (group F5, $9.1 \pm 1.6 \mu\text{U/mg}$; and group F25, $8.1 \pm 1.5 \mu\text{U/mg}$) (Fig. 3). The ratio of 8-OHdG positive nuclei was also significantly reduced in the gastrocnemius muscles of group F5 and F25, compared to those of group TB (Fig. 4). Likewise, expression of TNF- α and IL-6 was also significantly reduced in the gastrocnemius muscles of group F5 compared to those of group TB, and IL-6 was also significantly

inhibited in the gastrocnemius muscles of group F25 (Fig. 5). TNF- α and IL-6 correlated negatively with the wet weight of the gastrocnemius muscles of all mice of all the groups (Fig. 6).

Moreover, lung metastasis was macroscopically observed in mice of all the groups. The mean survival time was 49.8, 60.5, and 56.8 days for group TB, group F5, and group F25, respectively. There were insignificant differences in overall survival among the groups (Fig. 7).

Discussion

Cachexia is a secondary condition to chronic diseases, including cancer, diabetic mellitus, chronic obstructive pulmonary disease, and chronic heart disease. Although the pathophysiology of cachexia depends on the types of diseases, a common feature of these conditions is alteration of several factors in the plasma with destructive effects on the local tissues (25). Especially in the cachexic condition, caused by malignant tumors, tumor-derived factors, including inflammatory cytokines and exosome, have been reported to be involved in waste of the skeletal muscles, as a result of imbalance of the degradation and/or synthesis on myofibrillar proteins (26). Oxidative stress is one of the most common mechanisms in the signaling pathway of the imbalance under the cachexic condition in various diseases, and one of the important characteristics is increased ROS levels in the skeletal muscles of the cachexic patients (25, 27). This study showed that secretion products from LM8 osteosarcoma cells had a harmful effect on the C2C12 myotubes due to upregulation of ROS.

The administration of the antioxidants is considered as one of the therapeutic strategies for the treatment of waste of the skeletal muscles under the cachexic condition. In fact, a number of studies have reported the clinical uses of antioxidants in the treatment of the waste of skeletal muscles in various conditions (28–32). In this *in vitro* study, it was demonstrated that administration of febuxostat significantly reduces the generation of ROS in C2C12 myoblasts of LM8 osteosarcoma cells incubated in conditioned medium. Febuxostat is a clinically used drug for the treatment of hyperuricemia and could concomitantly suppress the generation of ROS in inhibition of xanthine oxidase. Thus, febuxostat is likely to have an ability of protection for skeletal muscles against deteriorated effects due to oxidative stress by tumors. In fact, our *in vivo* study clearly showed that the administration of febuxostat significantly inhibited the loss of body weight and wet weight of the gastrocnemius muscle in the mice injected with LM8 osteosarcoma cells. The mechanism seemed to be a direct inhibition of oxidative stress due to the increase in ROS generation in the skeletal muscles, since administration of febuxostat significantly reduced XO activity and expression of oxidative stress markers (8-OHdG) in the skeletal muscles of the LM8-induced mice.

The reduction of inflammatory cytokine expression was also observed in the skeletal muscles of this model by the administration of this drug, which is a selective XO inhibitor but not an anti-inflammatory agent. The inflammatory cytokine has been implicated as a critical mediator for progression of the cachexic condition of the skeletal muscles (33, 34). TNF- α can promote protein degradation through the transcription of ubiquitin proteasome E3, MurF1, and atrogin-1, of which expression had been shown in the skeletal muscles of cachexic individuals and the animal models of cachexia, which results in the

degradation of proteins in the skeletal muscles (21). IL-6 was also implicated as a critical regulator of inflammation-induced wasting of the skeletal muscles during cachexic progression (35). In fact, we found a significant correlation between reduction of wet weight of the gastrocnemius muscles and expression of TNF- α and IL-6. Besides, several studies showed anti-inflammatory effects by the administration of febuxostat in various organs, including liver, lung, and kidneys (36, 37). In these studies, suppressive effects on inflammation was likely to be a result of the inhibition of ROS production, which is known to induce the expression of inflammation-related cytokines. In addition, injured cells could induce the inflammation, resulting in further damage to the tissues. Thus, we suggested that the administration of the febuxostat could also protect the skeletal muscles exposed to secreted products from the LM8 osteosarcoma cells via an indirect mechanism by suppression of the inflammatory cytokines, as a result of reduced ROS generation.

