Original Article

TRANILAST INHIBITS PROLIFERATION OF PANCREATIC STELLATE CELLS AND ACTIVATION OF PANCREATIC FIBROSIS

Shinji Chikazawa[1], Yusuke Tando[2], Yuki Matsuhashi[1], Miyuki Yanagimachi[1], Teruo Nakamura[3], Toshihiro Suda[4], Makoto Daimon[1]

1 Department of Endocrinology and Metabolism, Hirosaki University Graduate School of Medicine

2 Department of Medical Life Science, Hirosaki University Graduate School of Health Science

3 Health Care Center Hirosaki Medical Association

4 Aomori Rosai Hospital

Inhibition of Pancreatic Fibrosis

Shinji Chikazawa

Department of Endocrinology and Metabolism, Hirosaki University Graduate School of Medicine

TEL 0172-39-5062, FAX 0172-39-5063 E-mail: sc0810@cc.hirosaki-u.ac.jp

Abstract

Chronic pancreatitis is histopathologically characterized by the progression of pancreatic fibrosis. Pancreatic stellate cells (PSCs) play a principal role in the development of pancreatic fibrosis. Tranilast suppresses collagen synthesis of fibroblasts derived from keloid hypertrophic scars and cell proliferation of fibroblasts. Hence, we hypothesize that tranilast suppresses fibrosis and is effective against pancreatic fibrosis. In this study. We examined the effect of tranilast on cell proliferation, activation, and collagen synthesis of cultured PSCs. Tranilast significantly inhibited the proliferation of PSCs when they were cultured with and without of platelet-derived growth factor (PDGF) and decreased the expression of alpha smooth muscle actin (α-SMA) in cultures with Transforming Growth Factor-β1 (TGF-β1) and PDGF. Tranilast did not cause a significant dose-dependent reduction in the amount of collagen in the medium. Neither did it significantly affect the expression of *Col-1* mRNA

compared to the control. Our results suggest that tranilast inhibits the development of pancreatic fibrosis, making it a potential therapeutic agents for chronic pancreatitis.

Key words: alpha smooth muscle actin; platelet-derived growth factor; pancreatic stellate cells; Transforming Growth Factor-β1; tranilast.

Introduction

Chronic pancreatitis is histopathologically characterized by the progression of pancreatic fibrosis. Pancreatic stellate cells (PSCs) play a principal role in the development of pancreatic fibrosis. It has also been revealed that activated PSCs are the main extracellular matrix-producing cells involved in collagen synthesis in pancreatic fibrosis[1)]. There is no cure for chronic pancreatitis, therefore, patients suffer from malabsorption and counteract malnutrition with enzyme replacement therapy[2)]. Pancreatic fibrosis could be inhibited by the inhibition of PSCs proliferation and activation, which are potential targets for the treatment of chronic pancreatitis. In other words, it may be possible to treat chronic pancreatitis with such a treatment. Tranilast, which is extracted from *Nandina domestica*, is a therapeutic drug for allergies and keloid hypertrophic scars. It was reported to suppress collagen synthesis of fibroblasts derived from keloid hypertrophic scars and cell proliferation of fibroblasts[3)]. Hence, we

hypothesize that tranilast suppresses fibrosis and is effective against pancreatic fibrosis. In this study, we examined the effect of tranilast on cell proliferation, activation and collagen synthesis of cultured PSCs.

Materials and Methods

Materials

Collagenase and pronase were purchased from Sigma-Aldrich, Inc. (St. Louis, MO, USA). DNase I was purchased from Roche (Basel, Switzerland). Bovine serum albumin (BSA) was purchased from Life Technologies Corporation (Carlsbad, CA, USA). Nycodenz was obtained from Axis-Shield PoC (Oslo, Norway). Platelet derived growth factor (PDGF)-BB was purchased from R & D systems, Inc. (Minneapolis, MN, USA). Transforming Growth Factor-β1 (TGF-β1) was purchased from PEPROTECH (Rocky Hill, CT, USA). Dulbecco's Modified Eagle Medium-GlutaMax was purchased from

Life Technologies Corporation (Carlsbad, CA, USA). Tranilast was provided by Kissei Pharmaceutical Co.,Ltd. (Nagano, Japan). Cell-Counting Kit-8 (CCK-8) was purchased from Dojin Chemical Laboratory Co., Ltd. (Kumamoto, Japan). Sircol™ Collagen Assay Kit was purchased from Biocolor Ltd. (County Antrim, UK).

Animals

One-year-old Wistar male rats were sacrificed and the pancreas of each rat was isolated under anesthesia (50 mg/kg pentobarbital IP). The experiment was performed in accordance with Guidelines for Animal Experimentation, Hirosaki University.

Cell culture

Rat PSCs were prepared following the methods of Apte et al.[4] and Shimizu et al.[5] with modifications. The procedure was as follows. Each rat pancreas was digested with

0.05% collagenase, 0.02% pronase and 0.05% DNase I dissolved in Gey's balanced salt solution (GBSS) for 30 min at 37°C in water. The digested tissues were filtered through a clinical gauze mesh. The cells were washed and resuspended in 9.5 ml GBSS containing 0.3% BSA. The cell suspension was mixed with 8 ml of 28.7% (wt/vol) Nycodenz gradient in GBSS and centrifuged for 20 min at 1400 *g*. Cells were harvested from just above the Nycodenz cushion and maintained in Dulbecco's Modified Eagle Medium-GlutaMax-I containing 10% fetal calf serum and antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin) at 37°C in a humidified atmosphere of 5% CO_2/95% air. The cells used for experiments were obtained passages 2, 3, and 4.

