Effect of topiroxostat on reducing oxidative stress in the aorta of streptozotocin-induced diabetic rats

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Abstract

Xanthine oxidoreductase exists both intracellularly and extracellularly and induces vascular injury by producing reactive oxygen species (ROS). Here, we investigated the effects and mechanism of action of topiroxostat, a xanthine oxidase inhibitor, on ROS using an animal model of type 1 diabetes with persistent hyperglycemia. Six-week-old male Sprague–Dawley rats were administered 50 mg/kg streptozotocin to induce diabetes; at 8 weeks of age, animals were administered topiroxostat (0.3, 1, or 3 mg/kg) for 2 weeks through mixed feeding after which the aorta was sampled. The production of superoxide, a type of ROS, was measured by chemiluminescence and dihydroethidium staining. Cytotoxicity was evaluated by nitrotyrosine staining. Topiroxostat at 3 mg/kg significantly decreased blood urea nitrogen, e-selectin, urinary malondialdehyde, and the urinary albumin/creatinine ratio compared with the streptozotocin group. Superoxide production by xanthine oxidase anchored to the cell membrane was significantly decreased by topiroxostat at both 1 mg/kg and 3 mg/kg compared with the streptozotocin group. Dihydroethidium staining revealed no significant effect of topiroxostat administration on superoxide production. The fluorescence intensity of nitrotyrosine staining was significantly suppressed by 3 mg/kg topiroxostat. Topiroxostat was found to inhibit the production of ROS in the thoracic aorta and suppress vascular endothelial damage. The antioxidant effect of topiroxostat appears to be exerted via the inhibition of anchored xanthine oxidase.

Keywords: topiroxostat, diabetic vascular disease, xanthine oxidase, oxidative stress
Introduction

People with diabetes are at increased risk of developing cardiovascular disease (CVD) and are also more likely to die from CVD.1-3 Oxidative stress is thought to be a cause of vascular damage in diabetic patients.4 Factors that instigate oxidative stress include increased production of progressive glycation end products, activation of the polyol pathway, increased protein kinase C and xanthine oxidase (XO), and uncoupling of endothelial nitric oxide synthase.4-7 Thus, suppressing oxidative stress is important for preventing the progression of vascular disease, although effective strategies for this are lacking.

Xanthine oxidoreductase (XOR) catalyzes the rate-limiting steps in the syntheses of xanthine from hypoxanthine and uric acid from xanthine.8 Under physiological conditions, XOR exists primarily as xanthine dehydrogenase (XDH), which uses nicotinamide adenine dinucleotide as an electron acceptor. However, under certain conditions, XDH is converted to XO through limited proteolysis. XO uses molecular oxygen as an electron acceptor to produce superoxide anion.9 XO has also been implicated in endothelial dysfunction, hypertension, and heart failure through the production of reactive oxygen species (ROS).10 XO exists both intracellularly and extracellularly, with the latter found in both a free form, which is present in circulating blood, or anchored, which is bound to proteoglycans on the surface of endothelial cells.11-13 The contributions (if any) of each XO form to disease pathogenesis are unknown.

The administration of allopurinol to cholesterol-loaded rabbits was shown to improve the vascular endothelial cell-dependent diastolic response, independent of serum uric acid concentration.14 It was also reported that XOR activity in pathological coronary arteries is increased in patients with coronary artery disease compared with that in healthy controls.15 These reports suggest that excessive activation of XOR is involved in the abnormal function of vascular endothelial cells in large arteries.

While topiroxostat, an XOR inhibitor, has been used to treat hyperuricemia and gout,
secondary effects other than lowering of uric acid have been reported. Administration of either febuxostat or topiroxostat to db/db mice resulted in the suppression of albuminuria, but only topiroxostat was found to have a dose-dependent effect which also correlated with plasma XOR activity. Plasma XOR can become anchored to proteoglycan on the surface of endothelial cells, leading to the local production of superoxide, which reacts with nitric oxide (NO) in the vasculature to produce cytotoxic peroxynitrite.

Topiroxostat is hypothesized to improve vascular endothelial function by inhibiting circulating XOR, anchored XOR, and vascular endothelial intracellular XOR; however, the extent of its involvement with each XOR type is unknown.

