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The protective effect of superoxide dismutase mimetic M40401 on balloon injury-related neointima formation: role of the lectin-like oxidized low-density lipoprotein receptor-1

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Abstract

Lectin-like oxidized low-density lipoprotein receptor-1 (LOX-1), the principal receptor for oxidized low-density lipoprotein (ox-LDL) in vascular endothelial cells (ECs), has recently been suggested to exert a pivotal role in atherogenesis, possibly by mediating ox-LDL-evoked endothelial dysfunction. On the other hand, LOX-1 expression seems to strongly correlate with the oxidative stress occurring in the vascular wall of experimentally injured blood vessels. Here we investigated LOX-1 expression and superoxide generation during neointima formation in a balloon injury rat carotid artery model. To test this, we employed M40401 [a manganese(II) complex with a bis(cyclo-hexylpyridine-substituted) macrocyclic ligand], a synthetic superoxide dismutase mimetic that is a selective scavanger of superoxide. The injury was performed inserting the balloon catheter through the rat common carotid artery and after 14 days a histopatological analysis revealed a significant re-stenosis with smooth muscle cell proliferation and neointima formation that was associated with an enhanced expression of LOX-1, nitrotyrosine (the footprint of peroxynitrite) staining and lipid peroxidation as assessed by malondialdehyde (MDA) formation. Pre-treatment of rats with M40401 (0.5-10 mg/Kg i.p. daily) reduced neointima formation, MDA accumulation, nitrotyrosine staining and LOX-1 expression. Here we show that removal of superoxide formation occurring in injured arteries reduces both neointima formation and LOX-1 expression and this may represent a novel therapeutical approach in the treatment of vascular disorders in which proliferation of vascular smooth muscle cells and ox-LDL-related endothelial cell dysfunction occur.

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Introduction

Vascular injury is accompanied by proliferation of sub-endothelial vascular smooth muscle cells (SMCs) and neointima formation leading to vascular occlusion and restenosis (Carter et al., 1994; Indolfi et al., 1995). Indeed, there is evidence that proliferation of SMCs is crucially involved in restenosis after procedures such as angioplasty (Holmes et al., 1984; McBride et al., 1988; Leimgruber et al., 1986; Guiteras et al., 1987) and arterial stenting (Hoffmann et al., 1996; Roubin et al., 1992).

Many factors have been implicated in the proliferation of SMCs subsequent to vascular injury including the disruption of the endothelial cell layer (Harker et al., 1974; Asahara et al., 1995), the release of growth factors via activation of circulating leukocytes and macrophages (Serrano et al., 1997; Kastrati et al., 2000; Danenberg et al., 2002; Ross, 1999) and the overproduction of reactive oxygen species (ROS), which activate redox-sensitive signalling pathways (for a review see ref. Griendling et al., 2000). All these steps lead to the remodelling of vascular architecture via induction of both cell proliferation and apoptosis (Nishio et al., 1997; Brown et al., 1999). However, the molecular and cellular mechanisms underlying these processes still remain to be elucidated.

ROS such as superoxide (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radicals (OH^-) can directly cause cell damage, induce the expression of proinflammatory genes, enhance the catabolism of nitric oxide (NO) via the formation of peroxynitrite ($ONOO^-$), and accelerate the oxidative modification of low-density lipoprotein (LDL) (see ref. Griendling et al., 2000). In particular, evidence exists that ox-LDL, induces endothelial expression of adhesion molecules, monocyte chemotactic protein-1 (MCP-1), E and P-selectin, V-CAM, I-CAM and SMC growth factors, and impairs endothelium-dependent vasorelaxation (Cushing et al., 1990; Li and Mehta, 2000). These events occur via the activation of LOX-1, which is the most relevant receptor for ox-LDL in vascular endothelial cells (ECs) (Sawamura et al., 1997), macrophages and activated SMCs (Yoshida et al., 1998; Chen et al., 2002). LOX-1 is a type II membrane protein with a C-type lectin-like structure at the C-terminus. It

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is not constitutively expressed, but previous *in vitro* studies revealed that the expression of LOX-1 is highly induced by stimuli relevant to atherosclerosis, such as cytokines, mechanical forces, angiotensin (Ang) II, and ox-LDL itself, hemodynamic stress and oxidative stress (Kume and Kita, 2001; Mehta and Li, 2002). Furthermore, LOX-1 has been shown to be upregulated in animal and human atheromatous lesions (Kume and Kita, 2001; Chen et al., 2002; Mehta and Li, 2002). These findings suggest a pivotal role for LOX-1 in atherogenesis, possibly by mediating ox-LDL-evoked ROS-generation and endothelial dysfunction, which in turn leads to uncontrolled proliferation of vascular SMCs.