Cell count

Four days after addition of tranilast, the number of PSCs stimulated by treatment with 25 ng/ml of PDGF was measured by WST-8. WST-8 detection was carried out using

CCK-8. PSCs were washed with PBS (-), and dipped in 0.025% Trypsin/0.01% EDTA for 15 min. They were then centrifuged for 5 min at 1000 rpm, and after the supernatant was discarded, the medium was added, and the cell suspension was prepared. One hundred μl of the suspension was dispensed into each well of the 96-well microplates, and 10 μl of CCK-8 was added to each well. The color development reaction in each well was conducted for 4 h in a CO_2 incubator. The absorbance was then measured at 450 nm.

Quantity of collagen in medium

The amount of collagen in the medium was quantified using Sircol™ Collagen Assay Kit, according to the manufacturer's instructions.

Western blotting

Proteins (10 μg) were separated on SDS polyacrylamide gels and electrophoretically transferred onto polyvinylidene fluoride membranes (Millipore, Bedford, MA, USA). Each membrane was blocked with 5% non-fat dried milk for 2 h and incubated overnight with primary antibodies against alpha smooth muscle actin (α-SMA) (1:200; PROGEN Biotechnik GmbH, Heidelberg, Germany), and β-actin (1:500; Santa Cruz Biotechnology, Dallas, TX, USA). After three washes with PBS-Tween (0.1%), the blots were incubated with goat anti-mouse IgG1-horseradish peroxidase (Santa Cruz Biotechnology) as secondary antibodies for 2 h at room temperature. Signals were detected using the ECL Western Blotting Detection Reagents (GE Healthcare UK Ltd, Amersham Place, UK).

Real time RT-PCR

Total RNA was extracted from PSCs using the RNeasy Mini Kit (Qiagen, Hilden,

Germany), according to the manufacturer's instructions. The extracted RNA was diluted and adjusted to 100 ng/μl. The RNA was reverse transcribed using High Capacity cDNA Transcription Kit (Applied Biosystems, Foster City, CA, USA) following the manufacturer's protocol. Relative quantification of *Col1* mRNA levels was performed by real-time PCR by StepOne and StepOnePlus Real Time PCR System (Applied Biosystems), using TaqMan primer sets (Col1a1 Rn01463848_m1, Gapdh Rn01775763_g1) purchased from Applied Biosystems.

1% $NaHCO_3$ (aq)

In this study, 1% $NaHCO_3$(aq) was used to dissolve tranilast. It was added to the medium in the control group.

Statistical analysis

Data are expressed as means ± standard deviations (S.D.). Statistical analysis was performed using GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego, CA, USA). Means were compared by one-way ANOVA, and Bonferroni corrections for post hoc tests were performed. P-values less than 0.05 were considered statistically significant.

Results

Cell culture

The primary culture cells obtained by the above methods are shown in Fig. 1a and 1b. The shape of the cells was similar to that reported in Apte et al's study[4]. These cells were considered PSCs.

Cell count

The effect of tranilast on the proliferation of PSCs was examined by a WST-8 assay for PSCs cultured with and without the addition of 25 ng/ml of PDGF-BB. In both conditions, 1×10^{-4} M and 3×10^{-4} M tranilast significantly inhibited the proliferation of PSCs (Fig. 2a and 2b).

Western blotting

The expression of α-SMA in PSCs was examined by western blot. When PSCs cultures were stimulated with 2 ng/ml of TGF-β1, the expression of α-SMA was decreased using 1×10^{-4} M and 3×10^{-4} M tranilast. On the other hand, when stimulated with 25 ng/ml of PDGF-BB, only 3×10^{-4} M tranilast, resulted in slightly decreased expression of α-SMA (Fig. 3).

Quantity of collagen in the medium

When the PSCs cultures were stimulated by 2 ng/ml of TGF-β1, the amount of collagen in the medium was quantified, and the data are shown relative to the control (%). The amount of collagen relative to the control was 121 ± 25.8% in 1×10^{-5} M, 71.4 ± 10.8% in 1×10^{-4} M, 78.8 ± 12.1% in 3×10^{-4} M tranilast. The amount was higher for 1×10^{-5} M tranilast, and lower for 1×10^{-4} M and 3×10^{-4} M tranilast compared to the amount observed in the control, but the differences were not significant (Fig. 4).

Real time RT-PCR

When the PSCs cultures were stimulated by 2 ng/ml of TGF-β1, the expression of *Col-1* mRNA relative to the control was analyzed. These relative expression levels were 1.17 ± 0.39 in 1×10^{-5} M, 0.74 ± 0.38 in 1×10^{-4} M, and 0.37 ± 0.24 in 3×10^{-4} M tranilast. The expression was inhibited in 1×10^{-4} M and 3×10^{-4} M tranilast, however, the differences between the treatments and the control were not significant (Fig. 5).

Discussion

In the healthy pancreas, PSCs are quiescent. In the event of pancreatic injury and inflammation, the quiescent PSCs become activated PSCs, which express α-SMA[4]. The PSCs proliferate via PDGF[6]. In this study, tranilast inhibited the proliferation of PSCs with and without stimulation with 25 ng/ml of PDGF-BB, and the inhibitory effect was stronger on stimulation with 25 ng/ml of PDGF than without it. Tranilast decreased the expression of α-SMA after treatment with 2 ng/ml of TGF-β1 and 25 ng/ml of PDGF-BB. These data indicate that tranilast can suppress the activation of PSCs. Furthermore, tranilast may exert an additional inhibitory effect in a pathologic environment, compared with normal conditions. Suzawa reported that tranilast did not influence cell proliferation for normal skin-derived fibroblasts[7]. Our results are consistent with this finding. It is likely that tranilast has similar effects in a pathologic