The aim of this study was to determine the effect and mechanism of action of topiroxostat on ROS in a rat model of type 1 diabetes. It was previously reported that XO was increased or activated in a streptozotocin-induced type 1 diabetic rat model, and ROS production was increased in a large blood vessel, the thoracic aorta. Therefore, we used the thoracic aorta of rats in this study.

Materials and Methods

Experimental Animals

This study was performed in accordance with the Guidelines for the Conduct of Animal Experiments issued by Nagoya City University and was approved by the Committee on the Ethics of Animal Experiments within that institution (H29-P-02). Sprague–Dawley rats were obtained from Japan SLC, Inc (Shizuoka, Japan) (total number used = 35). The rats were fed standard laboratory chow (CRF-1; Oriental Yeast Co., Tokyo, Japan) and were housed individually in a temperature- and light-controlled room (approximately 23 ± 2 °C, 12 h light/dark cycle) with free access to water. At 6 weeks of age, diabetes was induced by a single intravenous (i.v.) injection of 50 mg/kg streptozotocin prepared in saline solution; control rats
received a similar volume of saline solution (i.v.). The rats were monitored for the development of hyperglycemia for the next 7 days. At 7 weeks of age, blood was drawn from the tail vein to measure fasting blood glucose levels. Diabetic rats were divided into four groups matched for body weight and fasting blood glucose: control, streptozotocin, and three topiroxostat treatment groups (0.3, 1, or 3 mg/kg/day) based on the regime used in a previous report. From 8 weeks of age, the rats received either drug-containing chow or vehicle chow for 2 weeks. Prior to tissue collection, the rats were fasted overnight, anesthetized with sevoflurane (Maruishi Pharmaceutical Co., Osaka, Japan), and euthanized via exsanguination.

Blood samples were withdrawn from the heart and centrifuged at 3,000 g and 4 °C for 15 min to separate the plasma. The plasma samples were stored at −80 °C prior to use in assays. The aorta was immediately excised and placed in Krebs solution, and the connective tissue was removed. The aorta was stored in Krebs solution at 4 °C until use.

**Biochemical Analysis**

Whole blood was used for measuring glucose concentrations. Fasting blood glucose was measured by the glucose oxidase method using a glucose test meter (Sanwa Kagaku Kenkyusho Co., Ltd., Nagoya, Japan).

Blood urea nitrogen (BUN) in the plasma and creatinine concentration in the urine were measured with L-type Wako UN (catalog number: 416-55192, 412-55292, 419-41691, Fujifilm, Osaka, Japan) and L-type Wako Cre (catalog number: 469-07594, 465-07694, 413-41591, Fujifilm, Osaka, Japan) assays, respectively. Urinary albumin and malondialdehyde (MDA) concentration were measured using an enzyme-linked immunosorbent assay (ELISA) kit (catalog number: E111-125, Bethyl Laboratory, AL, USA) and an MDA assay kit (catalog number: NWK-MDA01, Northwest Life Science Specialties LLC, OR, USA), respectively. Urinary MDA and albumin concentrations were evaluated in ratio to urinary creatinine.
E-selectin concentrations were measured using an ELISA kit (catalog number: ELR-Eselectin, RayBiotech Life, GA, USA).

**Purine Bodies and Drug Concentrations**

Purine bases were measured using a previously described method.\(^{18}\) Plasma was added to Tris buffer (pH 8.5) containing NaCl, with \[^{15}\text{N}_2\]-xanthine and \[^{15}\text{N}_2\]-uric acid as internal standards, and then immediately heated at 95 °C for 5 min. The resulting suspensions were centrifuged at 15,000 g and 4 °C for 10 min. The supernatants were filtered through an ultrafiltration membrane (Amicon Ultra-0.5 centrifugal filter devices, 3 K, Millipore, MA, USA) and analyzed by LC/MS (LTQ-Orbitrap, Thermo Scientific, Kanagawa, Japan). To determine topiroxostat concentration, plasma was added to acetonitrile containing F10460 as internal standards and filtered through a membrane filter (Captiva ND plate, 0.22 mm, Agilent, CA, USA). The filtrate was evaporated and reconstituted with 10% methanol for LC/MS/MS analysis (TSQ-Quantum, Thermo Scientific).