The use of LM8 osteosarcoma cells was a major limitation of this study for the induction of cachexic condition, since this cell line has the characteristic of a high ability of pulmonary metastasis. Therefore, it was unclear whether ROS generation in this study was induced by oxidative stress due to pulmonary metastasis or secreted products from tumor cells. However, regardless of pulmonary metastasis in all mice inoculated with LM8 osteosarcoma cells, the mice were inhibited from experiencing a reduced body weight and wet weight of the gastrocnemius muscle by administration of the febuxostat. In addition, pulmonary metastasis as well as cachexia were observed in patients of the terminal phases. We believe that this study shows the effectiveness of the febuxostat for the quality of life of patients who are at advanced stage of malignant tumors, even though the lung metastasis and overall survival rates were ineffective.

In conclusion, febuxostat was not only effective against ROS generation in C2C12 myotubes incubated in the conditioned medium of LM8 osteosarcoma cells, but also inhibited the loss of the body weight and wet weight of the gastrocnemius muscles in the mice following the subcutaneous injection of LM8 osteosarcoma cells. The skeletal muscles in the LM8 injected mice showed significant increase in the oxidative stress markers and pro-inflammatory cytokines' expression, while administration of febuxostat significantly inhibited the increase of the expressions in skeletal muscles. Febuxostat could be a useful treatment for waste of the skeletal muscles caused by LM8 osteosarcoma, even though the drug has been ineffective for tumorigenesis.

Abbreviations

DCF: 2',7'-dichlorofluorescein

DCF-DA: 2',7'-dichlorofluorescein diacetate

DMEM: Dulbecco's Modified Eagle's Medium

FBS: fetal bovine serum

PBS: phosphate-buffered saline

ROS: reactive oxygen species

TB:tumor bearing

XO: xanthine oxidase

8OHdG:8-hydroxy-2'-deoxyguanosine

Declarations

Ethics approval and consent to participate

All procedures involving animals were approved by the committee of animal research at Mie University.

Consent for publication

Not applicable

Availability of data and material

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Funding

Not applicable

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

TT and MT designed the research and wrote the paper. TI and TA participated in the experimental design and techniques. All authors read and approved the final manuscript.