environment, which are beneficial in terms of drug safety. However, in our study, collagen in the medium could not detected by western blotting (data not shown), and tranilast did not cause a dose-dependent reduction in the amount of collagen in the medium. This might be attributed to the wide variability resulting from a smll size (n=5). Similar results were obtained for *Col-1* mRNA. The relative expression of *Col-1* mRNA indicated a small but non-significant dose-dependent reduction. The variability was clearly high owing to the small sample size (n=4). Some materials have been reported to have an inhibitory effect on pancreatic inflammation and the activation of PSCs. Curcumin, a polyphenol compound, inhibits PDGF-induced PSCs proliferation, and decreases the expression of α-SMA and collagen[8]. Candesartan, an angiotensin II receptor antagonist alleviates chronic pancreatitis and fibrosis by suppressing TGF-β1, preventing PSC activation[9]. Tranilast was reported to prevent the progression of diabetic nephropathy[10]. Diabetic nephropathy is characterized by the progression of

renal fibrosis. In other words, tranilast prevents renal fibrosis. Tranilast has also been reported to inhibit myocardial TGF-β1 expression, fibrosis in rats post-myocardial infarction and collagen production in cardiac fibrosis[11]. Therefore, tranilast may inhibit pathological fibrosis in various organs. In conclusion, although therapies targeting the inhibition of pancreatic fibrosis have not yet been realized, they show promise for inhibiting the progression of chronic pancreatitis. Tranilast is one such potential therapeutic agents for chronic pancreatitis.

Acknowledgments

We thank Mr. Kousuke Kasai for his assistance.

We also thank Kissei Pharmaceutical Co.,Ltd. for providing the tranilast used in this study.

References

1) Masamune A, Watanabe T, Kikuta K, Shimosegawa T. Roles of pancreatic stellate cells in pancreatic inflammation and fibrosis. Clin Gastroenterol Hepatol. 2009;7: s48-s54.

2) Nakamura T, Takeuchi T. Pancreatic steatorrhea, malabsorption, and nutrition biochemistry: a comparison of Japanese, European, and American patients with chronic pancreatitis. Pancreas. 1997;14:323-333.

3) Suzawa H, Kikuchi S, Ichikawa K, Koda A. Inhibitory action of tranilast, an anti-allergic drug, on the release of cytokines and PGE_2 from human monocytes-macrophages. Jpn.J.Pharmacol. 1992;60:85-90.

4) Apte M V, Haber P S, Applegate T L, Norton I D, McCaugan G W, Korsten M A, Pilola R C et al. Periacinar stellate shaped cells in rat pancreas:identification,

isolation, and culture. Gut. 1998;43:128-133.

5) Shimizu K, Shiratori K, Kobayashi M, Kawamata H. Troglitazone inhibit the progression of chronic pancreatitis and the profibrogenic activity of pancreatic stellate cells via a PPARγ-independent mechanism. Pancreas. 2004;29:67-74.

6) Apte M V, Haber P S, Applegate T L, Norton I D, McCaugan G W, Korsten M A, Pilola R C et al. Pancreatic stellate cells are activated by proinflammatory cytokines: implications for pancreatic fibrogenesis. Gut. 1999;44:534-541.

7) Suzawa H, Kikuchi S, Ichikawa K, Arai N, Tazawa S, Tsuchiya O, Momose Y et al. Effect of tranilast, an anti-allergic drug, on the human keloid tissues. Nihon Yakurigaku Zasshi. 1992;99:231-239.

8) Masamune A, Suzuki N, Kikuta K, Satoh M, Satoh K, Shimosegawa T. Curcumin blocks activation of pancreatic stellate cells. J Cell Biochem. 2006;97:1080-1093.

9) Yamada T, Kuno A, Masuda K, Ogawa K, Sogawa M, Nakamura S, Ando T et al. Candesartan, an angiotensin II receptor antagonist, suppresses pancreatic inflammation and fibrosis in rats. JPET. 2003;307:17-23.

10) Akahori H, Ota T, Torita M, Ando H, Kaneko S, Takamura T. Tranilast prevents the progression of experimental diabetic nephropathy through suppression of enhanced extracellular matrix gene expression. JPET. 2005;314:514-521.

11) See F, Watanabe M, Kompa A R, Wang B H, Boyle A J, Kelly D J, Gilbert R E et al. Early and delayed tranilast treatment reduces pathological fibrosis following myocardial infarction. Heart Lung Circ. 2013;22:122-132.

Figure Legends

Fig. 1a Light micrograph of PSCs after 7 days in primary culture from rat pancreas (×100 objective).

Fig. 1b Light micrograph of PSC after 7 days in primary culture from rat pancreas (×400 objective). The PSCs showing lipid droplets in the cytoplasm.

Fig. 2a Effect of tranilast on proliferation of PSCs using WST-8 for PSCs cultured without stimulation. The number of PSCs is shown as the % relative to the control on the *Y* axis. Tranilast concentration is shown on the X axis. Data are shown as means ± S.D. (n=5). **P < 0.01, ***P < 0.001 compared to control.

Fig. 2b Effect of tranilast on proliferation of PSCs using WST-8 for PSCs cultured with

stimulation with 25 ng/ml of PDGF-BB. The number of PSCs is shown as the % relative to the control on the *Y* axis. Tranilast concentration is shown on the *X* axis. Data are shown as means ± S.D. (n=4). ***P < 0.001 compared to control

Fig. 3 Effect of tranilast on the expression of α-SMA by western blotting, when PSC cultures were stimulation with 2 ng/ml of TGF-β1 and 25 ng/ml of PDGF-BB. β-actin was used as an internal control.