**XOR Activity in Plasma**

XOR activity was measured using a previously described method.\(^{18}\) Plasma was added to a mixture containing \[^{15}\text{N}_2\]-xanthine (0.8 mmol/L), nicotinamide adenine dinucleotide (1 mmol/L), and oxonate (0.013 mmol/L) in 20 mmol/L Tris buffer (pH 8.5) and incubated at 37 °C for 30 min. Next, \[^{13}\text{C}_2,^{15}\text{N}_2\]-uric acid was added as internal standard and the mixture was heated for 5 min at 95 °C and then centrifuged at 15,000 g and 4 °C for 10 min. Supernatants were filtered through ultrafiltration membranes (Amicon Ultra-0.5) and the \[^{15}\text{N}_2\]-uric acid concentration was measured by LC/MS (LTQ-Orbitrap). Activity was expressed as \[^{15}\text{N}_2\]-uric acid nmol/min/mg protein.
Anchored XOR Activity

The effect of topiroxostat on anchored XOR activity was investigated by chemiluminescence as previously described.\textsuperscript{14} Aortas (3-mm-long ring segments) were examined using the superoxide-sensitive chemiluminescent dye 8-amino-5-chloro-7-phenylpyrrole[3,4-d] pyridazine-1,4-(2H,3H) dione sodium salt (L-012, Wako Pure Chemical Industries Ltd., Osaka, Japan) and a luminometer (Glomax®-Multi Detection System, Promega, WI, USA). Briefly, aortas were equilibrated for 30 min at 37 °C in Krebs solution gassed with 95% O\textsubscript{2}/5% CO\textsubscript{2}. A scintillation vial containing modified Krebs–HEPES buffer solution with L-012 (100 µM) and xanthine (50 µM) was placed into the luminometer to determine the background signal. Arterial segments were then placed individually into scintillation vials and chemiluminescence signals were traced continuously for 30 min at 37 °C. Arterial segments were retrieved, dried at 90 °C for 24 h, and weighed. The blank signal was subtracted from the chemiluminescence signal in the presence of sample, and the difference was used to calculate the amount of luminescence per minute. Chemiluminescence was expressed as a ratio to the standard (5 µU/mL XO) and corrected for dry weight.

The effects of heparin (1000 U/mL) and superoxide dismutase (SOD; 200 U/mL, Wako Pure Chemical Industries Ltd.) were evaluated to confirm whether these agents inhibited the effects of ROS.

Intracellular XOR Activity

The effect of topiroxostat on intracellular XOR activity was investigated by oxidative fluorescence staining with dihydroethidium, as previously described.\textsuperscript{24,25} Segments of aorta stored in Optimal Cutting Temperature (OCT) compound were used for these experiments. A frozen segment of embedded aorta was cut into 8-µm thicknesses under a cryostat and then mounted on MAS-coated glass slides. Arterial sections were incubated with Krebs–Henseleit
buffer (pH 7.4) in a CO₂ incubator for 20 min at 37 °C in the presence or absence of xanthine (50 µM) and then incubated with Krebs–Henseleit buffer (pH 7.4) containing dihydroethidium (1 µM) in a CO₂ incubator for 30 min at 37 °C. Images were obtained using a confocal laser scanning microscope system (LSM 510, Carl Zeiss, Jena, Germany). Fluorescence intensity in each section was measured from eight randomly selected regions (10×10 pixels) and averaged using digital image analyzer software (ImageJ, National Institutes of Health, Bethesda, MD, USA).

**Cytotoxicity Assay**

*Preparation of Tissue Sections*

Aortas cut into 3-mm sections were fixed with 4% paraformaldehyde and incubated in 10% sucrose and 15% sucrose (4 h each), followed by overnight incubation in 20% sucrose. Specimens were then embedded using OCT compound and snap-frozen in liquid nitrogen. Frozen segments of embedded aorta were cut into 6-µm thicknesses under a cryostat and mounted on MAS-coated glass slides (Matsunami Glass, Osaka, Japan).