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Figures

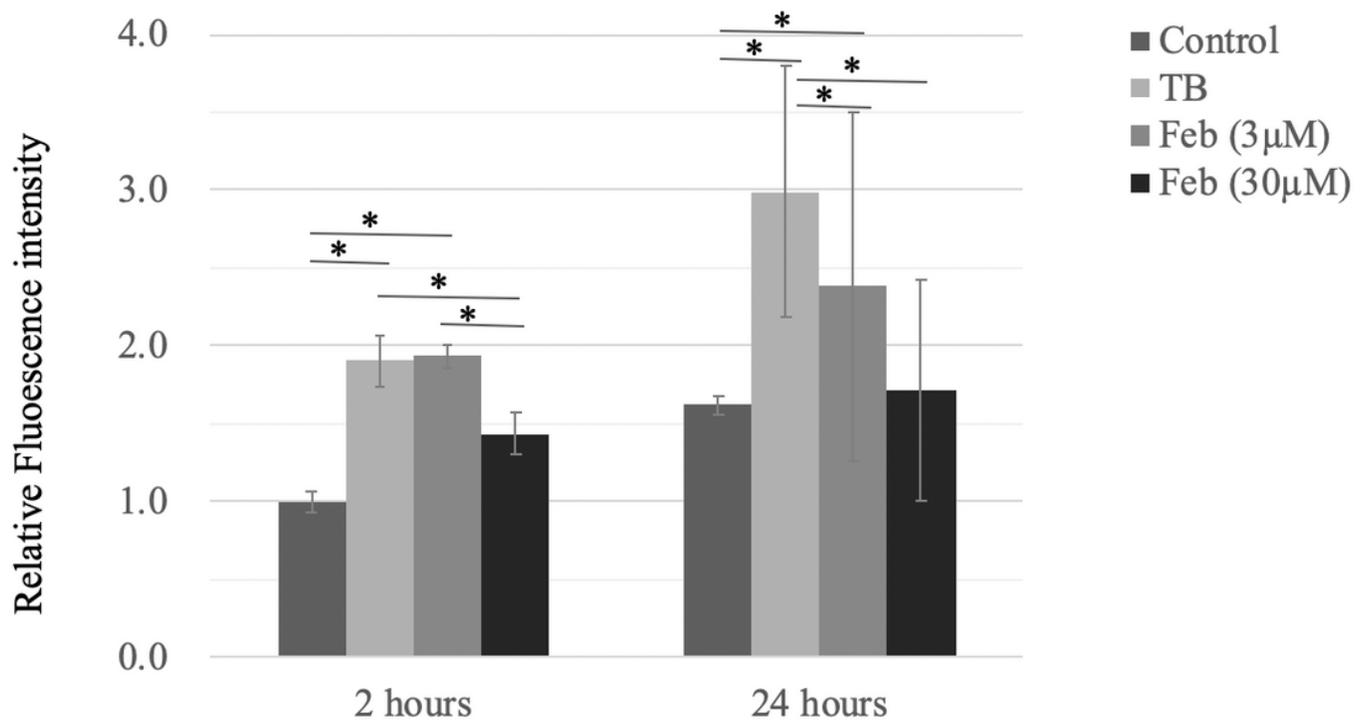


Figure 1

A 2',7'-dichlorofluorescein diacetate (DCF-DA) assay on C2C12 myotubes. ROS generation was significantly upregulated in C2C12 myotubes at 2 h after exposure to the conditioned medium for the LM8 osteosarcoma cells, while ROS generation was significantly inhibited by the addition of febuxostat with a dose of 30 µM. In addition, ROS generation was predominant at 24 h, which is when the febuxostat doses were effective at both levels (3 µM and 30 µM). LM8: addition of conditioned medium from LM8 osteosarcoma cells. Feb: administration of febuxostat. * p<0.05 (among each group).

