Mizoribine suppresses the progression of experimental peritoneal fibrosis in a rat model

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Abstract

Background/Aims: Peritoneal fibrosis is a serious complication of peritoneal dialysis (PD). It has been reported that administration of mizoribine, an effective immunosuppressant, ameliorated renal fibrosis in a rat model of unilateral ureteral obstruction. We therefore examined the effects of mizoribine in an experimental model of peritoneal fibrosis.

Methods: Twenty-four rats were given a daily intraperitoneal injection of chlorhexidine gluconate and ethanol dissolved in saline. The rats were divided into three groups (n=8 per group) that received either vehicle or mizoribine at a dose of 2 mg/kg or 8 mg/kg once a day. Twenty-eight days after the start of the treatments the rats were sacrificed and peritoneal tissue samples collected. Macrophage infiltration (ED1), myofibroblast accumulation (α-smooth muscle actin: SMA) and expression of type III collagen, transforming growth factor (TGF)-β and monocyte chemotactic protein-1 (MCP-1) were examined by immunohistochemistry.

Results: Mizoribine significantly suppressed submesothelial zone thickening and reduced macrophage infiltration. Mizoribine also reduced collagen III$^+$ area and decreased the number of α-SMA$^+$, TGF-β$^+$ and MCP-1$^+$ cells. The magnitude of the changes observed was dose-dependent.

Conclusion: The administration of mizoribine prevented the progression of peritoneal fibrosis in this rat model. Mizoribine may represent a novel therapy for peritoneal sclerosis in patients undergoing long-term PD.

Key words: macrophage; mizoribine; peritoneal dialysis; peritoneal fibrosis; rat

Introduction

Peritoneal dialysis (PD) has been used for more than two decades as an attractive treatment for end-stage kidney disease. However, long-term PD treatment is associated with histopathological alterations in the

peritoneum [1]. Continuous exposure to bioincompatible dialysis fluids and repeated episodes of bacterial peritonitis play a major role in the alteration of peritoneal function and structure that occurs with time [2, 3]. The characteristic pathologic findings in the peritoneum of patients on long-term continuous ambulatory peritoneal dialysis (CAPD) therapy include marked peritoneal fibrosis with marked accumulation of collagen and loss of peritoneal mesothelial cells [4]. Indeed, a minority of patients may develop the serious complication of encapsulating peritoneal sclerosis (EPS) that is associated with a high mortality and characterized by severe progressive peritoneal fibrosis [5]. Therapeutic strategies for EPS are limited and include the appropriate use of steroids [6]. However, there is no experimental model for peritoneal fibrosis that is similar to the fibrosis that develops in patients on CAPD.

Suga et al. developed an experimental model of peritoneal fibrosis in rats induced by the peritoneal injection of chlorhexidine gluconate (CG) [7]. CG-induced peritoneal fibrosis in rats is very similar to that seen in patients with EPS as many of the pathologic findings in the peritoneum of CAPD patients including the increased expression of type III collagen, α-smooth muscle actin (α-SMA) and macrophage infiltration were also observed in the peritoneum of animals injected with CG [8].

Mizoribine is an imidazole nucleoside isolated from *Eupenicillium brefeldianum* and is an orally administered immunosuppressive agent. Mizoribine inhibits the conversion of inosine 5'-nucleotide to guanosine 5'-nucleotide in the purine nucleotide biosynthetic pathway and has similar immunosuppressive effects upon both humoral and cellular immunity to mycophenolate mofetil (MMF) [9, 10, 11]. The efficacy of this agent has been demonstrated in patients with diverse conditions including renal transplant recipients [12] and patients with rheumatoid arthritis [13], Sjögren's syndrome [14], lupus nephritis [15] and primary nephrotic syndrome [16]. Moreover, the incidence of adverse effects including myelosuppression, hepatotoxicity and nephrotoxicity is lower with this drug than other immunosuppressive agents. Furthermore, recent studies have demonstrated that mizoribine improves renal tubulointerstitial fibrosis in unilateral ureteral obstruction (UUO) in the rat [17] in a dose-dependent manner [18].

In this study, we examined the therapeutic efficacy of mizoribine in a rat model of peritoneal fibrosis induced by the administration of CG.

Methods

Animals

Fifty-two Wistar rats weighing 190 to 200 g were obtained from Charles River Laboratories Japan (Yokosuka, Japan). The animals were housed in the animal facility of Hiroshima University with free access to food and water. The Institutional Animal Care and Use Committee at Hiroshima University (Hiroshima, Japan) approved all the animal protocols and the experiments were performed in accordance with the National Institutes of Health Guidelines on the use of Laboratory Animals.

Experimental protocol

Peritoneal fibrosis was induced by the intraperitoneal injection of 0.1% chlorhexidine gluconate (CG) in 15% ethanol dissolved in saline as described previously [19]. Briefly, rats received a daily intraperitoneal injection of 0.1% CG in 15% ethanol dissolved in 2 mL of saline for a period of 28 days. The intraperitoneal injection of CG was performed under anesthesia with isoflurane in order to ensure the accuracy of the injection. Intraperitoneal injections of CG were performed in the left portion of the abdomen, whereas the right portion of the peritoneum

was processed for histological evaluation in order to avoid mechanical damage of the peritoneum caused by repeated injections confounding the findings.

The rats were divided into three groups that received either: (1) vehicle buffer (CG + vehicle group, N=8), (2) mizoribine at 2 mg/kg body weight (CG + 2 mg mizoribine group, N=8), (3) mizoribine at 8 mg/kg body weight (CG + 8 mg mizoribine group, N=8).

Mizoribine (Asahi Kasei Pharma Corporation, Tokyo, Japan) was dissolved in 0.5 ml of distilled water and administered by daily oral gavage just before the CG injection. Control rats received a daily intraperitoneal injection of vehicle only (15% ethanol dissolved in 2 ml of saline) for 28 days (control group, N=8). Rats were killed 28 days after starting CG injection and the parietal peritoneal tissues were carefully dissected prior to fixation in 10% formalin and embedding in paraffin.

Histological analysis

Formalin-fixed, paraffin-embedded sections (4 μm) were stained with hematoxylin and eosin (H&E) for light microscopic observation. Cross-sections of the abdominal wall were examined and the thickness of the submesothelial collagenous zone above the abdominal muscle layer was defined as the peritoneal thickness [20]. The extent of peritoneal thickening was determined by analysis of digitized images using image analysis software NIS-Elements D (Nikon Corporation, Tokyo, Japan). The image was transformed into a matrix of 1280 × 960 pixels and viewed at ×100 magnification. We selected a width of 840 μm in the examined field under the microscope and measured the area of the submesothelial layer within this selected width. For each sample, eight such areas were selected and the average area of the submesothelial layer was determined.

Immunohistochemistry analysis

Immunohistochemical analyses were performed using 4 μm tissue sections as described previously [21, 22]. The following primary antibodies were used: (1) mouse monoclonal anti-rat ED1 antibody as a macrophage marker (1:200 dilution, MCA341R; Serotec, Oxford, UK); (2) polyclonal rabbit anti-rat type III collagen antibody (1:500 dilution, AB757P; Chemicon International Inc., Temecula, CA, USA); (3) mouse monoclonal anti-α-smooth muscle actin (α-SMA) (1:1000 dilution, A2547; Sigma, St Louis, MO, USA); (4) polyclonal rabbit anti-mouse TGF-β1 antibody (1:1000 dilution, sc-146; Santa Cruz, CA, USA); (5) polyclonal rabbit anti-rat monocyte chemotactic protein-1 (MCP-1) antibody (1:250 dilution, FL-148; Santa Cruz, CA, USA). Tissue sections were placed in 0.01M citrate buffer (pH 6.0) and heated for 10 minutes in a microwave oven. This treatment was used for ED1, TGF-β1, and MCP-1 staining. Sections were blocked in 5% fetal calf serum (FCS), 5% bovine serum albumin (BSA) and 10% normal goat serum in phosphate-buffered saline (PBS) for 60 minutes and then incubated overnight at 4^0C with primary antibody diluted in 10% normal goat serum and 5% normal rat serum. After washing, endogenous peroxidase activity was blocked by incubating tissue sections in 0.6% H_2O_2 in methanol for 20 min. For ED1 and α-SMA immunostaining, tissue sections were incubated with goat anti-mouse immunoglobulin-G (IgG) conjugated with horseradish peroxidase (HRP, P0447; DAKO, Glostrup, Denmark, diluted 1/50) for 45 minutes at room temperature followed by a complex of HRP-conjugated mouse anti-HRP IgG (P0850; DAKO, diluted 1/50) for 45 minutes at room temperature. For type III collagen and MCP-1 immunostaining, tissue sections were incubated with goat anti-rabbit IgG conjugated with HRP (P0448; DAKO, diluted 1/50) for 45 minutes at room temperature followed by a complex of HRP-conjugated rabbit anti-HRP IgG (Z0113; DAKO diluted 1/50) for 45 minutes at room temperature. Immunostaining for

TGF-β1 was conducted using the Vectastain ABC Elite reagent kit (Vector Labs, Burlingame, CA, USA) according to the manufacturer's protocol. Goat anti-rabbit IgG (1:250 dilution, 65-6140; ZYMED, Carlsbad, CA, USA) was used as a secondary antibody. Specific antibody binding was detected by color development following reaction with H_2O_2 and 3-3 diaminobenzidine tetrahydrochloride. In each peritoneal sample, the numbers of ED1 positive cells, α-SMA positive cells, TGF-β positive cells and MCP-1 positive cells were counted in 10 fields (×400 magnification). In order to assess the area positive for type III collagen immunostaining, the image files (1280 × 960) at ×200 magnification were analyzed using Lumina Vision software (Mitani, Fukui, Japan). The positive area was shown as the mean of diaminobenzidine positive pixel values obtained from five image files in each section.