*Fluorescent Immunostaining*

Antigen activation was performed using HistoVT One solution (Nacalai Tesque, Kyoto, Japan). Sections were rinsed with phosphate-buffered saline (PBS) and blocked with Blocking One Histo (Nacalai Tesque) before overnight incubation at 4 °C with two primary antibodies: mouse monoclonal antibody against 3-nitrotyrosine (3-NT, SC32757; Santa Cruz Biotechnology 1:50) and rabbit monoclonal antibody against CD31, an endothelium marker (Abcam, ab222783, 1:100). The next day, sections were rinsed with PBS and incubated for 1 h at room temperature with the secondary antibodies goat anti-mouse IgG H&L (Alexa Fluor® 488 ab150117 1:500) to track 3-NT (green) and goat anti-rabbit IgG H&L (Alexa Fluor 647...
(antibody ab150083 1:500) to track CD31 (red). For the secondary antibody-only control, PBS was used instead of primary antibody. Finally, sections were mounted and stained with 4,6-diamidino-2-phenylindole (DAPI) using the Vector TrueVIEW Autofluorescence Quenching Kit with DAPI (Vector Laboratories, CA, USA). Images were obtained using a confocal laser scanning microscope system (LSM 800, Carl Zeiss). Fluorescence intensity in each section was measured from four randomly selected regions (overlapping areas of CD31 and 3-NT fluorescence) and averaged using ImageJ software.

**Chemicals**

**Drugs and Materials**

Topiroxostat was provided by Sanwa Kagaku Kenkyusho (Inabe, Japan). XO (from cow’s milk) and streptozotocin were purchased from Sigma Aldrich (St. Louis, MO, USA). SOD (from bovine erythrocytes) and L-012 were purchased from Wako Pure Chemical Industries. All other reagents used were commercially available.

Dihydroethidium was purchased from Molecular Probes (OR, USA). Mouse monoclonal antibody against 3-NT was purchased from Santa Cruz Biotechnology (CA, USA). Rabbit monoclonal antibody against CD31, goat anti-mouse IgG H&L (Alexa Fluor 488 ab150117), and goat anti-rabbit IgG H&L (Alexa Fluor 647 ab150083) were purchased from Abcam (Cambridge, UK).

**Solutions**

The composition of the Krebs solution was as follows: 137.4 mM NaCl, 5.9 mM KCl, 1.2 mM MgSO₄, 2.5 mM CaCl₂, 15.5 mM NaHCO₃, 1.2 mM KH₂PO₄, and 11.5 mM glucose. The solution was bubbled with 95% oxygen and 5% carbon dioxide to obtain pH 7.3–7.4. Modified Krebs–HEPES buffer contained 99 mM NaCl, 4.7 mM KCl, 1.9 mM CaCl₂, 1.2 mM MgSO₄,
20 mM HEPES, 1.03 mM K$_2$HPO$_4$, 25 mM NaHCO$_3$, and 11.1 mM glucose (pH 7.4). Krebs–Henseleit buffer contained 118.3 mM NaCl, 4.7 mM KCl, 2.5 mM CaCl$_2$, 1.2 mM MgSO$_4$, 1.2 mM KH$_2$PO$_4$, 25 mM NaHCO$_3$, and 11 mM glucose (pH 7.4).

**Statistical Analysis**

All results were reported as means ± standard deviation, and n values represented the number of rats used (each rat provided only one segment for a given experiment). Multiple comparisons were analyzed using one-way analysis of variance followed by Tukey’s post hoc tests. Statistical significance was defined as p<0.05.

**Results**

**Biochemical Profile**

At 10 weeks of age, body weights were markedly higher in the control rats than in the streptozotocin-treated rats. Fasting blood glucose was significantly increased in the streptozotocin-treated rats compared with that in the control group. BUN concentrations were significantly elevated in the streptozotocin group compared with those in the control group, but were significantly decreased in the topiroxostat 3 mg/kg group compared with those in the streptozotocin group. Urinary MDA concentrations corrected for creatinine were significantly elevated in the streptozotocin group compared with those in the control group, but were significantly decreased in the topiroxostat 1 mg/kg and 3 mg/kg groups compared with those in the streptozotocin group. Urinary albumin to creatinine ratio (UACR) was significantly elevated in the streptozotocin group compared with that in the control group. Topiroxostat showed dose-dependent and significant decreases in UACR.