Recent studies have demonstrated that gene expression of LOX-1 is upregulated by superoxide anions, H_2O_2, Ang II and homocysteine in *in vitro* and the *in vivo* settings (Nagase et al., 2001). The enhanced expression of LOX-1 can be inhibited by antioxidants, indicating that restoring the antioxidant defenses in vascular tissues may be relevant in endothelial dysfunction associated with oxidative stress and enhanced LOX-1 expression (Nagase et al., 2001). However the clinical use of free radical scavengers such as recombinant human superoxide dismutase (SOD) and catalyse enzyme has shown limited effect, perhaps due to their short half life and to their very low penetration in vascular tissues (Flaherty et al., 1994). Recently, a class of stable, non peptidyl low-molecular weight molecules proven to possess selective catalytic rate towards superoxide comparable to the native SOD has been reported (Riley et al., 1996; Salvemini et al., 1999; Muscoli et al., 2003). These new SOD mimetics (SODm) represent a breakthrough in chemical design since they are stable *in vivo*, penetrate cells readily, have wide tissue distribution in rats, is excreted intact with no detectable dissociation and is recovered in urine and feces intact (Salvemini et al., 1999) and do not interact with other free radicals such as peroxynitrite (Muscoli et al., 2003). The use of the SODm has been suggested for treatment of diseases characterized by superoxide overproduction (Muscoli et al., 2003; Salvemini et al., 2002). In particular, evidence has been presented that M40401, a SODm (Figure 1) exerts protective effect in

many disease states, including ischemia/reperfusion injury and neurodegenerative disorders such as Parkinson and AIDS dementia complex (Mollace et al., 2002; Mollace et al., 2003a; Mollace et al., 2003b). The present study was designated to evaluate 1) the relationship between carotid artery injury, ROS formation, expression of LOX-1 receptor and SMC proliferation and 2) the effect of novel selective SOD mimetic M40401 on both LOX-1 expression and neointina formation subsequent to vascular injury.

Methods

Male Wistar rats (350-400 g, Charles River, Italy) were used for these studies. All animals were housed and cared for in accordance with the guidelines of the Institutional Animal Care and Use Committee of the University of Catanzaro "Magna Graecia"and in accordance with NIH guidelines on laboratory animal welfare. All rats were maintained under identical conditions of temperature (21 ± 1°C), humidity (60 ± 5%) and light-dark cycle and chow and water were available *ad libitum*.

Vascular injury induced by balloon angioplasty

The rats were anaesthetized with intramuscular 100 mg/kg ketamine (Sigma Chimica, Milan, Italy) and 5 mg/kg xylazine (Sigma Chimica). Carotid artery was injured using a balloon embolectomy catheter, as previously described and validated (Indolfi et al., 1995). In brief, the balloon catheter (2F Fogarty, Baxter Corporation, USA, Santa Ana, California) was introduced through the right external carotid artery into the carotid artery and the balloon was inflated at 1.5 atmosphere pressure using a calibration device (Indeflator Plus 20, Advanced Cardiovascular System, Inc., Temecula, California), and pulled three times. To keep the duration of the injury that might influence the vascular SMC proliferation constant, we maintained constant the time of balloon inflation to 18 s. In an additional group of rats (Sham, n=10), the effects of the anaesthesia and the surgical procedure (without the balloon injury) were also assessed.

Drug dosage and administration

The SOD mimetic M40401 used in this study was synthesized as previously described (Salvemini et al., 1999) and was dissolved in buffered saline (pH 7.4). M40401 (0.5-10 mg/kg) or

saline was given daily intraperitoneally (i.p.; 0.3 ml) for 14 days after balloon injury. The same drug administration protocol was performed in sham animals.

Morphological evaluation of the carotid artery

At the indicated time, animals were anesthetized with an intramuscular injection of 100 mg/kg ketamine and 5 mg/kg xylazine, and the carotid arteries were fixed by transcardiacal perfusion at 120 mm Hg with 100 ml of phosphate-buffered saline (PBS, pH 7.2), followed by 150 ml 4% paraformaldehyde (pH 7.2). The carotid arteries were removed and six cross sections were cut (each 6 micronm thick) from the approximate mid-portion of the artery. Three sections were stained with hematoxylin-eosin to demarcate cell types; and the remaining three sections were stained with aldehyde fuchsin and counterstained with Van Gieson's solution to demarcate the internal elastic lamina. The sections were photographed under low power, videodigitized and stored in the image analysis system (Mipron, Kontron Electronics, Eching, Germany) in a 512 3 512 matrix with an eight-bit gray scale and a 12-field view. The media, neointima and vessel wall were traced carefully, and the ratios between the neointima and media were calculated as previously shown (Indolfi et al., 1995). The intraobserver variability was minimal (Indolfi et al., 1995).