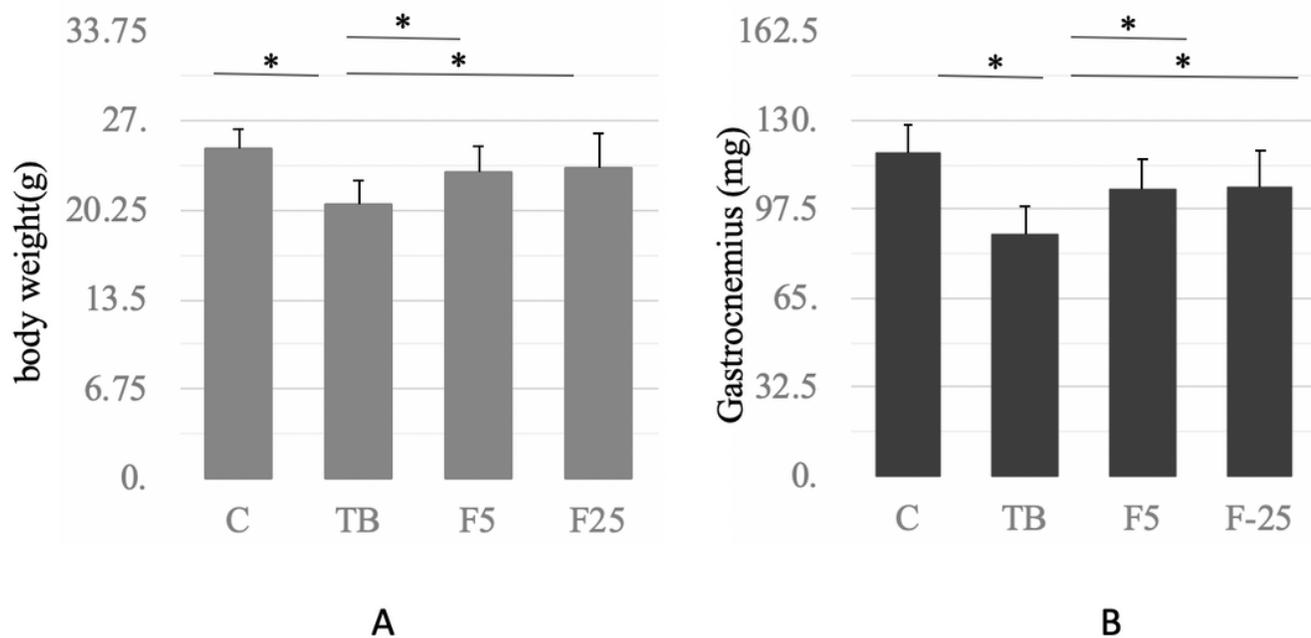


Figure 2

(A) Body weight, (B) Wet weight of the gastrocnemius muscles. Administration of febuxostat with doses of 5 $\mu\text{g/ml}$ and 25 $\mu\text{g/ml}$ significantly inhibited reduction of body weight and muscle wet weight due to the inoculation of the LM8 osteosarcoma cells. Group TB, LM8-bearing mice without febuxostat; group F5, LM8-bearing mice with febuxostat 5 $\mu\text{g/ml}$; group F25, tumor-bearing mice with febuxostat 25 $\mu\text{g/ml}$; and group C, control mice. * $p < 0.05$ (among each group).

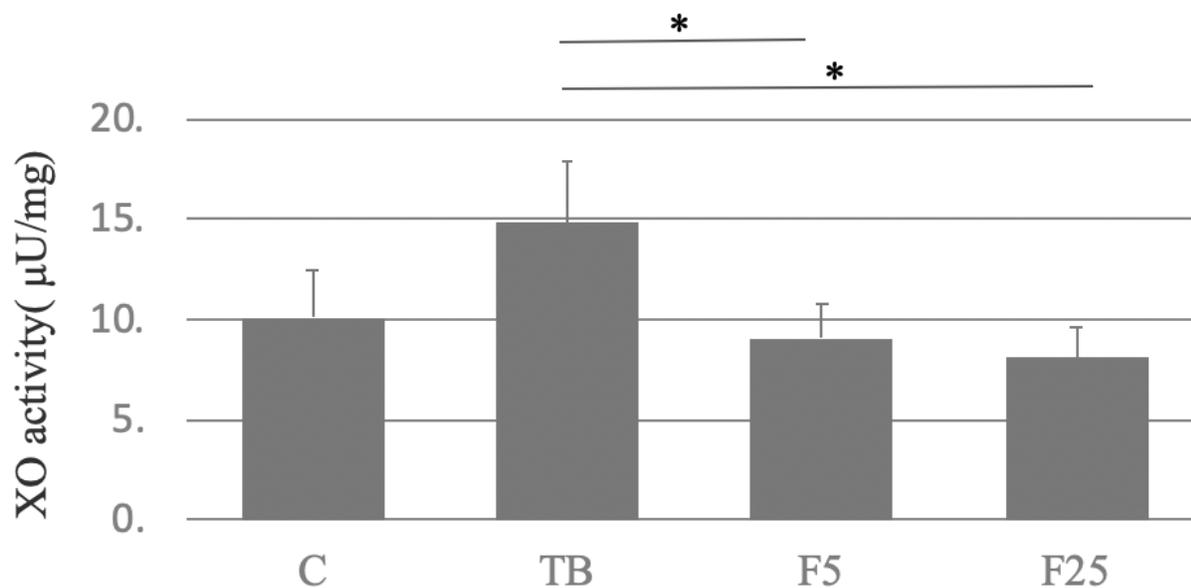


Figure 3

Xanthine oxidase (XO) activity in the muscles. The XO activity was significantly increased in the skeletal muscles of group TB compared to group C. The activity was clearly reduced by the administration of febuxostat. Group TB, LM8-bearing mice without febuxostat; group F5, LM8-bearing mice with febuxostat 5 µg/ml; group F25, tumor-bearing mice with febuxostat 25 µg/ml; and group C, control mice. * p<0.05 (among each group).