Fig. 4 Effect of tranilast on the amount of collagen in the medium quantified using the Sircol™ Collagen Assay Kit, when PSC cultures were stimulated with 2 ng/ml of TGF-β1. The amount of collagen in the medium was shown as the % relative to the control on the *Y* axis. Tranilast concentration is shown on the *X* axis. Data are shown as means ± S.D. (n=5).

Fig. 5 Effect of tranilast on *Col-1* mRNA expression by real time RT PCR, when PSCs were cultured with stimulation by 2 ng/ml of TGF-β1. Relative expression of *Col-1* mRNA is shown on the *Y* axis. Tranilast concentration is shown on the *X* axis. Data are shown as means ± S.D. (n=4)

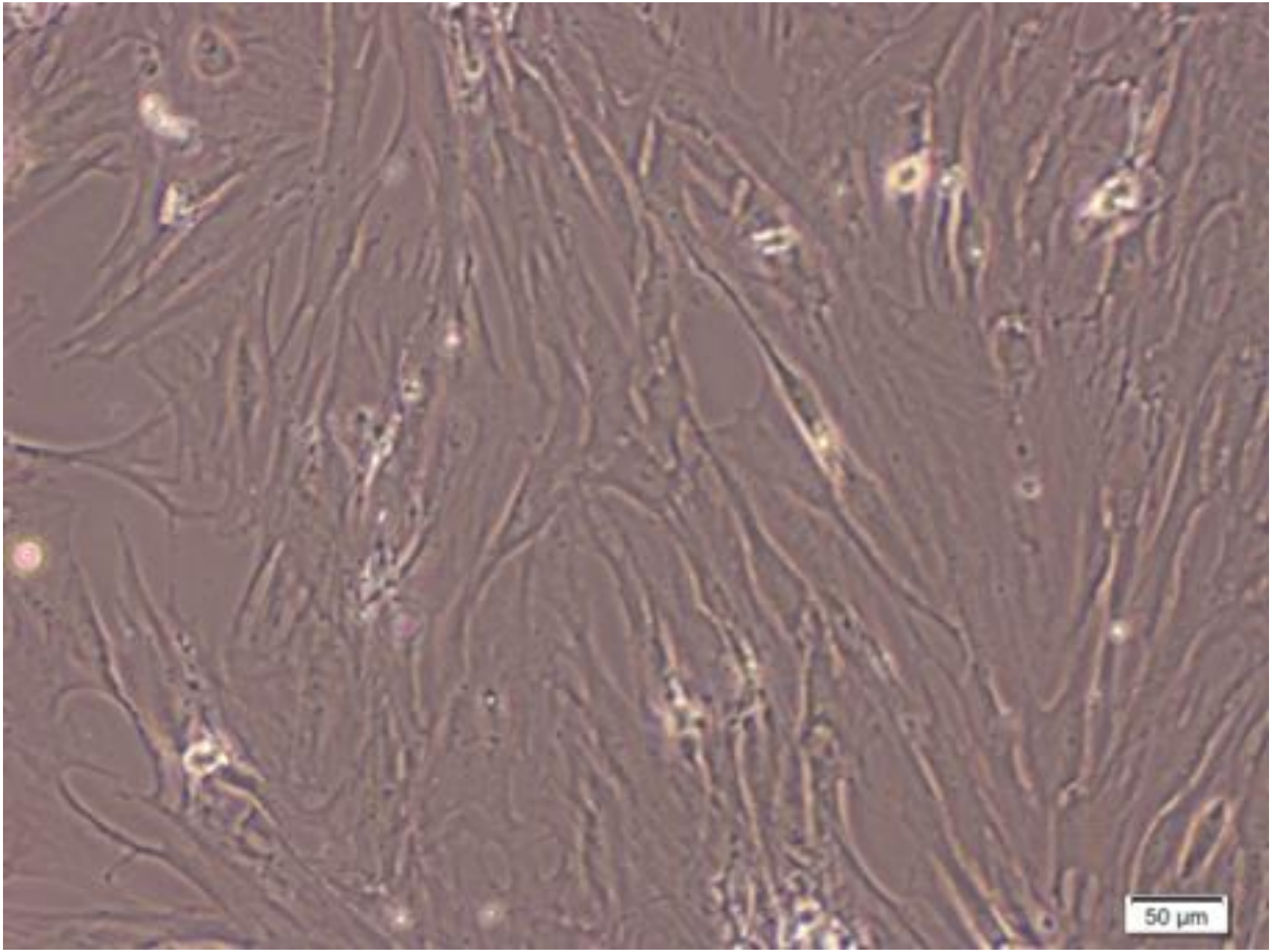

Fig. 1a

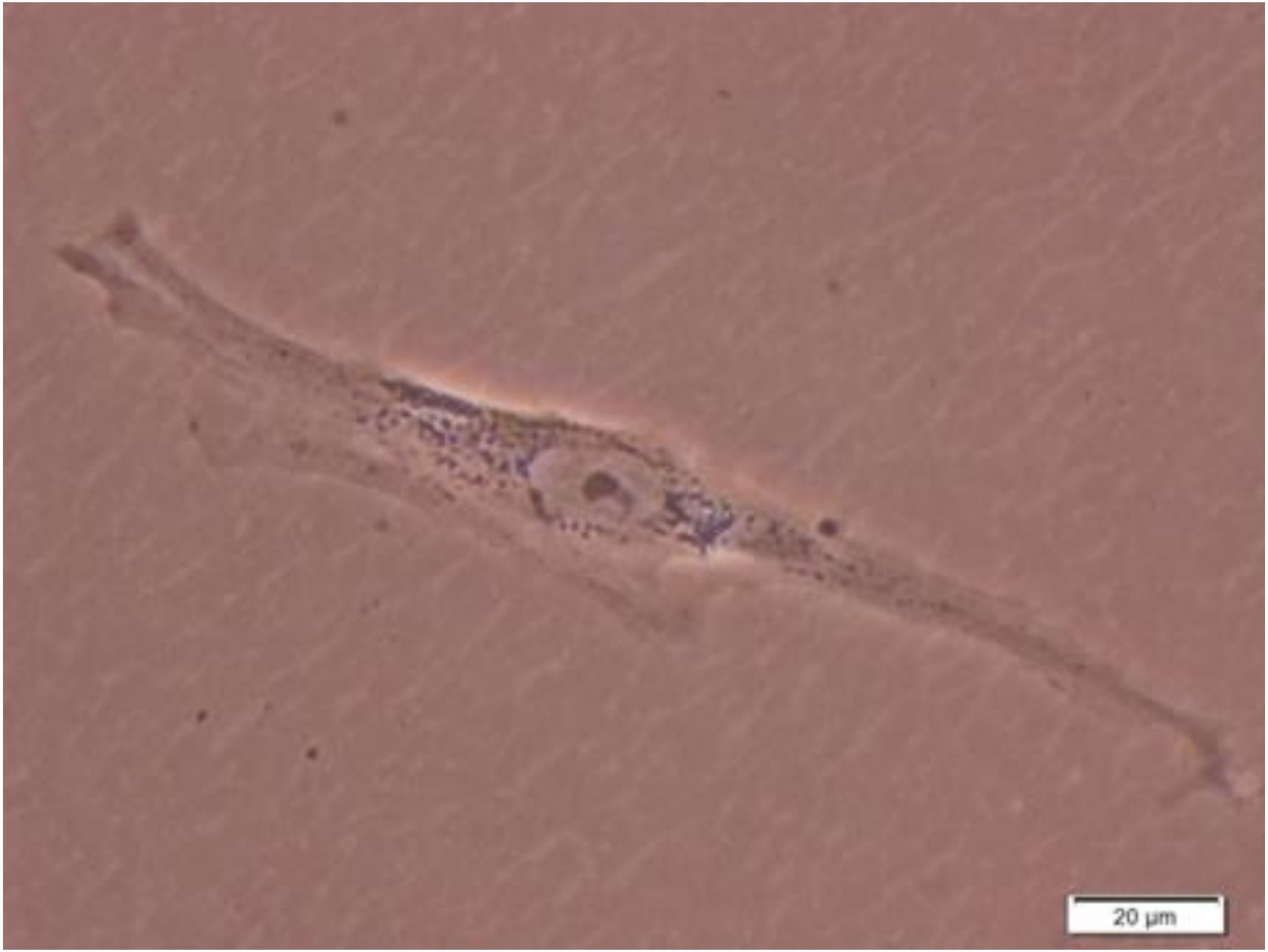

Fig. 1b

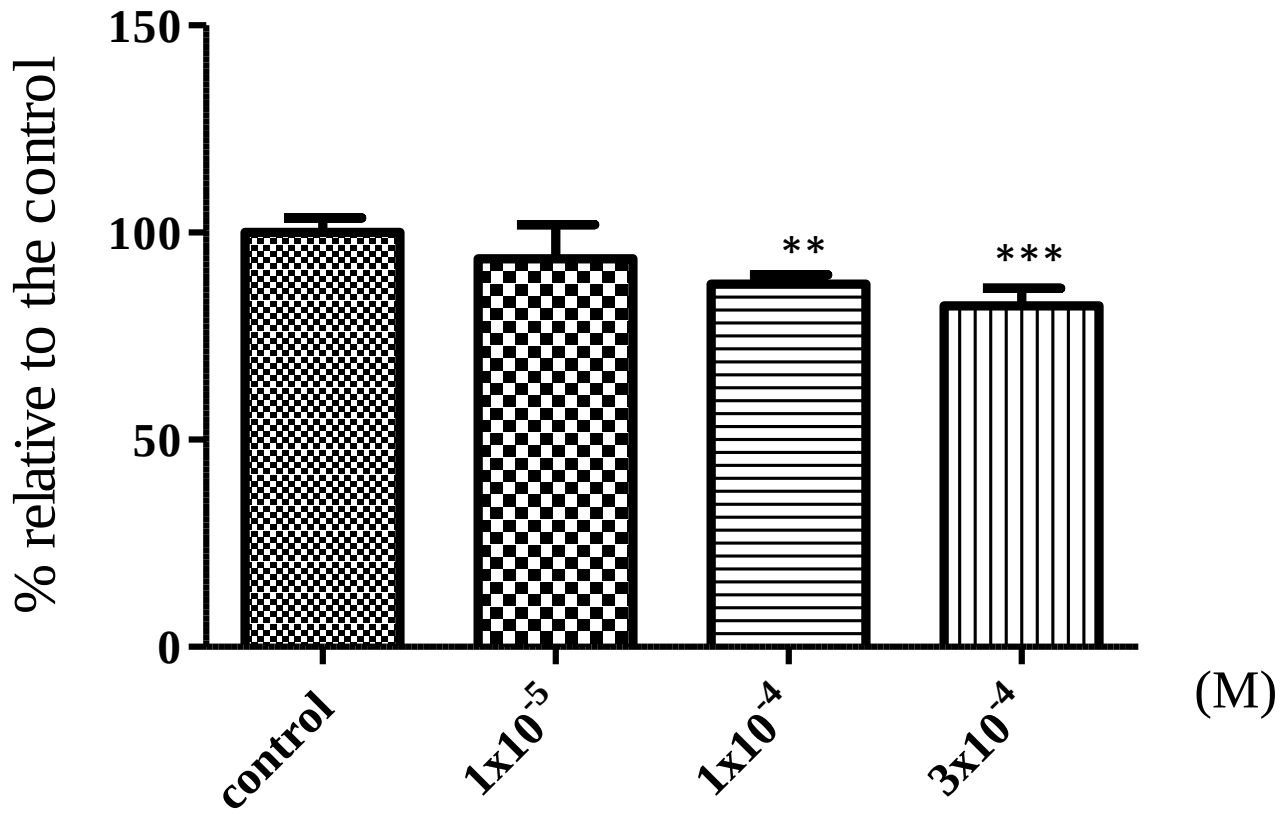
150
100
50
0
% relative to the control
**
***
control
1x10-5
1x10-4
3x10-4
(M)