Two color immunostaining was used to detect colocalisation of ED1 and TGF-β. After staining for TGF-β and development with diaminobenzidine to give a brown color, the sections were placed in 0.01M citrate buffer (pH 6.0) and heated for 10 minutes in a microwave oven. The sections were then blocked as described above, incubated overnight at 4°C with the monoclonal anti-ED1 antibody and incubated further with goat anti-mouse HRP for 45 minutes at room temperature. This was followed by incubation for 45 minutes at room temperature in a complex of HRP-conjugated mouse anti-HRP IgG and development with Vector SG to give a blue/gray color.

Pharmacokinetic analysis

Twenty female Wistar rats weighing 190 to 200g were used for the pharmacokinetic analysis. Rats were divided into two groups with 10 rats receiving mizoribine at 2 mg/kg body weight by oral gavage (2 mg mizoribine group) and the remaining 10 rats receiving mizoribine at 8 mg/kg body weight by oral gavage (8 mg mizoribine group). In each group, blood samples were collected from 5 rats at 0.5, 2 and 4 hours after administering mizoribine whilst the blood samples of the remaining 5 rats were collected 1, 3 and 6 hours after administering mizoribine. To minimize the influence of blood loss upon the pharmacokinetic analysis, we collected blood three times per rat. The serum concentration of mizoribine was determined by high-performance liquid chromatography (HPLC) [23]. The simulation values of pharmacokinetics analysis were performed with the statistical software WinNonlin Ver5.2 (Pharsight Corporation, USA) and fitted to the one-compartment model.

Statistical analysis

Results are expressed as means ± standard error (SE) for each group. Statistical analysis was performed with analysis of variance by Tukey's post-hoc test. Data differences were deemed significant at $P<0.05$.

Results

Morphologic Examination

Morphologic changes were assessed by H&E staining. A monolayer of mesothelial cells was observed in normal rats covering the peritoneal surface without any thickening of the peritoneum (Fig. 1a). The appearance of the peritoneum of control rats receiving intraperitoneal vehicle only (15% ethanol dissolved in saline) was similar to that of the normal rats (Fig. 1b). The daily intraperitoneal injection of CG for 28 days resulted in marked thickening of the submesothelial compact zone associated with increased cellularity (Fig. 1c). The administration of mizoribine suppressed both the thickness of the submesothelial zone and the increased cellularity induced by CG (Fig. 1d and 1e). Morphological evaluation revealed a significant inhibitory effect of mizoribine on CG-induced peritoneal

thickening with this anti-fibrotic effect being dose-dependent (control group 17.4 ± 1.7×10^3μm^2; CG + vehicle group 222.7 ± 8.5×10^3μm^2; CG + mizoribine 2 mg group 128.5 ± 4.1×10^3μm^2; CG + mizoribine 8 mg group 85.8 ± 3.3×10^3μm^2, Fig. 1f).

Macrophage infiltration

We examined the peritoneal expression of the macrophage marker ED1. ED1$^+$ cells were rarely observed in the control group (1.4 ± 0.6 ED1$^+$ cells/10 fields, Fig. 2a). The number of ED1$^+$ cells in the peritoneum was significantly increased in rats receiving injections of CG (491.1 ± 34.5 ED1$^+$ cells/10 fields, p<0.01, Fig. 2b and 2e) compared with the control group. Daily treatment with 2mg of mizoribine significantly suppressed macrophage infiltration of the peritoneum induced by CG injection compared with the group receiving CG and vehicle (189.8 ± 7.7 vs 491.1 ± 34.5 ED1$^+$ cells/10 fields, p<0.01, Fig. 2c and 2e). In addition, the number of ED1$^+$ cells in the peritoneum was further decreased in rats treated with 8mg of mizoribine per day compared to rats treated with 2mg of mizoribine daily thereby indicating a dose-dependent effect (93.0 ± 8.4 vs 189.8 ± 7.7 ED1$^+$ cells /10 fields, p<0.01, Fig. 2d and 2e). The number of ED1$^+$ cells per submesothelial area (mm^2) was also analyzed and the results were similar to those expressed as the number of ED1$^+$ cells per field with mizoribine exerting a significant anti-inflammatory and therapeutic effect (Table 1). These data suggested that the marked reduction in the number of ED1$^+$ macrophages was not merely a reflection of reduced submesothelial tissue volume. These results demonstrate that treatment with mizoribine significantly inhibits infiltration of the peritoneum by ED1$^+$ macrophages following the administration of CG, with this action possibly being related to the potent immunosuppressive effect of mizoribine.

Immunohistochemical analysis of type III collagen

To examine the effects of mizoribine on peritoneal fibrosis induced by CG, we next examined the expression of type III collagen. Marked expression of type III collagen in the submesothelial zone was observed in rats receiving CG compared to the control group receiving vehicle alone (573.2 ± 28.6 vs 6.6 ± 1.4 ×10^3 pixels, p<0.01, Fig. 3a, 3b and 3e). Daily treatment with 2 mg of mizoribine significantly reduced the peritoneal expression of type III collagen induced by CG compared to rats receiving CG alone (306.8 ± 15.5 vs 573.2 ± 28.6 ×10^3 pixels, P<0.01, Fig. 3c and 3e). In addition, the expression of type III collagen in the submesothelial zone was further and significantly decreased in rats treated with 8mg of mizoribine per day compared to rats treated with 2mg of mizoribine per day thereby indicating a dose-dependent therapeutic and anti-fibrotic effect (181.7 ± 11.8 vs 306.8 ± 15.5 ×10^3 pixels, P<0.01, Fig. 3d and 3e).

Immunohistochemical analysis of α-SMA expression

We also determined the number of α-SMA$^+$ myofibroblasts in the peritoneum associated with peritoneal fibrosis. In the control group, the expression of α-SMA was only observed in vascular smooth muscle cells (6.9 ± 2.5 α-SMA$^+$ cells/10 fields, Fig. 4a). In rats administered CG, α-SMA expression was found in myofibroblasts in addition to vascular smooth muscle cells with numerous α-SMA$^+$ myofibroblasts evident in the thickened peritoneal tissues (707.8 ± 36.1 α-SMA$^+$ cells/10 fields, Fig. 4b). Treatment with mizoribine significantly inhibited the increase in peritoneal α-SMA$^+$ myofibroblast number induced by CG compared to rats administered CG alone (291.0 ± 24.1 vs 707.8 ± 36.1 α-SMA$^+$ cells/10 fields, P<0.01, Fig. 4c and 4e). The increase in the number of α-SMA$^+$ cells in the peritoneum was further and significantly suppressed in rats treated with 8 mg mizoribine daily compared rats

treated with 2mg mizoribine (140.8 ± 15.5 vs 291.0 ± 24.1 α-SMA$^+$ cells/10 fields, P<0.01, Fig. 4d and 4e). The number of α-SMA$^+$ cells per submesothelial area (mm^2) was also determined and similar results were obtained (Table 1) thereby indicating that the reduction in the number of α-SMA$^+$ cells in mizoribine treated rats was not merely a reflection of reduced submesothelial tissue volume.

Immunohistochemical analysis of TGF-β

We next examined the peritoneal expression of the profibrotic growth factor TGF-β that is associated with peritoneal fibrosis. In the control group, TGF-β$^+$ cells were rarely observed (6.0 ± 1.9 TGF-β$^+$ cells/10 fields, Fig. 5a) and immunoreactivity for TGF-β was observed in the submesothelial area and in the fibrotic layer. The number of peritoneal TGF-β$^+$ cells was increased markedly in rats receiving CG alone compared to the control group (617 ± 27.6 vs 6.0 ± 1.9 TGF-β$^+$ cells/10 fields, P<0.01, Fig. 5b and 5e). Daily treatment with 2mg of mizoribine significantly suppressed the increase in the number of peritoneal TGF-β$^+$ cells compared to rats administered CG alone (249.4 ± 11.3 vs 617 ± 27.6 TGF-β$^+$ cells/10 fields, P<0.01, Fig. 5c and 5e). The number of peritoneal TGF-β$^+$ cells was further reduced in rats treated with 8mg mizoribine daily compared to rats receiving 2mg of mizoribine (127.6 ± 7.6 vs 249.4 ± 11.3 TGF-β$^+$ cells/10 fields, P<0.01, Fig. 5d and 5e). The number of TGF-β$^+$ cells per submesothelial area (mm^2) was also determined and similar results were observed (Table 1) suggesting that the reduction in the number of TGF-β$^+$ cells was not merely a reflection of reduced submesothelial tissue volume.