Malondialdehyde Determinations

Malondialdehyde (MDA; used as a biochemical marker for lipid peroxidation) was measured by a method previously described (Salvemini et al, 2002). MDA was measured 3,7 and 14 days after induction of balloon injury in carotid artery of either untreated or M40401-treated rats. Briefly, injured carotid artery of rat was surgically identified, removed and then frozen in liquid nitrogen, and homogenized in potassium chloride (1.15%). Chloroform (2 ml) was then added to each homogenate

and then spun for 30 min. The organic layer of the sample was removed and dried under nitrogen gas and re-constituted with 100 µl of saline. MDA generation was evaluated by the assay of thiobarbituric acid (TBA)-reacting compounds. The addition of a solution of 20 µl of sodium dodecyl sulphate (SDS; 8.1%), 150 µl of 20% acetic acid solution (pH3.5), 150 µl of 0.8% TBA and 400 µl of distilled water, produced a chromogenic product which was extracted in n-butanol and pyridine. Then, the organic layer was removed and MDA levels read at 532 nm and expressed as nmol MDA/g wet tissue.

Immunohistochemistry

Immunohistochemistry for LOX-1 and nitrotyrosine was carried out as described previously (Mehta et al, 2002). After transcardiacal perfusion, the carotid arteries were fixed in 4% paraformaldehyde. Cryosections (8 µm thick) were incubated with the primary anti-mouse LOX-1 (1:2000; gifted by Professor T. Sawamura, Osaka, Japan) and anti-nitrotyrosine antisera (1:2000; Cayman Chemicals) overnight at 4°C, treated with the secondary biotinylated goat anti-mouse IgG antibody (Chemicon) 1h at RT, followed by peroxidase-conjugated avidin-biotin-complexes (Vectastain Elite ABC-Peroxidase kit; Vector Laboratories). The reaction was visualized using a metal enhanced DAB kit (Pierce). Quantitation of staining was then performed by densitometry using ImageQuant 5.2 software by Molecular Dynamics (Molecular Dynamics, CA).

Western blot analysis

Carotid artery lysates from each experiment (30 µg per lane) were separated by 10% SDS–PAGE and transferred to nitrocellulose membranes. After incubation in blocking solution (4% dry non-fat milk, Sigma, St. Louis, MO), membranes were incubated with anti-LOX-1 (1:10.000, gifted by Professor T. Sawamura, Osaka, Japan) overnight at 4°C. Membranes were rinsed and then incubated

with anti-mouse IgG antibody (Amersham) for 1 h at RT, and the specific complex was detected by an enhanced chemiluminescence detection system (ECL, Amersham) and relative intensities of protein bands were analyzed by MSF-300G Scanner.

Statistical analysis

Results are shown as mean ± s.e.m. for n animals. Unless specified, statistical analysis was done using ANOVA followed by post hoc Tukey's test. A P value ≤0.05 was considered significant

Results

In rats undergoing balloon injury of left carotid artery, a significant proliferation of sub-endothelial vascular SMCs occurred when compared to sham operated animals (Figure 2). Indeed, the mechanical injury, was accompanied by disruption of EC layer of injured blood vessels with active proliferation of SMCs and subsequent neointima formation (Figure 2). In particular, cross sectional area of vessel wall increased significantly 7 days after injury, reaching its maximum 14 days after balloon angioplasty (n= 20; Figure 3), an effect accompanied by similar changes occurring in the intima/media ratio (Figure 2). Early phases of neointima formation were characterized by an intense production of MDA and nitrotyrosine staining in vascular tissues of injured rats (Figure 4 and 5; n=20), indicating overproduction of ROS in carotid arteries with balloon injury. In particular, both MDA accumulation and nitrotyrosine staining in proliferating tissue occurred early in the post-injury period (day 3) and remained at high levels during development of neo-intima (day 7 and 14 ; Fig. 4 and 5). A similar effect was found when LOX-1 expression in neointima of injured carotid artery was examined. Indeed, both immunohistochemical staining of LOX-1 and Western blot analysis revealed significant expression of LOX-1 receptor in proliferating SMCs by day 3 after injury (Figure 6; n=20). This effect was also found on days 7 and 14 after injury, suggesting that an active expression of LOX-1 receptor accompanied the process of restenosis (Figure 6).