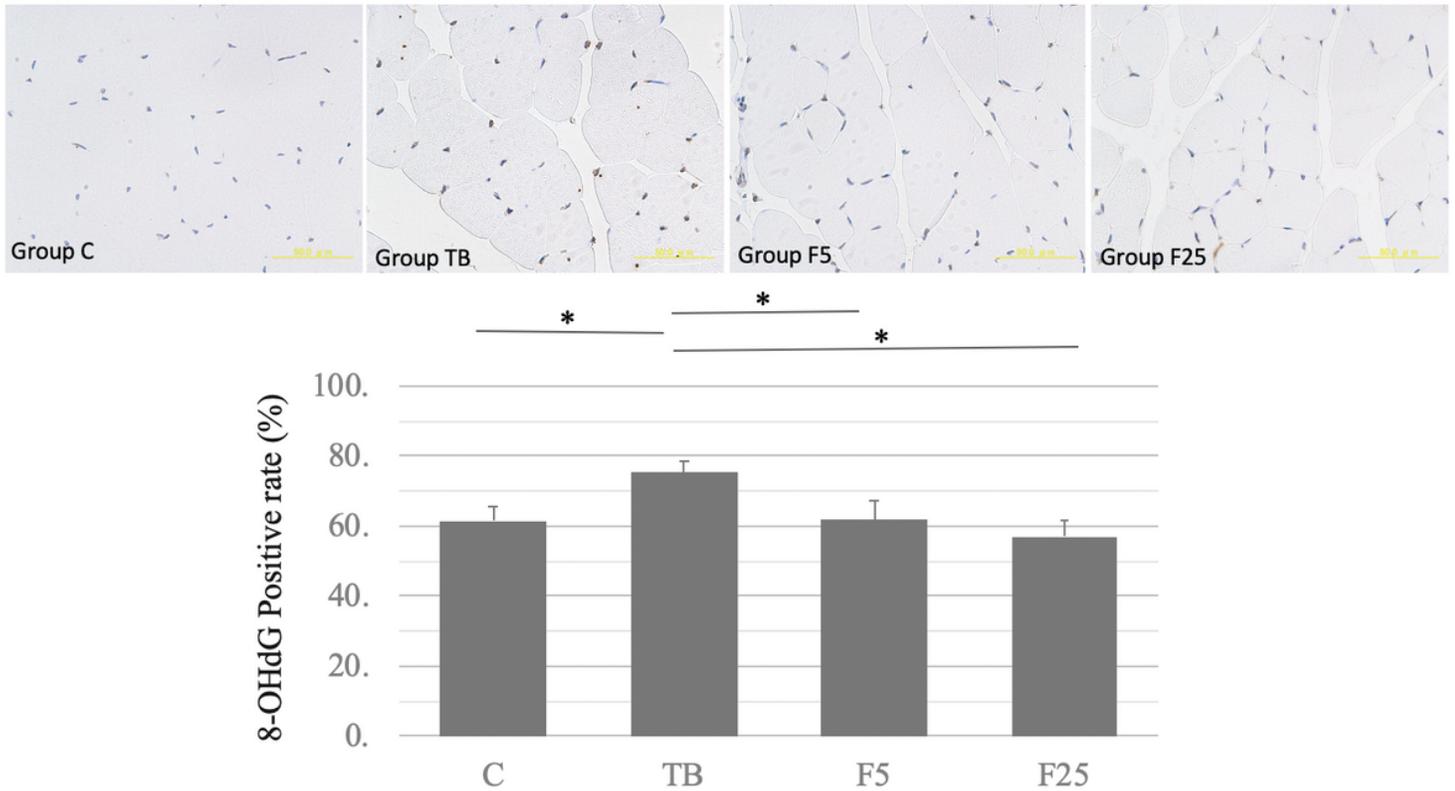
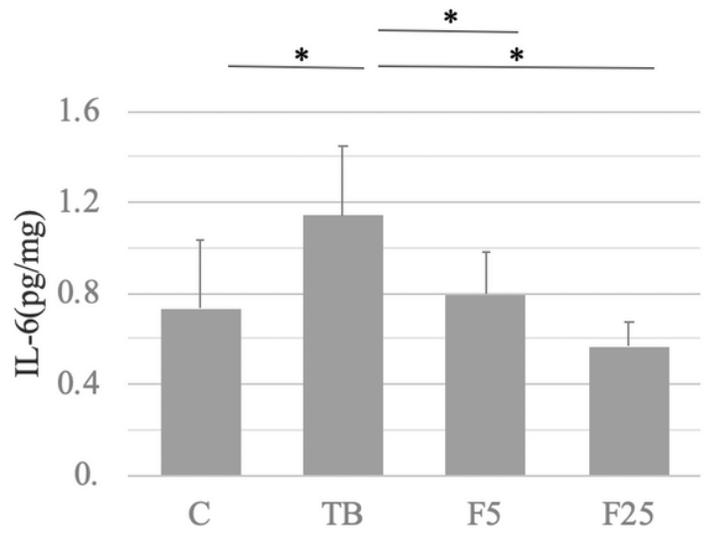
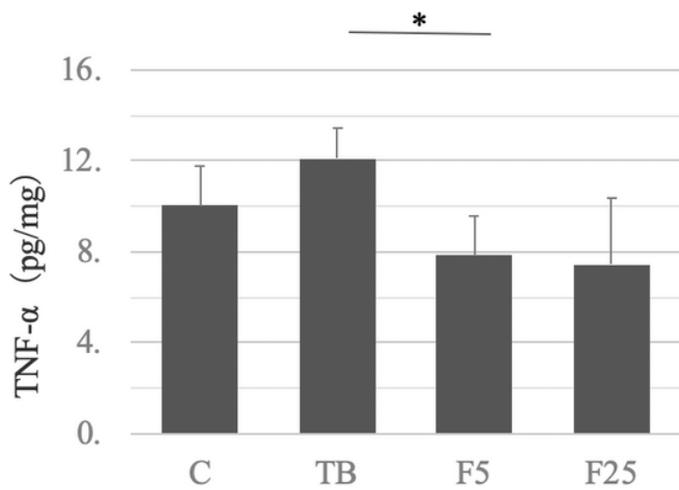