Immunohistochemical analysis of MCP-1

We next examined the peritoneal expression of MCP-1, a chemotactic factor which attracts monocytes. MCP-1$^+$ cells were observed rarely in the control group (29.0 ± 11.3 MCP-1$^+$ cells/10 fields, Fig. 6a). The number of peritoneal MCP-1$^+$ cells was increased markedly in rats receiving CG alone compared to the control group (1338.4 ± 102.4 vs 29.0 ± 11.3 MCP-1$^+$ cells/10 fields, P<0.01, Fig. 6b and 6e). Daily treatment with 2mg of mizoribine significantly suppressed the increase in the number of peritoneal MCP-1$^+$ cells compared to rats administered CG alone (476.5 ± 50.4 vs 1338.4 ± 102.4 MCP-1$^+$ cells/10 fields, P<0.01, Fig. 6c and 6e). The number of peritoneal MCP-1$^+$ cells was further reduced in rats treated with 8mg mizoribine daily compared to rats receiving 2mg of mizoribine (228.3 ± 28.1 vs 476.5 ± 50.4 MCP-1$^+$ cells/10 fields, P<0.01, Fig. 6d and 6e). The number of MCP-1$^+$ cells per submesothelial area (mm^2) was also determined and similar results were observed (Table 1) suggesting that the reduction in the number of MCP-1$^+$ cells was not merely a reflection of reduced submesothelial tissue volume.

Double staining of ED1 and TGF-β

We also performed double staining of ED1 and TGF-β. The majority of ED1$^+$ macrophages in the submesothelial compact zone showed immunoreactivity for TGF-β (Fig. 7), demonstrating colocalisation of ED1 and TGF-β.

Pharmacokinetic parameters

The change of serum concentration of mizoribine over time is shown in Fig. 8 and the pharmacokinetic profile of mizoribine was analysed using this data (Table 2.). The time to maximum serum concentration (Tmax) of mizoribine was 1.04 hr in the group treated with 2mg mizoribine and 1.21 hr in the 8mg mizoribine group. The maximum serum concentration (Cmax) of mizoribine was 0.514 μg/mL in the group treated with 2mg mizoribine and 2.214 μg/mL in the 8mg mizoribine group. The elimination half-life (T1/2) of mizoribine was

1.42 hr in the group treated with 2mg mizoribine and 1.68 hr in the 8mg mizoribine group. The area under the serum concentration-time curve infinity (AUCinf) of mizoribine was 1.61 μg･hr/mL in the group treated with 2mg mizoribine and 8.46 μg･hr/mL in 8mg mizoribine group.

Discussion

In the present study, we demonstrated that administration of mizoribine markedly reduced macrophage infiltration in the experimental rat model of peritoneal fibrosis induced by CG with this effect being dose-dependent. Moreover, treatment with mizoribine diminished collagen accumulation in the thickened submesothelial area. These findings indicate that mizoribine might be useful in preventing the progression of peritoneal fibrosis and implicate macrophage infiltration, at least in part, in the development of peritoneal fibrosis.

The inhibitory effect of mizoribine upon the number of $ED1^+$ macrophages in the thickened submesothelial area may be mediated via the potent immunosuppressive action of mizoribine. Mizoribine inhibits both humoral and cellular immunity via the inhibition of *de novo* purine biosynthesis [9]. Sato et al. reported that treatment with mizoribine significantly ameliorated tubulointerstitial fibrosis and infiltration by macrophages and T lymphocytes in rats with unilateral ureteral obstruction [18]. Kikuchit et al. also showed mizoribine inhibited renal macrophage accumulation and prevented the progression of glomerulosclerosis and interstitial fibrosis in non-insulin-dependent diabetic rats. [24]. It was reported that macrophages can synthesize extracellular matrix protein such as collagen and fibronectin which may cause fibrosis [25, 26]. In this regard, the infiltrating macrophages secrete important soluble factors such as TGF-β [27] and it is therefore possible that the decrease in submesothelial area caused by administration of mizoribine may be associated closely with the inhibitory effect upon macrophage infiltration.

Characteristic histological changes of peritoneal fibrosis such as thickening of the submesothelial area and the accumulation of type III collagen were significantly inhibited by treatment with mizoribine. The pivotal role of TGF-β1 in the development of peritoneal fibrosis results from the effect of TGF-β1 upon human peritoneal mesothelial cells (HPMC) including the induction of epithelial mesenchymal transition and the *de novo* synthesis of type III collagen [28, 29]. Immunohistochemical analysis indicated a significant inhibitory effect of mizoribine on the number of $TGF\text{-}\beta^+$ cells in the peritoneum. Thus, the inhibitory effect of mizoribine on the accumulation of type III collagen induced by administration of CG may be partly mediated via its inhibitory effect upon TGF-β expression. Immunoreactivity for α-SMA and TGF-β1 was observed in the submesothelial area in rats administered CG. Treatment with mizoribine suppressed the increase in the number of peritoneal $\alpha\text{-SMA}^+$ cells in rats administered CG in parallel with its inhibitory effect on TGF-β expression and accumulation of extracellular matrix. The *de novo* synthesis of α-SMA by HPMC after TGF-β1 stimulation has been reported [29] and it is possible that the therapeutic effect of mizoribine on peritoneal fibrosis may partly result from its regulatory role upon the conversion of peritoneal mesothelial cells to myofibroblasts.

The expression of MCP-1, a chemotactic factor which attracts monocytes, was inhibited significantly by treatment with mizoribine. Mizoribine inhibited upregulation of chemokines such as MCP-1, as well as suppressing the proliferation of macrophages. As macrophage accumulation in the thickened submesothelial area was suppressed, the production of

TGF-β may also have been inhibited, resulting in prevention of peritoneal fibrosis. The colocalisation of ED1 and TGF-β observed with double immunostaining supports this hypothesis.

For the analysis of immunohistochemical findings, we also determined the number of $ED1^+$, α-SMA^+ ,$TGF\text{-}\beta^+$ and $MCP\text{-}1^+$ cells per submesothelial area (mm^2). The results were similar to the measurement of cell number per field thereby demonstrating that the significant therapeutic effects of mizoribine did not merely reflect the differences in submesothelial thickening between experimental groups.

No adverse events were observed in this study despite the fact that the higher dose of mizoribine used was greater than the conventional dose of mizoribine used (up to 5 mg/kg body weight). Stypinski et al. reported that the daily administration of mizoribine up to 12mg/kg body weight caused no significant adverse events in healthy male volunteers except for a slight elevation in serum uric acid [30]. Tanaka et al. reported that a peak serum level of mizoribine of at least 2.5-3.0 μg/ml is necessary to achieve satisfactory clinical efficacy of the drug in the treatment of lupus nephritis [31]. Pharmacokinetic analysis of the rats in this study indicated that the Cmax was lower than 2.5-3.0 μg/ml when the dose of mizoribine administered was 8mg/kg body weight. Moreover, rats receiving 8mg mizoribine daily exhibited a more marked amelioration in peritoneal fibrosis than rats receiving 2mg mizoribine. Therefore, the administration of mizoribine at a dose of 8mg/kg body weight is optimal for preventing the progression of peritoneal fibrosis in rats.

In the present study, we induced peritoneal fibrosis by the intraperitoneal injection of CG. It is true that CG-induced peritoneal fibrosis does not fully replicate the peritoneal sclerosis or encapsulating peritoneal sclerosis observed in patients on long-term PD. However, there is not an ideal experimental model that simulates long-term PD and CG-induced peritoneal fibrosis undoubtedly exhibits key features of peritoneal fibrosis.

In conclusion, the administration of mizoribine prevented the progression of peritoneal fibrosis in a rat model. Mizoribine is a potentially useful therapy for peritoneal sclerosis in patients undergoing long-term PD. As mizoribine has lower nephrotoxicity than other immunosuppressive agents, its benefit may be in preserving residual renal function of PD patients in comparison with other immunosuppressive agents.