Plasma E-selectin concentrations were significantly elevated in the streptozotocin group compared with those in the control group but were significantly decreased in the topiroxostat
1 mg/kg and 3 mg/kg groups compared with those in the streptozotocin group (Table 1).

**Purine Bodies**

No difference in uric acid concentration was observed between the control and streptozotocin groups, but concentrations were significantly lower in the topiroxostat 3 mg/kg group compared with those in the streptozotocin group. Hypoxanthine and xanthine were detected only in the topiroxostat 1 mg/kg and 3 mg/kg groups as Fig. 1A-C.

**XOR Activity in Plasma**

XOR activity in plasma was significantly higher in the streptozotocin group compared with that in the control group but was significantly decreased in the topiroxostat 1 mg/kg and 3 mg/kg groups compared with that in the streptozotocin group (Fig. 1D).

**Anchored XOR Activity**

Superoxide production was significantly increased in the streptozotocin group compared with that in the control group and was significantly suppressed in the topiroxostat 1 mg/kg and 3 mg/kg groups compared with that in the streptozotocin group. Heparin treatment in each group significantly suppressed superoxide production in the control, streptozotocin, topiroxostat 0.3 mg/kg, and topiroxostat 1 mg/kg groups (Fig. 2). SOD treatment also suppressed superoxide production (results not shown).

**Intracellular XOR Activity**

Superoxide production was significantly increased in the streptozotocin group compared with that in the control group, but was unchanged with xanthine and topiroxostat (Fig. 3).
Cytotoxicity Assay

The fluorescence intensity of nitrotyrosine was significantly increased in the streptozotocin group compared with that in the control group and was significantly decreased in the topiroxostat 3 mg/kg group compared with that in the streptozotocin group (Fig. 4).

Discussion

Streptozotocin-treated rats had hyperglycemia, albuminuria, and high BUN. Topiroxostat administration improved UACR and BUN values. Because there was no improvement in blood glucose levels, these changes were not related to blood glucose control. Given the improvement in urinary MDA concentrations, these changes may be mediated by the suppression of oxidative stress, a hypothesis consistent with previous reports.\textsuperscript{26-30} The activity of circulating XOR was significantly suppressed by topiroxostat compared with the activity observed in the streptozotocin group. Correspondingly, uric acid concentrations were significantly decreased and hypoxanthine was detected. In contrast, topiroxostat did not inhibit increased intracellular XO-derived ROS. In addition to xanthine oxidase, several enzyme systems can produce ROS in the vessel wall; these include nicotinamide adenine dinucleotide phosphate oxidase, enzymes of the mitochondrial respiratory chain, and endothelial nitric oxide synthase uncoupling.\textsuperscript{31} Topiroxostat alone may not sufficiently inhibit intracellular ROS production arising from the oxidative stress caused by these complex factors.

Importantly, we found that topiroxostat inhibited ROS derived from XO anchored to the cell membrane in the aorta of the diabetic animal model. XO has been shown to exist both intracellularly and extracellularly, and forms of extracellular XO have been reported. Namely, there is a free form that exists in circulating blood and an anchored form that is bound to proteoglycans on the surface of endothelial cells.\textsuperscript{11-13,32} However, the differences in the
contribution of each XO type to disease pathogenesis are unknown. Dose-dependent inhibition of albuminuria in db/db mice treated with topiroxostat has previously been reported, correlated with plasma XOR activity.\(^{18}\) Topiroxostat is hypothesized to ameliorate vascular damage as a result of inhibiting circulating blood XO, anchored XO, and intracellular XO. In the current study, chemiluminescence results indicated that topiroxostat inhibited the increase in ROS production by anchored XOR, however, oxidative fluorescence staining with dihydroethidium showed that the increase in ROS production by intracellular XO was not changed by topiroxostat. The results of the current study suggest that topiroxostat may ameliorate vascular damage by inhibiting anchored XO.