Treatment of rats with M40401 (0.5-10 mg/Kg given i.p. daily after balloon injury) significantly antagonized balloon-induced neointima formation (n= 10 for each dose; Figure 2 and 3). Indeed, both cross sectional area of injured carotid artery and intima/media ratio (data not shown) were reduced dose-dependently by daily administration of the M40401 SOD mimic. In addition, treating rats over the post-injury period with M40401 (0.5-10 mg/Kg given i.p. daily after angioplasty; n = 10 for each dose) significantly reduced MDA formation, nitrotyrosine staining and LOX-1 expression in vascular tissue (Figure 4, 5 and 6; Table 1). Treatments with M40401 (10 mg/kg given i.p. daily after

angioplasty, n = 10) did not modified the serum cholesterol and triglyceride levels or the serum lipoproteins such as HDL, LDL, IDL and VLDL (data not shown). All these effects occurred by day 3, indicating that oxidative stress and LOX-1 expression are early events in the biochemical changes which can be found in vascular tissue after induction of injury, and that restoring antioxidant status by treating rats with M40401 antagonized both free radical formation and LOX-1 expression and, finally, neointima formation.

Discussion

The present data show that balloon injury of the rat carotid artery is accompanied by proliferation of sub-endothelial SMCs and that this effect is associated with oxidative stress and overexpression of ox-LDL receptor LOX-1. Furthermore, balloon-induced vascular injury, SMC proliferation and expression of LOX-1 and nitrotyrosine are all inhibited by M40401, a novel non peptidyl SOD mimic, suggesting that removal of superoxide generated in the injured vascular tissues leads to potent protective effect against SMC proliferation following angioplasty.

This *in vivo* study indicates that superoxide anion generation is a crucial step in activating proliferation of sub-intimal SMCs, which follows vascular injury and that LOX-1 appears to be involved in this process, which leads to the reactive neointima formation.

It is known that intracellular and extracellular production of ROS and the consequent activation of specific signaling pathways and induction of redox-sensitive genes coordinate several integrated physiological responses in vascular tissues, including growth of SMCs, induction of an inflammatory response, and impairment of endothelium-dependent relaxation (Griendling et al, 2000). In particular, production of superoxide is up-regulated by hormone-sensitive enzymes such as the vascular NAD(P)H oxidases, and its metabolism is kept under tight control by the endogenous antioxidant system such as SOD, catalase, and glutathione peroxidase. ROS serve as second messengers to activate multiple intracellular proteins and enzymes, including the epidermal growth factor receptor, c-Src, p38 mitogen-activated protein kinase, Ras, and Akt/protein kinase B (Griendling et al, 2000). Activation of these signaling cascades and redox-sensitive transcription factors leads to induction of many genes with important functional roles in the pathophysiology of vascular cells (Griendling et al, 2000). Thus, ROS participate in vascular SMC growth and migration, modulation of EC function, expression of a proinflammatory phenotype, and modification of the extracellular matrix. All these events play

important roles in vascular diseases, such as hypertension and atherosclerosis, suggesting that ROS and the associated signaling pathways may represent important therapeutic targets.

Our data indicate that LOX-1 may be a crucial link between ROS generation and activation of redox-sensitive genes involved in SMC proliferation. Previous *in vitro* studies have indicated that LOX-1 expression is upregulated by a host of stimuli including inflammatory cytokines, mechanical forces and ox-LDL (Kume et al., 1998; Aoyama et al., 1999; Kunsch and Medford, 1999). All these result in production of ROS, which have been shown to upregulate LOX-1 in the reperfused tissues (Li et al., 2003). In addition, evidence exists that ROS can activate redox-sensitive transcription factors such as NF-kB and AP-1 (Kunsch and Medford, 1999) and that the ROS-related activation of NF-kB and AP-1 leads to activation of the promoter region of the LOX-1 gene (Cominacini et al., 2000). Finally, it has recently been reported that ROS are generated upon stimulation of LOX-1 by ox-LDL, which subsequently activate the transcription factor NF-kB, which in turn upregulated Ang II type 1 receptor with subsequent overexpression of LOX-1 receptor in a positive feedback fashion (Li et al., 2000). Thus, it can be suggested that the resultant ROS would further upregulate LOX-1 expression, leading to the formation of a feedback loop (Fig. 7).