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Tables

Table 1. Morphometric analysis of immunohistochemical findings

	CG + vehicle	CG + 2 mg mizoribine	CG + 8 mg mizoribine
$ED1^+$ cells (/mm^2)	88.3±5.1	59.0±1.3[a]	43.1±3.1[a,b]
α-SMA^+ cells (/ mm^2)	128.0±7.2	89.9±5.6[a]	65.6±6.7[a,b]
TGF-β^+ cells (/ mm^2)	112.6±8.0	78.2±4.8[a]	59.5±2.2[a,b]
MCP-1^+ cells (/ mm^2)	239.5±15.0	151.1±11.5[a]	119±19.7[a,b]

All values are mean ± SE. n=8 in each group. The number of positive cells was counted in 10 fields and represented as per mm^2 of submesothelial fibrotic tissue by measuring the fibrotic area using NIS-Elements D software. [a]P<0.05 vs CG + vehicle group. [b]P<0.05 vs CG + 2 mg mizoribine group.

Table 2. Pharmacokinetic parameter (one compartment model).

	Mizoribine 2mg/kg	Mizoribine 8mg/kg
Tmax (hr)	1.04	1.21
Cmax (μg/mL)	0.514	2.214
$T_{1/2}$ (hr)	1.42	1.68
AUCinf (μg·hr/mL)	1.61	8.46

Pharmacokinetics analysis was performed with the statistical software WinNonlin Ver5.2 (Pharsight Corporation, USA). Cmax, the maximum concentration of mizoribine in serum; Tmax, the time to maximum concentration of mizoribine in serum; T1/2, elimination half-life of mizoribine; AUCinf, the area under the serum concentration-time curve infinity of mizoribine.

Fig. 1.

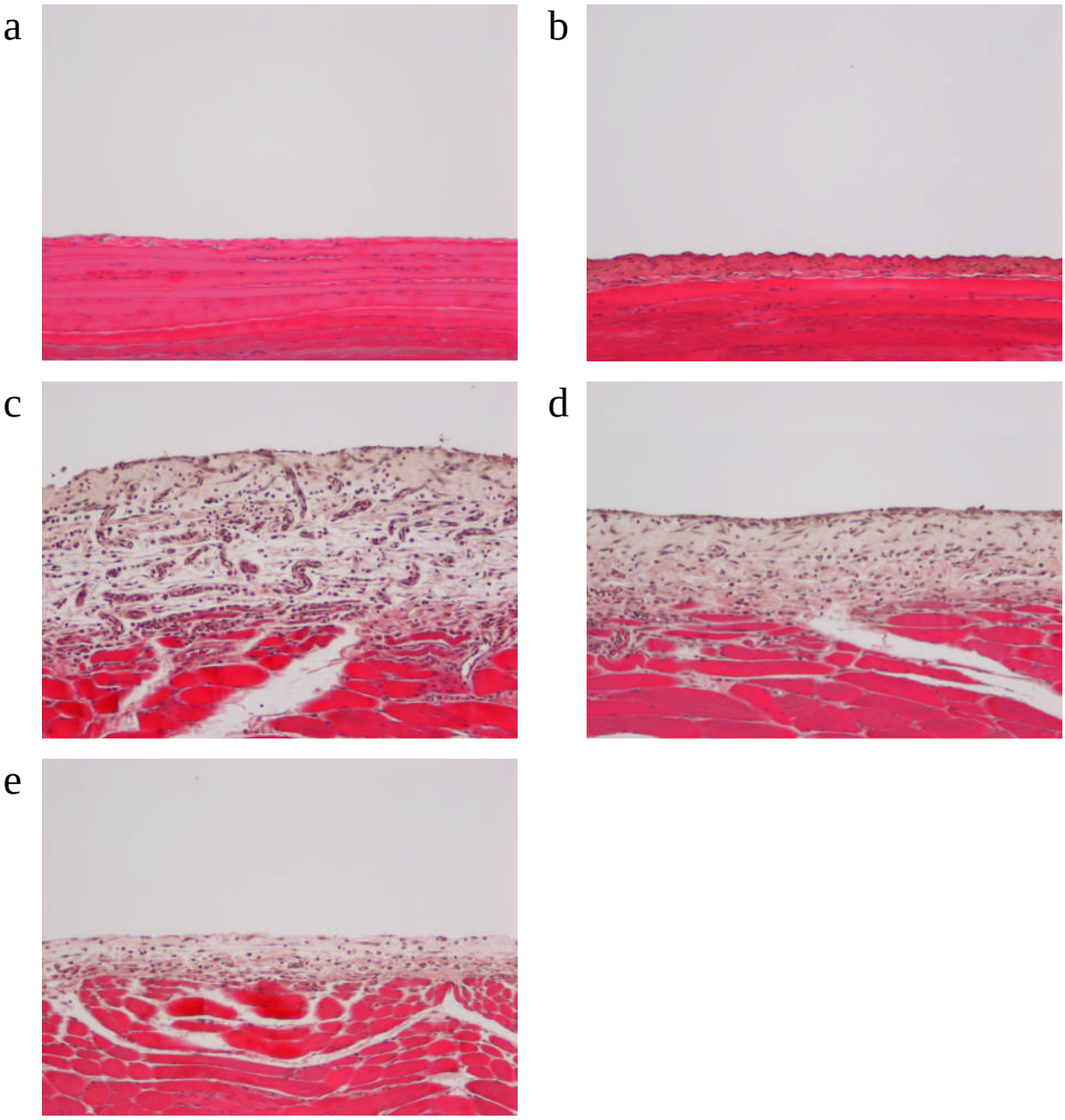
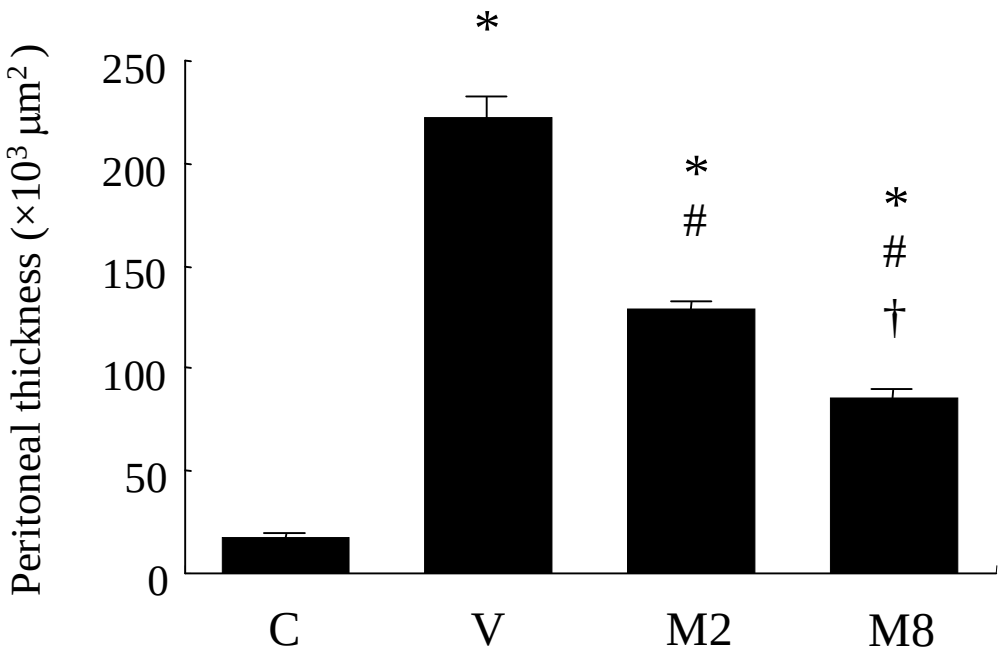


Fig. 1.