Oxidative stress is a major contributing factor to vascular damage in diabetes. XOR activation promotes the uptake of low-density lipoprotein into macrophages, indicating that XOR is directly involved in the progression of atherosclerosis.\(^{33}\) The results of nitrotyrosine staining in the present study suggest that endothelial cell damage in the aorta may be improved by topiroxostat administration. E-selectin concentrations were also improved by topiroxostat administration. These findings suggest that topiroxostat may inhibit ROS and reduce vascular damage in the aorta, changes that may have beneficial effects in controlling CVD in patients with diabetes. In the CARES trial, febuxostat was associated with increased mortality compared with allopurinol in patients with CVD.\(^{34}\) In the Beyond UA trial, topiroxostat had no significant effect on atherosclerosis in patients with hyperuricemia associated with hypertension, but did lower early morning systolic blood pressure and significantly suppressed plasma XOR activity and UACR compared with febuxostat.\(^{35}\) The mechanism by which topiroxostat binds to XOR is comparable with that of febuxostat and allopurinol, and although some studies have examined its effects on vascular endothelial cells, the present study suggests that it may have beneficial effects. A large artery was required to perform the chemiluminescence experiments. Therefore, the current study used the aorta for these
experiments. However, it is unclear whether small blood vessels, such as resistance and cerebral arteries, show the same response as the aorta. This issue will be examined in future research.

Circulating XO released from XO-rich organs can bind to glycosaminoglycans on the surface of endothelial cells and become endocytosed, inhibiting nitric oxide-dependent vascular smooth muscle cell relaxation. This suggests that not only endogenously produced XO in endothelial cells but also circulating and anchored XO released from XO-rich organs are important sources of ROS that lead to endothelial dysfunction. Anchored XO, which originates from XO released from organs such as the liver and intestinal tract, and inhibiting it can reduce ROS not only in vascular endothelial cells but also in blood vessels throughout the body.

The present study had four limitations. First, the activity of circulating XOR was measured using LC/MS, but because XO and XDH were mixed, the effect of XO-derived oxidative stress could not be selectively evaluated. Second, the maximum dose of topiroxostat administered was 3 mg/kg. The maximum tolerated dose might be greater than 3 mg/kg, but this was not investigated. The paucity of reports on the repeated administration of topiroxostat in rats indicates that further evaluation at higher doses is needed. Furthermore, only a type 1 diabetes model was used in this study. However, it has been reported that XO was also activated in Zucker diabetic rats, a type 2 diabetes model. Other diabetic models should also be considered in future studies. In addition, we used only the thoracic aorta for the current experiments, and did not use other small vessels, such as resistant and cerebral vessels, which will be examined in future studies.

In conclusion, topiroxostat inhibits anchored XO and thus ameliorates vascular endothelial damage in the aorta of a diabetic animal model. The results of this study suggest that topiroxostat may exert a vasoprotective effect in patients with diabetes-induced macrovascular
disease. As a next step, prospective clinical studies in patients with diabetes-induced macrovascular disease should be carried out to evaluate the vasoprotective effect of topiroxostat.
Conflict of Interest

Chigusa Kikuchi and Tamihide Matsunaga received a research grant from Sanwa Kagaku Kenkyusho. Takashi Nakamura and Takayo Murase are employees of Sanwa Kagaku Kenkyusho. The remaining authors have no conflicts of interest.
References