Figure 4

Immunohistochemical analysis of 8-OHdG on gastrocnemius muscles. The pictures showed that the stained nuclei were markedly increased in group TB compared to group C. In fact, the number of stained nuclei was significantly increased due to inoculation of the LM8 osteosarcoma cells, whereas the number was decreased in the skeletal muscles of group F5 and group F25 compared to group TB. Group TB, LM8-bearing mice without febuxostat; group F5, LM8-bearing mice with febuxostat 5 µg/ml; group F25, tumor-bearing mice with febuxostat 25 µg/ml; and group C, control mice. * p<0.05 (among each group).

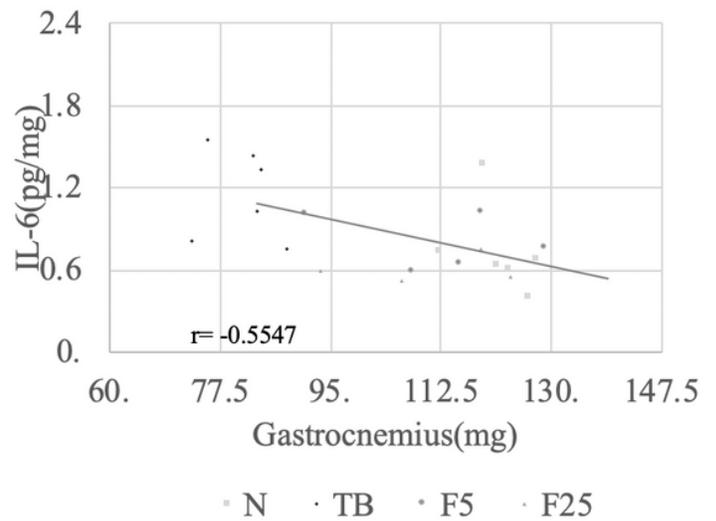
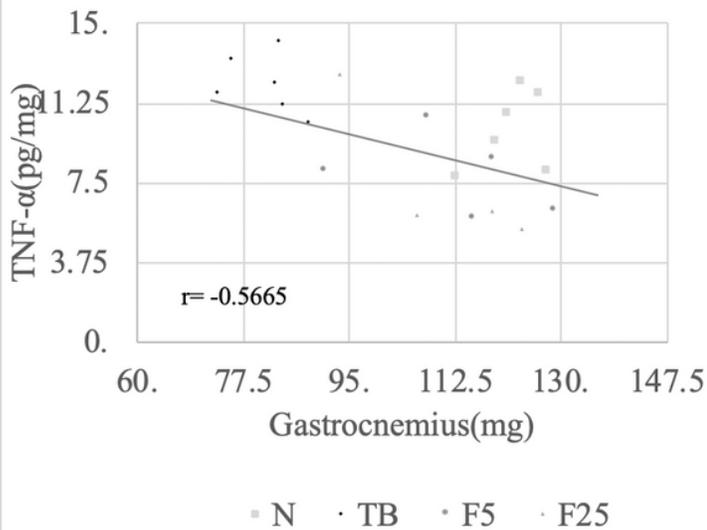