f



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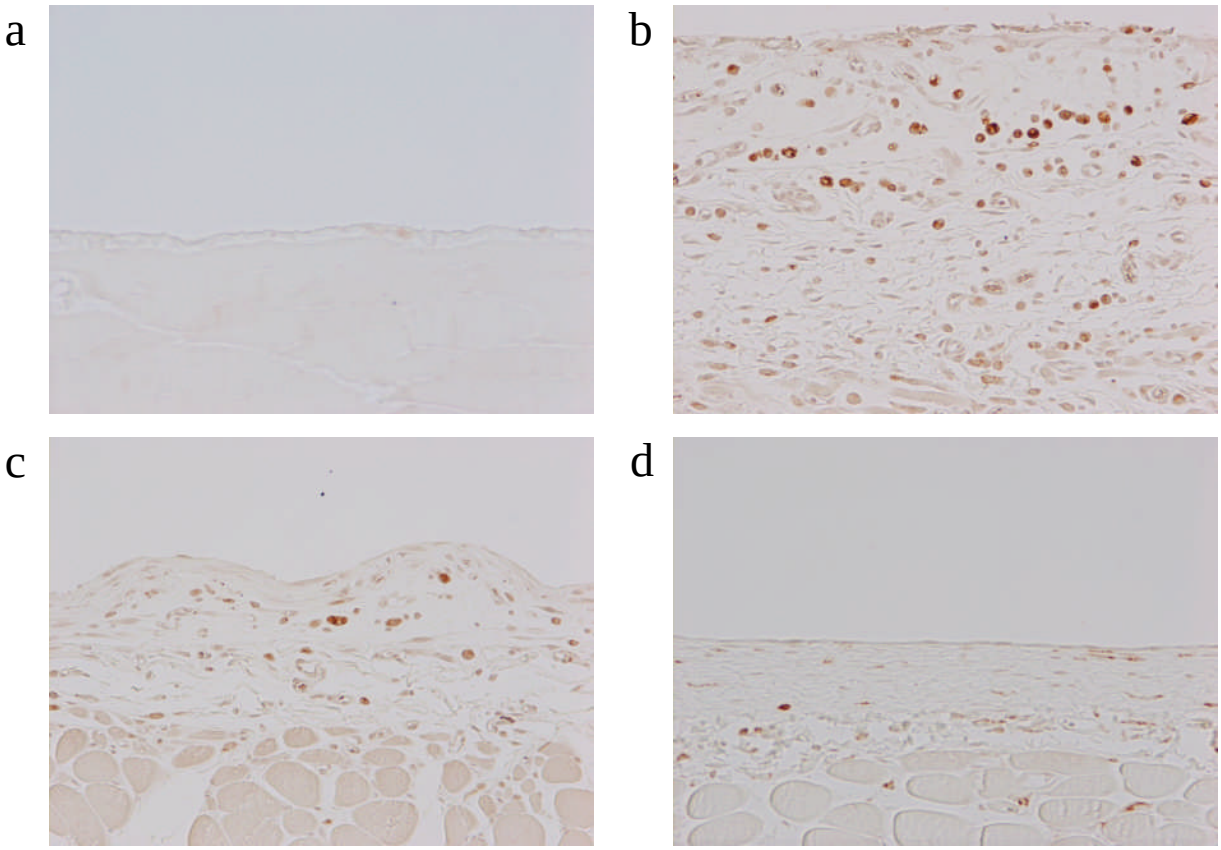

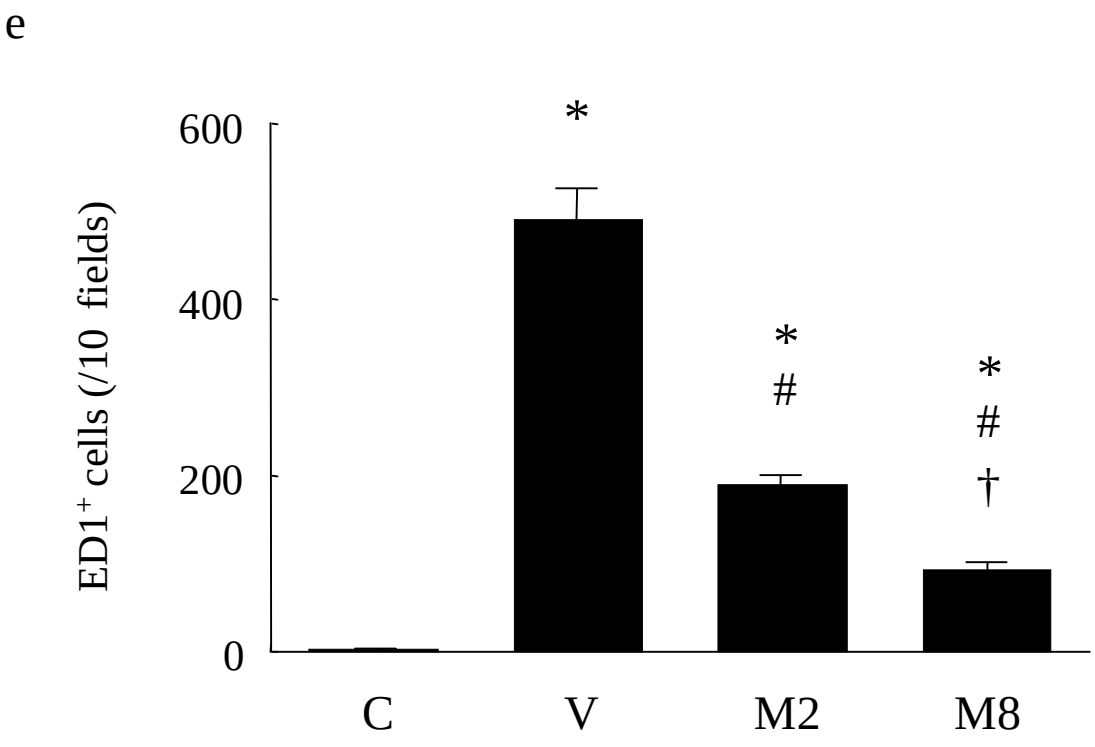

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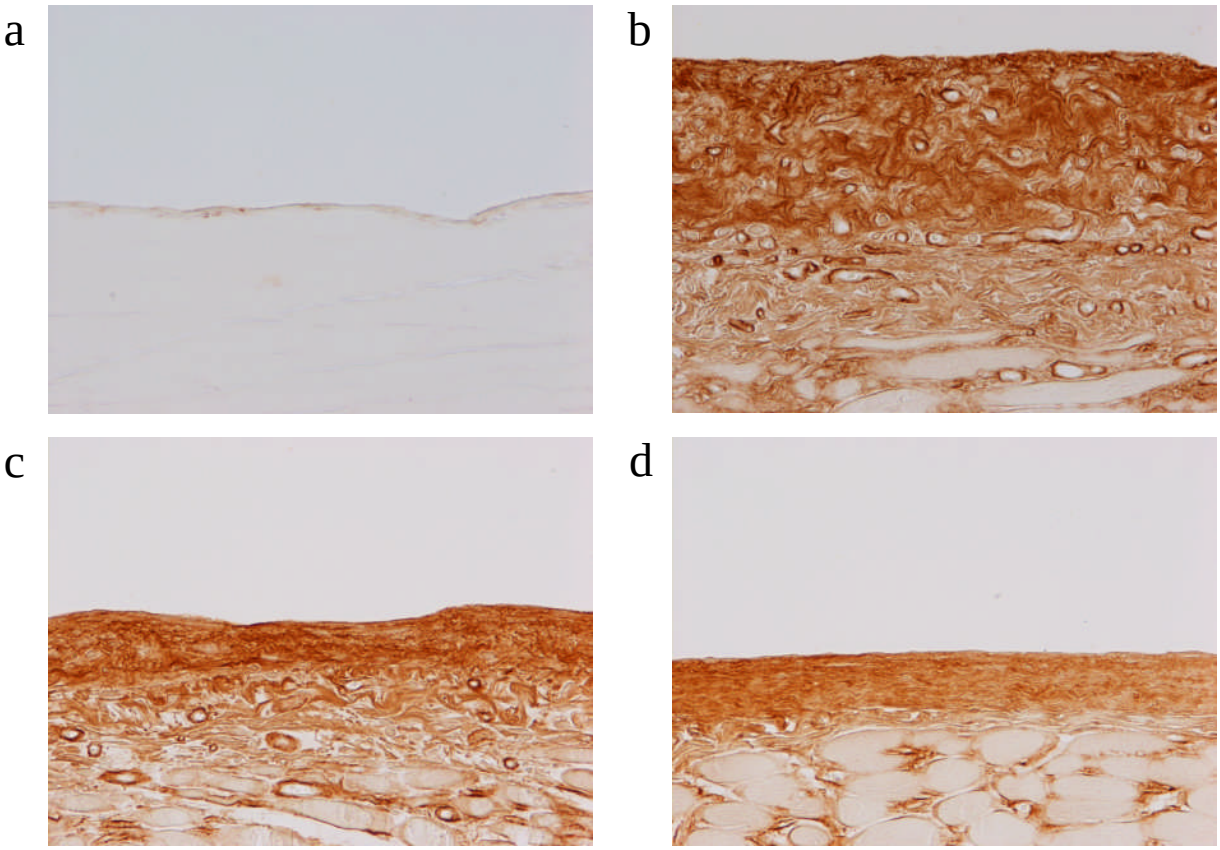