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Table 1

Biochemical profile of rats treated with topiroxostat

<table>
<thead>
<tr>
<th></th>
<th>Body weight (g)</th>
<th>Blood glucose (mg/dL)</th>
<th>BUN (mg/dL)</th>
<th>Urinary MDA (µM/mg creatinine)</th>
<th>UACR (µg/mg creatinine)</th>
<th>E-selectin (ng/mL)</th>
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<tbody>
<tr>
<td>Control</td>
<td>337.2±25.7</td>
<td>127.8±59.0</td>
<td>18.8±5.8</td>
<td>0.13±0.05</td>
<td>209.8±119.8</td>
<td>12.5±3.5</td>
</tr>
<tr>
<td>STZ</td>
<td>219.1±39.6 a</td>
<td>479.7±44.6 a</td>
<td>84.7±17.5 a</td>
<td>0.48±0.19 a</td>
<td>2050.2±411.8 a</td>
<td>29.8±3.4 a</td>
</tr>
<tr>
<td>Tpr 0.3 mg/kg</td>
<td>223.7±33.0</td>
<td>466±171.1</td>
<td>95.5±19.9</td>
<td>0.34±0.09</td>
<td>1276.8±399.8 b</td>
<td>29.3±3.4</td>
</tr>
<tr>
<td>Tpr 1 mg/kg</td>
<td>214.8±24.0</td>
<td>471.1±97.3</td>
<td>88.8±18.2</td>
<td>0.28±0.05 b</td>
<td>745.1±391.8 b</td>
<td>19.8±6.4 b</td>
</tr>
<tr>
<td>Tpr 3 mg/kg</td>
<td>222.2±35.1</td>
<td>419.2±137.4</td>
<td>40.4±15.4 b</td>
<td>0.19±0.05 b</td>
<td>272.6±87.08 b</td>
<td>13.5±3.2 b</td>
</tr>
</tbody>
</table>

Data are expressed as the mean ± standard deviation. N=7.

a p < 0.05 vs. control, b p < 0.05 vs. STZ. (Multiple comparisons were analyzed using one-way analysis of variance followed by Tukey’s post hoc tests)

STZ, streptozotocin; Tpr, topiroxostat; BUN, blood urea nitrogen; MDA, malondialdehyde; UACR, urinary albumin to creatinine ratio.
Figure 1. Concentration of purine bodies in plasma; uric acid (A), hypoxanthine (B), xanthine (C). Xanthine oxidoreductase activities in plasma (D). Data are the mean ± standard deviation (N=7), a$p < 0.05$ vs. control, b$p < 0.05$ vs. STZ (one-way analysis of variance followed by Tukey’s post hoc tests).

STZ, streptozotocin.
**Figure 2.** Evaluation of anchored xanthine oxidoreductase (XOR) activity. The effect of topiroxostat on anchored XOR was studied by chemiluminescence. The graph shows the difference between the luminescence in the presence of the sample and the blank, expressed as a ratio to the standard (5 µU/mL xanthine oxidase) and corrected for dry weight.

Black bar: heparin-free; Gray bar: 1000 U/mL heparin added.

Data are the mean ± standard deviation (N=7), \(^a p < 0.05\) vs. control, \(^b p < 0.05\) vs. STZ, \(^c p < 0.05\) vs. heparin free (one-way analysis of variance followed by Tukey’s post hoc tests).

STZ, streptozotocin.
Figure 3. Evaluation of intracellular xanthine oxidoreductase (XOR) activity. The effect of topiroxostat on intracellular XOR was studied by oxidative fluorescence staining with dihydroethidium.
(A) Dihydroethidium fluorescence signal in aorta histological sections observed under a confocal microscope. (B) Quantification of fluorescence intensity of the superoxide-sensitive dye dihydroethidium in the intimal region. (C) Quantification of dihydroethidium fluorescence intensity in the media region. Black bar: xanthine-free; Gray bar: 50 µM xanthine added.

Data are the mean ± standard deviation (N=7), *p < 0.05 vs. control + xanthine (one-way analysis of variance followed by Tukey’s post hoc tests).

STZ, streptozotocin; Tpr, topiroxostat.
Figure 4. Evaluation of cytotoxicity.

The effect of topiroxostat on cytotoxicity was studied by fluorescence immunostaining.
Frozen sections of aorta were immunostained with antibodies against 3-NT and CD31 and co-stained with DAPI. (A) Fluorescence signal of 3-NT (green), CD31 (red), and DAPI (blue) in aorta histological sections observed under a confocal microscope. (B) Quantification of fluorescence intensity of the overlapping areas of CD31 and 3-NT fluorescence. Scale bar, 50 μm. Data are the mean ± standard deviation (N=7), $^a p < 0.05$ vs. control, $^b p < 0.05$ vs. STZ (one-way analysis of variance followed by Tukey’s post hoc tests).

3-NT, 3-nitrotyrosine; STZ, streptozotocin; Tpr, topiroxostat.