Fig. 2a

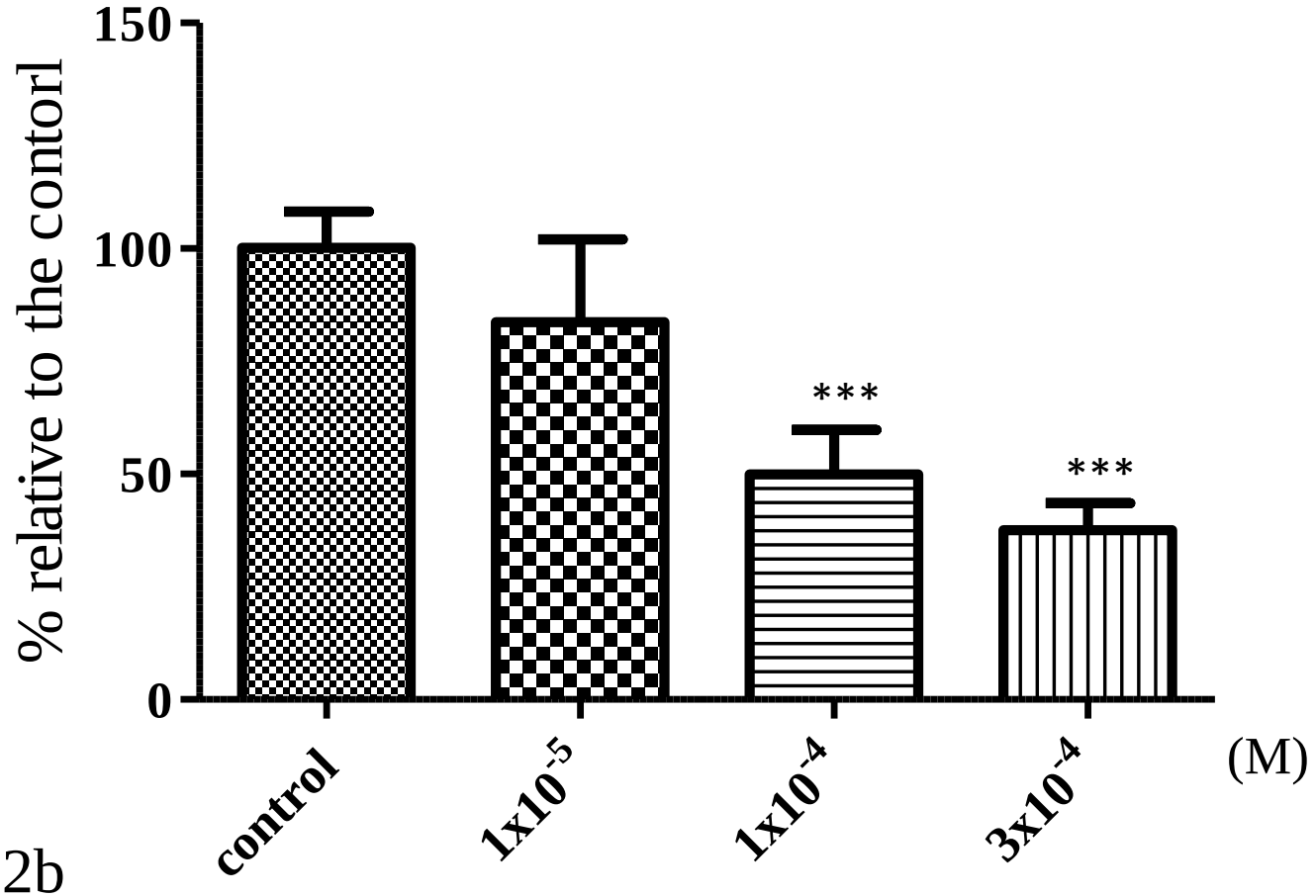
150
100
50
0
% relative to the contorl
***
***
control
1x10-5
1x10-4
3x10-4
(M)