a
b
c
d

e

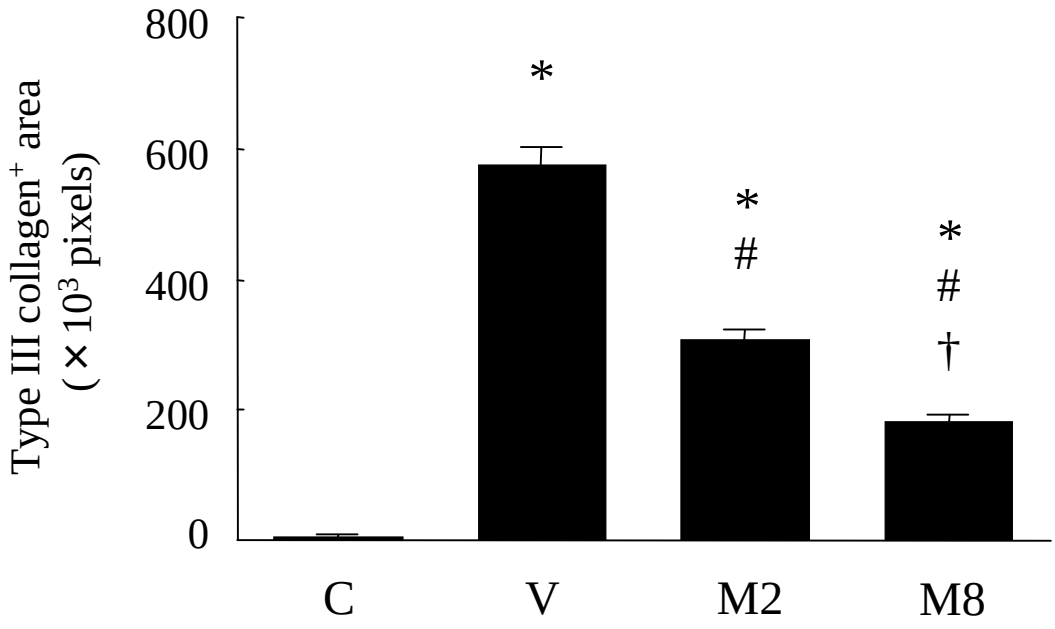
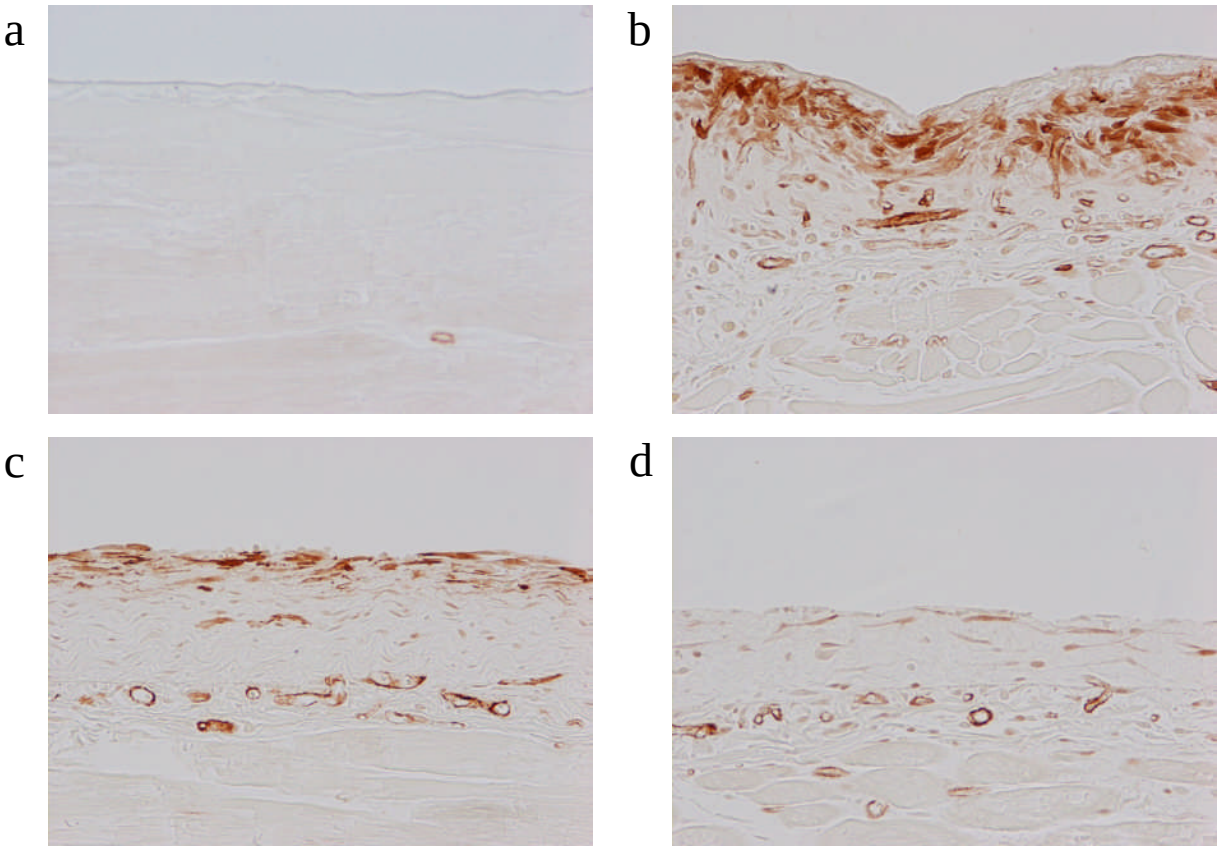
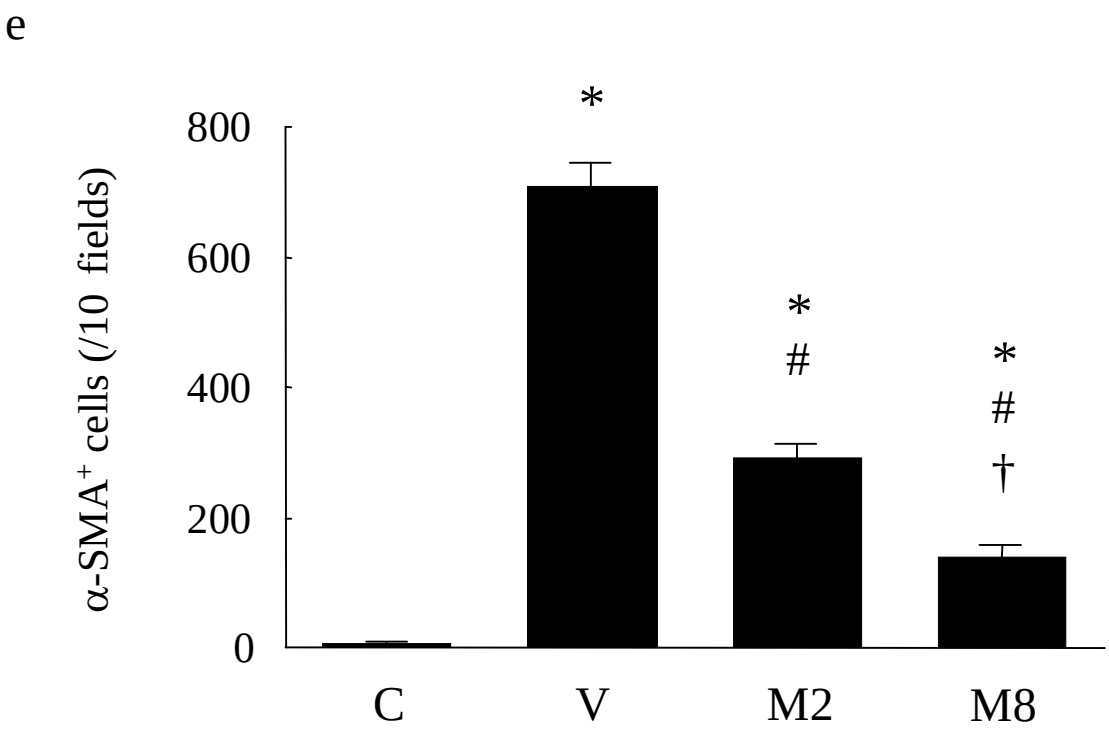

Type III collagen+ area
(× 10³ pixels)
800
600
400
200
0
*
*
#
*
#
†
C
V
M2
M8

Fig. 4.