A

B

Figure 5

TNF- α (A) and IL-6 (B) in the gastrocnemius muscles. TNF- α in the skeletal muscles were significantly reduced in group F5, along with the tendency in group F25. IL-6 was also upregulated due to the injection of LM8 osteosarcoma cells, and was significantly inhibited in both groups F5 and F25. Group TB, LM8-bearing mice without febxostat; group F5, LM8-bearing mice with febxostat 5 μ g/ml; group F25, tumor-bearing mice with febxostat 25 μ g/ml; and group C, control mice. * $p < 0.05$ (among each group).



A

B

Figure 6

(A) The wet weight of muscle and TNF- α , (B) the wet weight of muscle and IL-6 of each gastrocnemius collected from the mice of all groups are depicted in the scatter plot. TNF- α and IL-6 correlated negatively with the wet weight of the gastrocnemius muscles in all mice across groups. The correlation coefficient for TNF is -0.5665, and -0.5547 for IL-6.

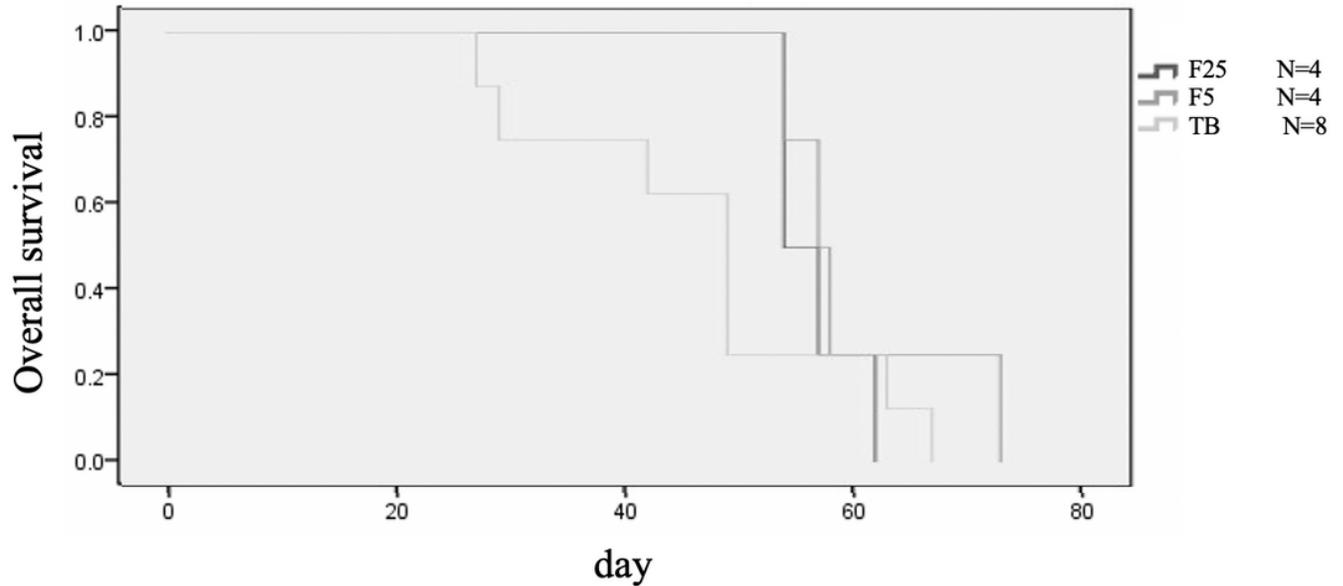


Figure 7

Overall survival of the mice with inoculation of LM8 osteosarcoma cells. Mean duration values of the overall survival were 49.8, 60.5, and 56.75 days in group TB, group F5, and group F25, respectively. No differences among groups were found. Group TB, LM8-bearing mice without febuxostat; group F5, LM8-bearing mice with febuxostat 5 $\mu\text{g}/\text{ml}$; and group F25, tumor-bearing mice with febuxostat 25 $\mu\text{g}/\text{ml}$