Fig. 2b

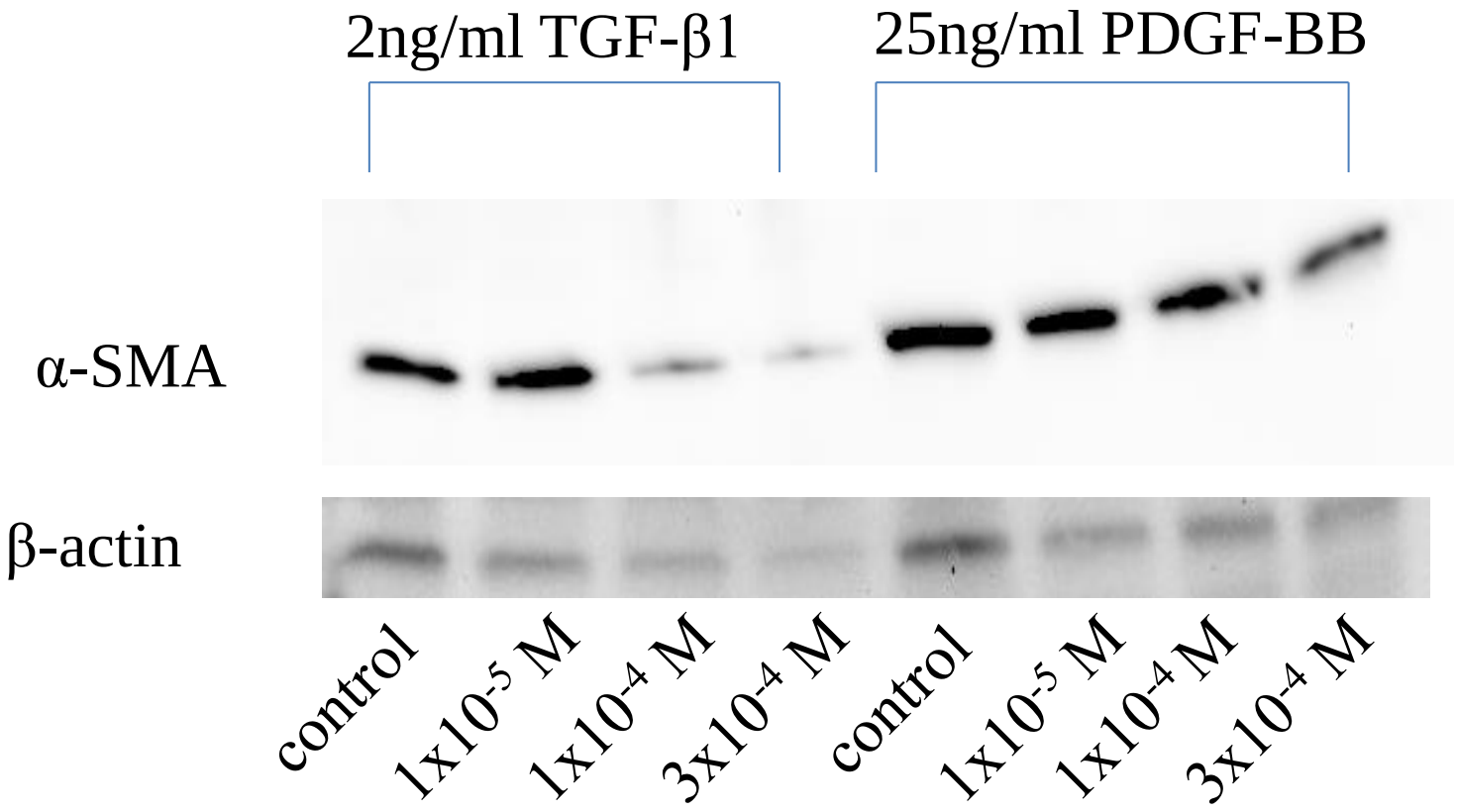
2ng/ml TGF-β1
25ng/ml PDGF-BB
α-SMA
β-actin
control
1x10-5 M
1x10-4 M
3x10-4 M
control
1x10-5 M
1x10-4 M
3x10-4 M

Fig. 3

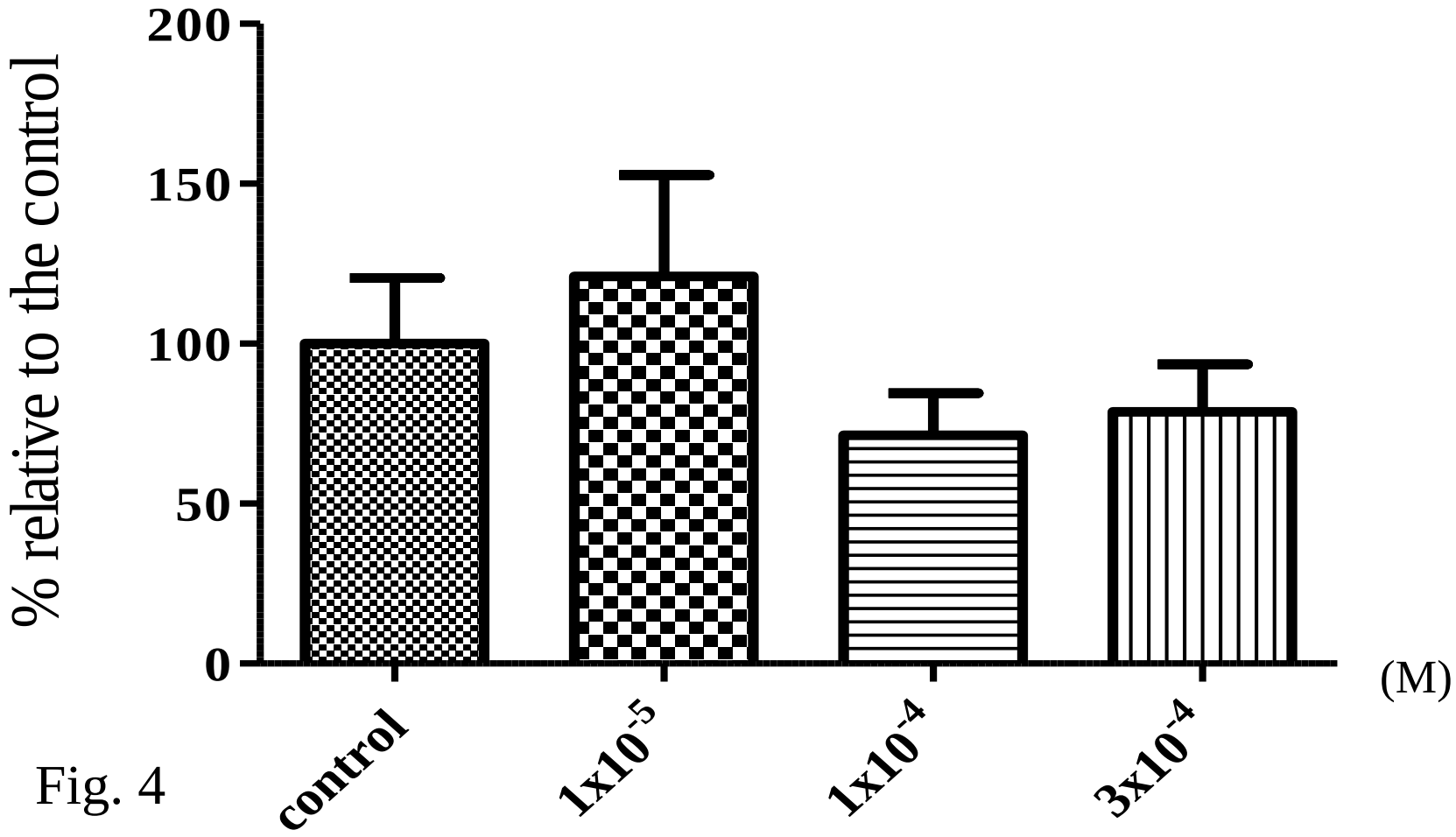
% relative to the control
200
150
100
50
0
control
1x10-5
1x10-4
3x10-4
(M)