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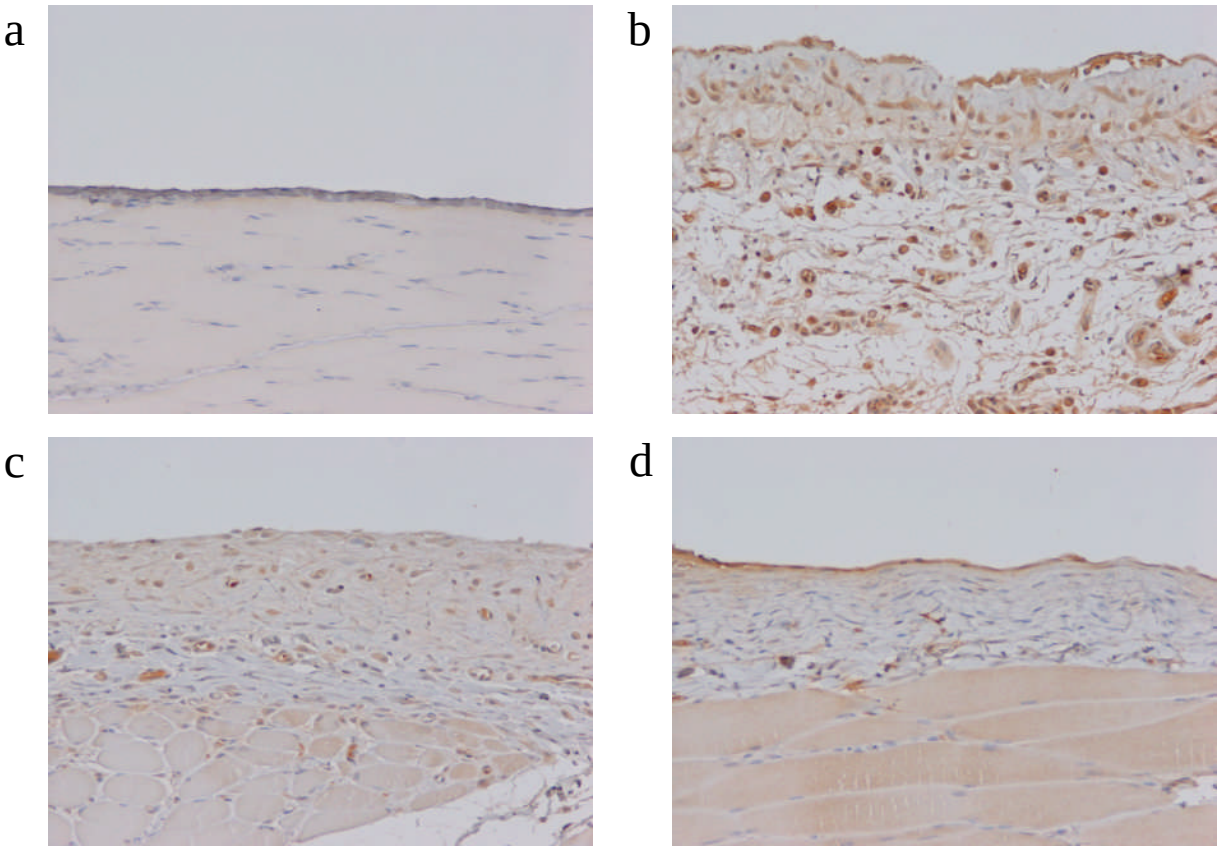

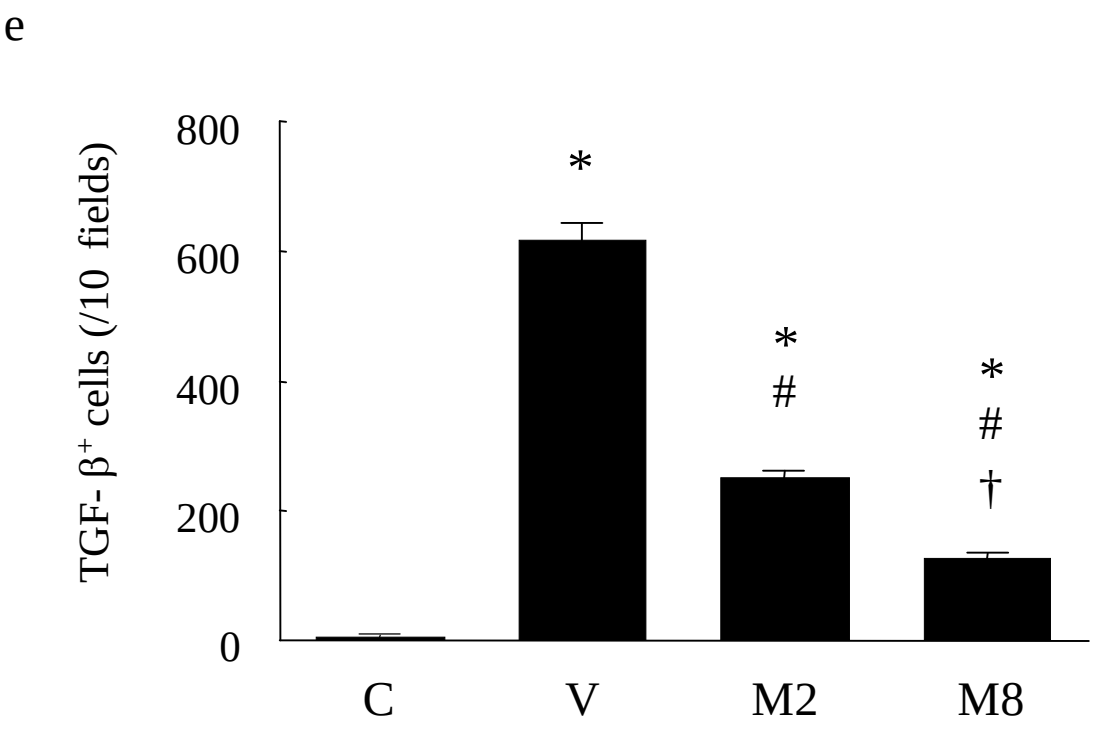

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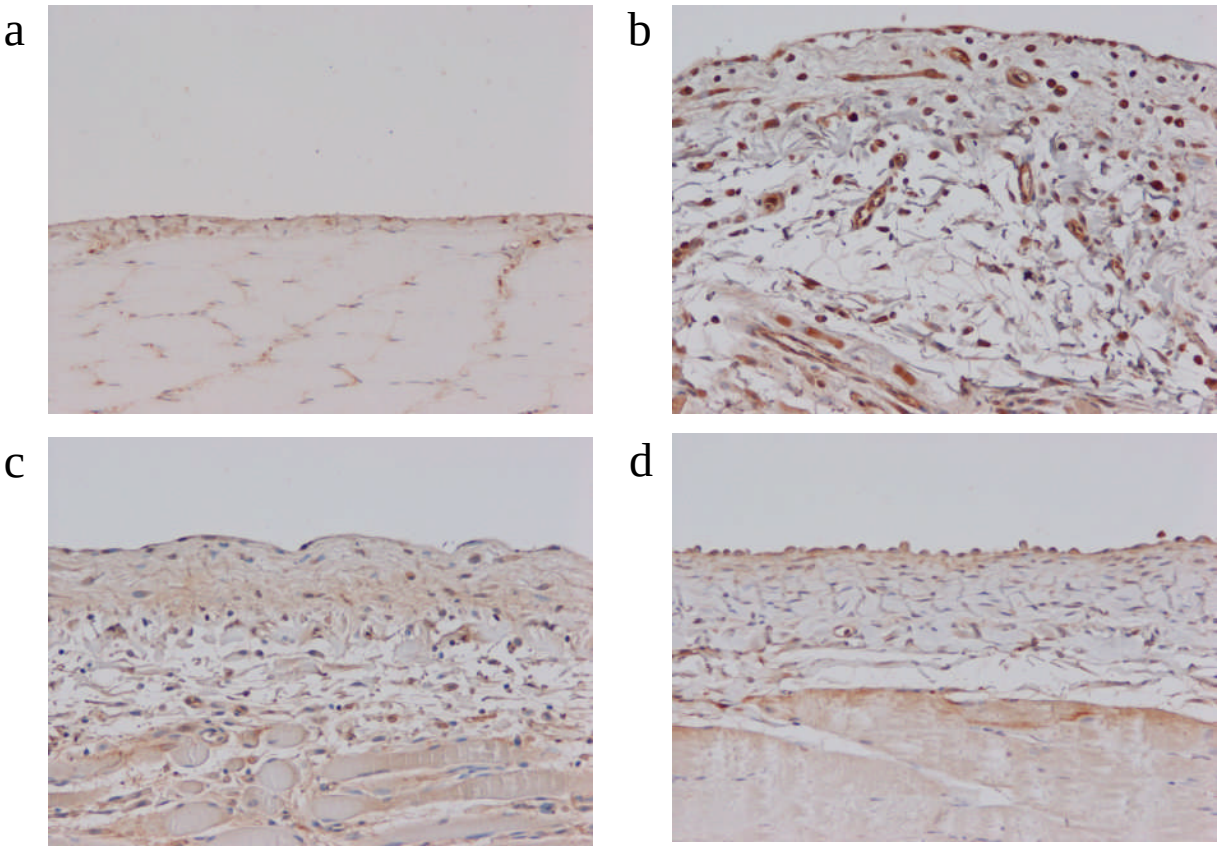
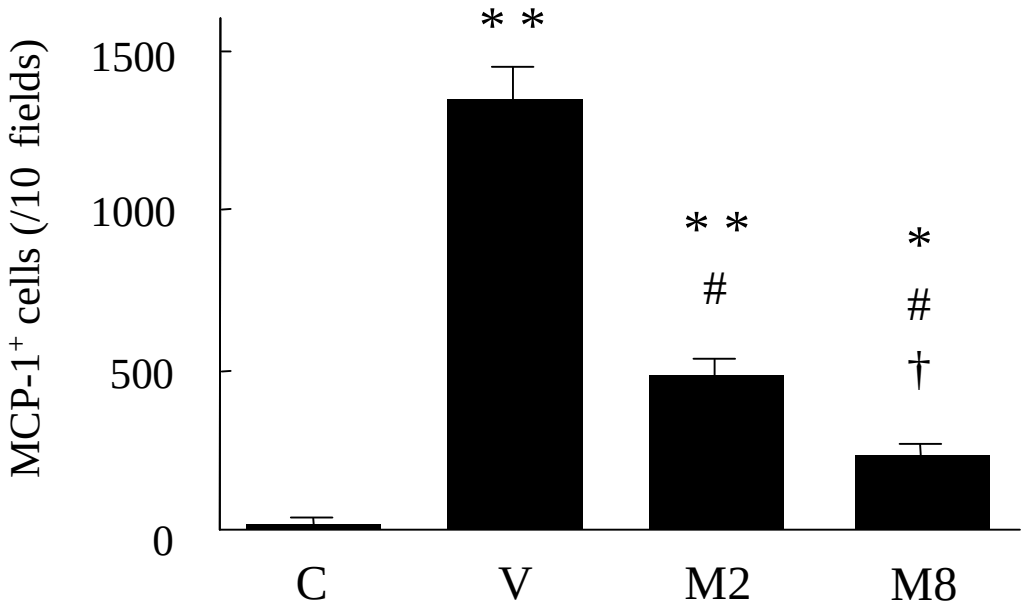