This concept is in accordance with our data. Indeed, both generation of ROS, as shown by the nitrotyrosine staining, the footprint of the highly reactive peroxynitrite, in vascular tissues and LOX-1 expression occurred earlier than the neo-intima formation, which reached its peak at two weeks after balloon injury. On the other hand, M40401, which selectively inhibits superoxide formation, reduced ROS generation (as shown by the decrease in MDA formation and nitrotyrosine staining in injured vessels), LOX-1 expression and, finally, neo-intima formation suggesting that oxidative stress triggers the cascade of events which generates, via inflammation of vascular wall and LOX-1 expression, the proliferation of SMC which accompanied balloon injury.

In conclusion, our studies suggest that oxidative stress occurring in injured arteries triggers both LOX-1 expression and neointima formation, and this may be relevant in the treatment of vascular disorders in which proliferation of vascular smooth muscle cells and ox-LDL-related EC dysfunction occur.

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Figure 1- Chemical structure of M40401

Figure 2. Balloon injury is accompanied by neointima formation of carotid artery, compared to sham operated rats. Cross sectional area (A) and neointima/media ratio (N/M ratio; C) are increased in injured vessel as shown by representative histological examination (B). M40401 (10 mg/Kg given i.p. daily in the post-angioplasty period (14 days), reversed this effect. * $P<0.05$ untreated *vs* M40401 treated rats.

Figure 3. The time-dependent (3, 7 and 14 days; A) effect of balloon injury (BI) in cross sectional area of carotid artery is reversed by M40401 (0.5-10 mg/Kg given i.p. daily for 14 days), dose-dependently reversed the restenosis of the injured vessel (B). † $P<0.001$ when compared to sham; * $P<0.05$, ** $P<0.001$ treated *vs* M40401-untreated rats.

Figure 4. Balloon injury (BI) is accompanied by malondialdehyde (MDA nmol/g $^{-1}$wet tissue) accumulation in carotid artery 3, 7 and 14 days after injury, compared to sham operated rats. M40401 (10 mg/Kg) was given given i.p. daily in the post-angioplasty period (A). M40401 (0.5-10 mg/Kg given i.p. daily in the post-angioplasty period (14 days), reversed this effect (B). * $P<0.05$, ** $P<0.001$ untreated *vs* M40401 treated rats.

Figure 5. Balloon injury (BI) is accompanied by time-dependent nitrotyrosine staining in carotid artery 3, 7 and 14 days after injury, compared to sham operated rats. M40401 (10 mg/Kg given i.p. daily during the post-angioplasty period) reversed this effect. * $P<0.05$ untreated *vs* M40401 treated rats.

Figure 6. Neo-intima formation in carotid artery 3, 7 and 14 days after balloon injury is accompanied by intense expression of LOX-1 receptor as shown by both immunohistochemical examination and

western blotting analysis. M40401 (10 mg/Kg given i.p. daily during the post-angioplasty period) reversed this effect. * P<0.05 untreated *vs* M40401 treated rats.

Figure 7. LOX-1 expression, triggered by ROS after balloon injury, leads to furter generation of free radical species which, in turn, activate redox-sensotive genes which contribute in restenosis. M40401, via inhibition of superoxide anions selectively, blocks these LOX-1 mediated events which follow balloon injury in rats.

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Table 1. The effects of M40401 on LOX-1 expression and nitrotyrosine staining after ballon injury.

	LOX-1	**Nitrotyrosine staining**
Sham	0.130 ± 0.015	0.142 ± 0.012
BI 14dd	0.503 ± 0.011†	0.638 ± 0.023†
BI 14dd +M40401 0.5 mg/kg	0.424 ± 0.025*	0.416 ± 0.17
BI 14dd +M40401 1 mg/kg	0.331 ± 0.018**	0.270 ± 0.014*
BI 14dd +M40401 5 mg/kg	0.180 ± 0.021**	0.168 ± 0.018**
BI 14dd +M40401 10 mg/kg	0.150 ± 0.022**	0.125 ± 0.020**

Data are expressed as densitometry units and represent the mean ± sem of 6 independent experiments.
† P<0.001 when compared to sham; * P<0.01, ** P<0.001 treated *vs* M40401-untreated rats.