Fig. 4

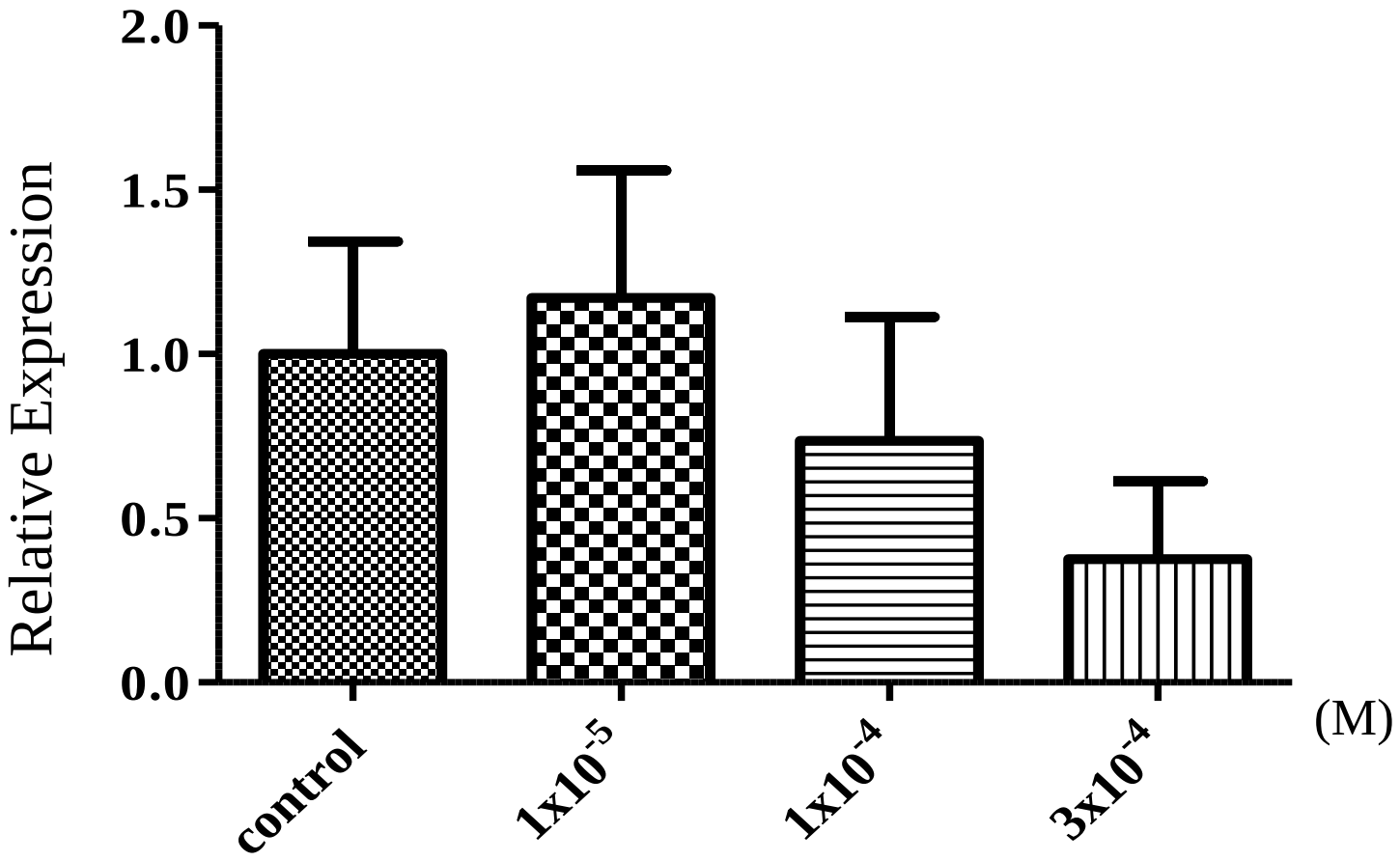
Relative Expression
2.0
1.5
1.0
0.5
0.0
control
1x10-5
1x10-4
3x10-4
(M)

Fig. 5