e



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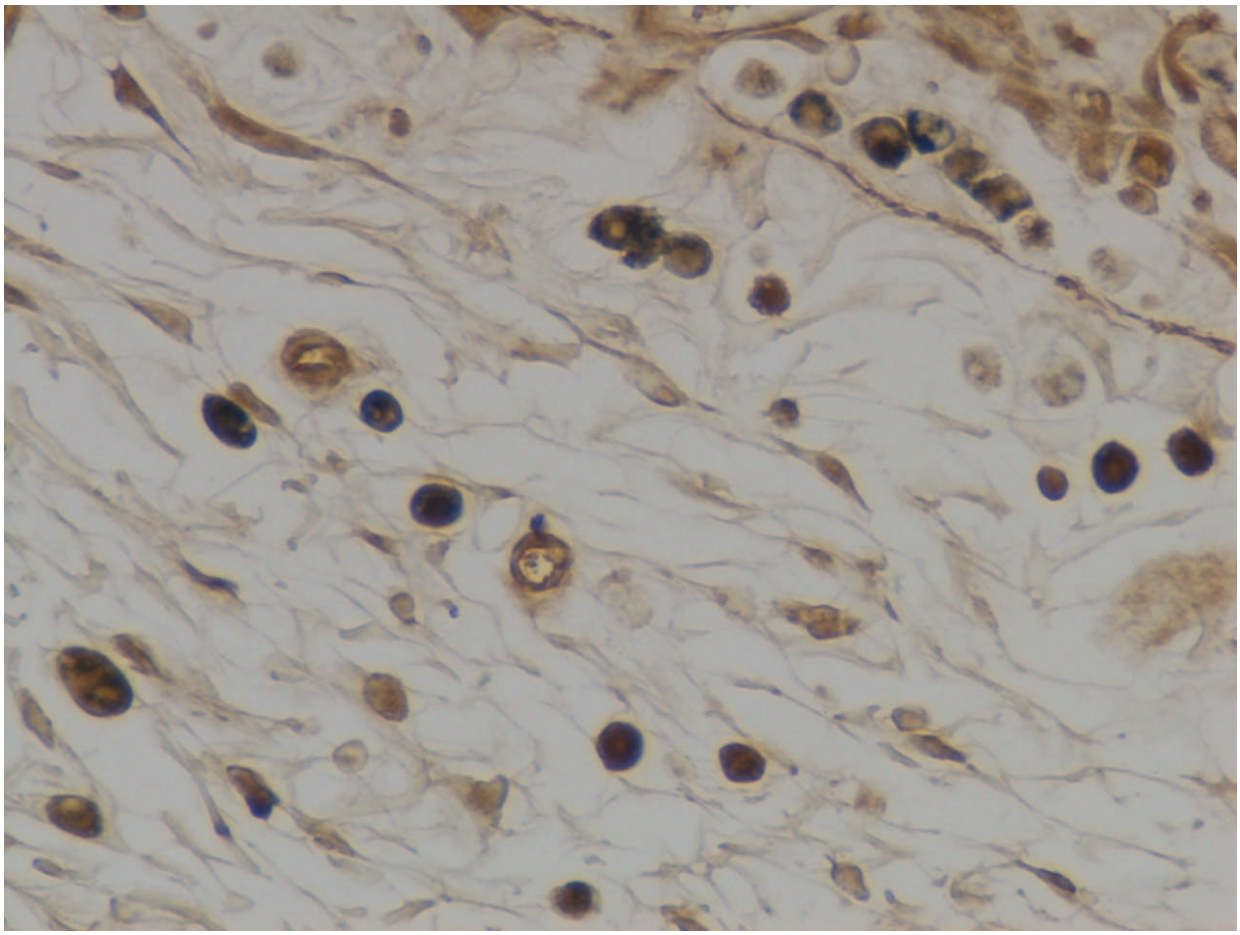

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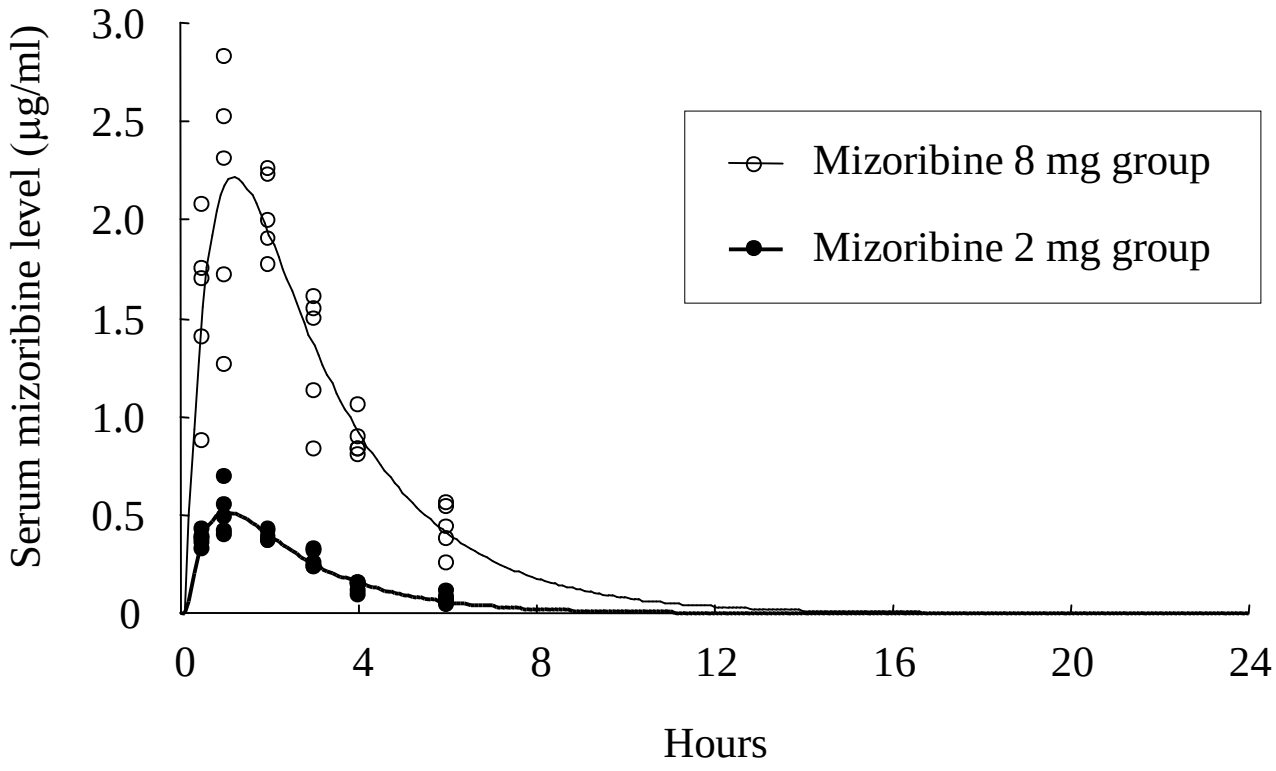
Serum mizoribine level (μg/ml)
3.0
2.5
2.0
1.5
1.0
0.5
0
0
4
8
12
16
20
24
Hours
Mizoribine 8 mg group
Mizoribine 2 mg group