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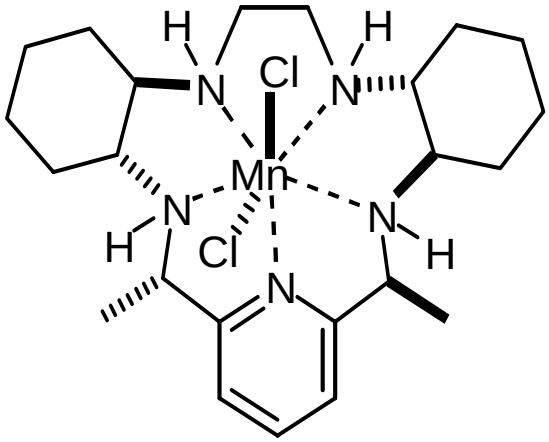
Figure 1



M40401

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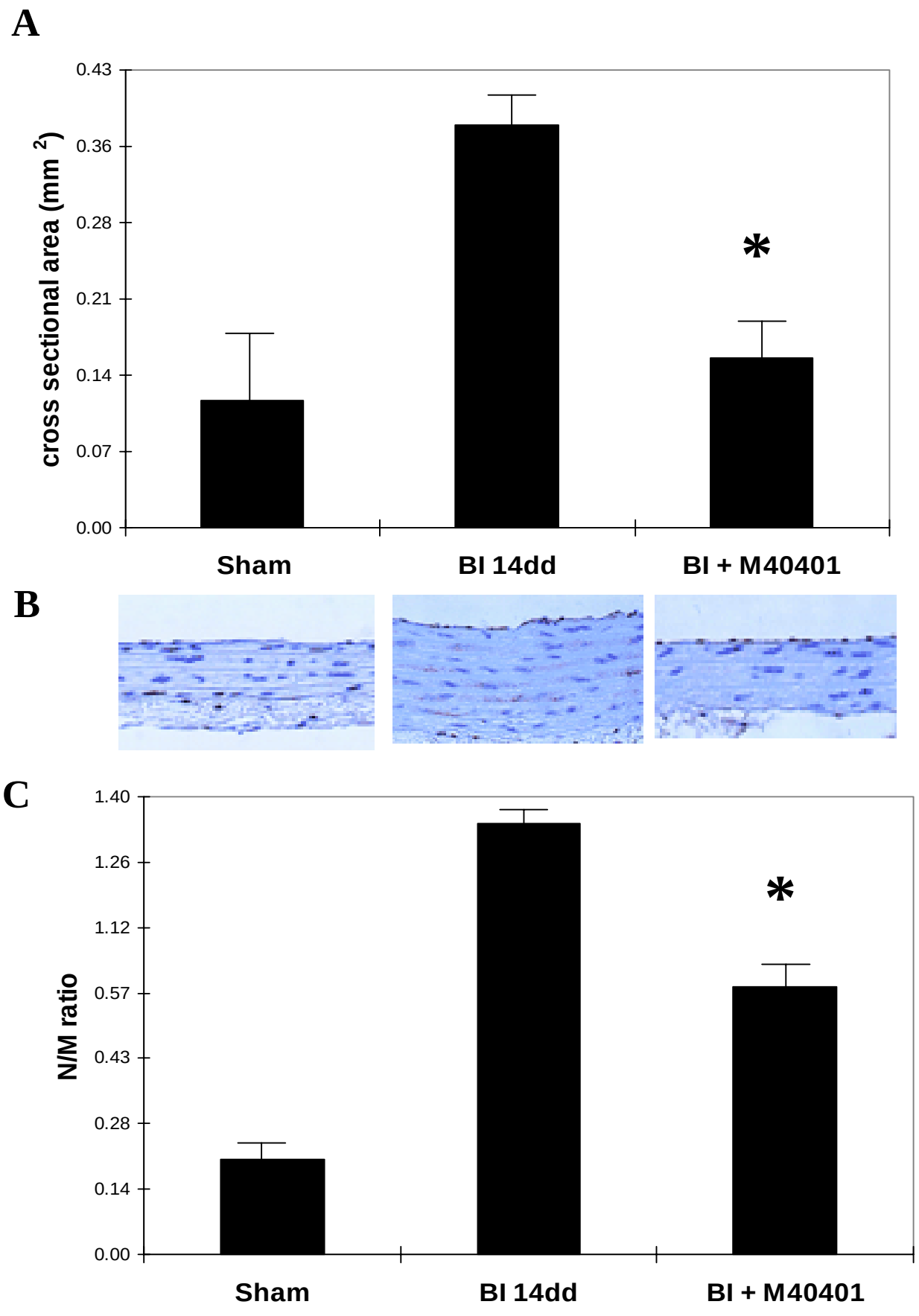

Figure 2

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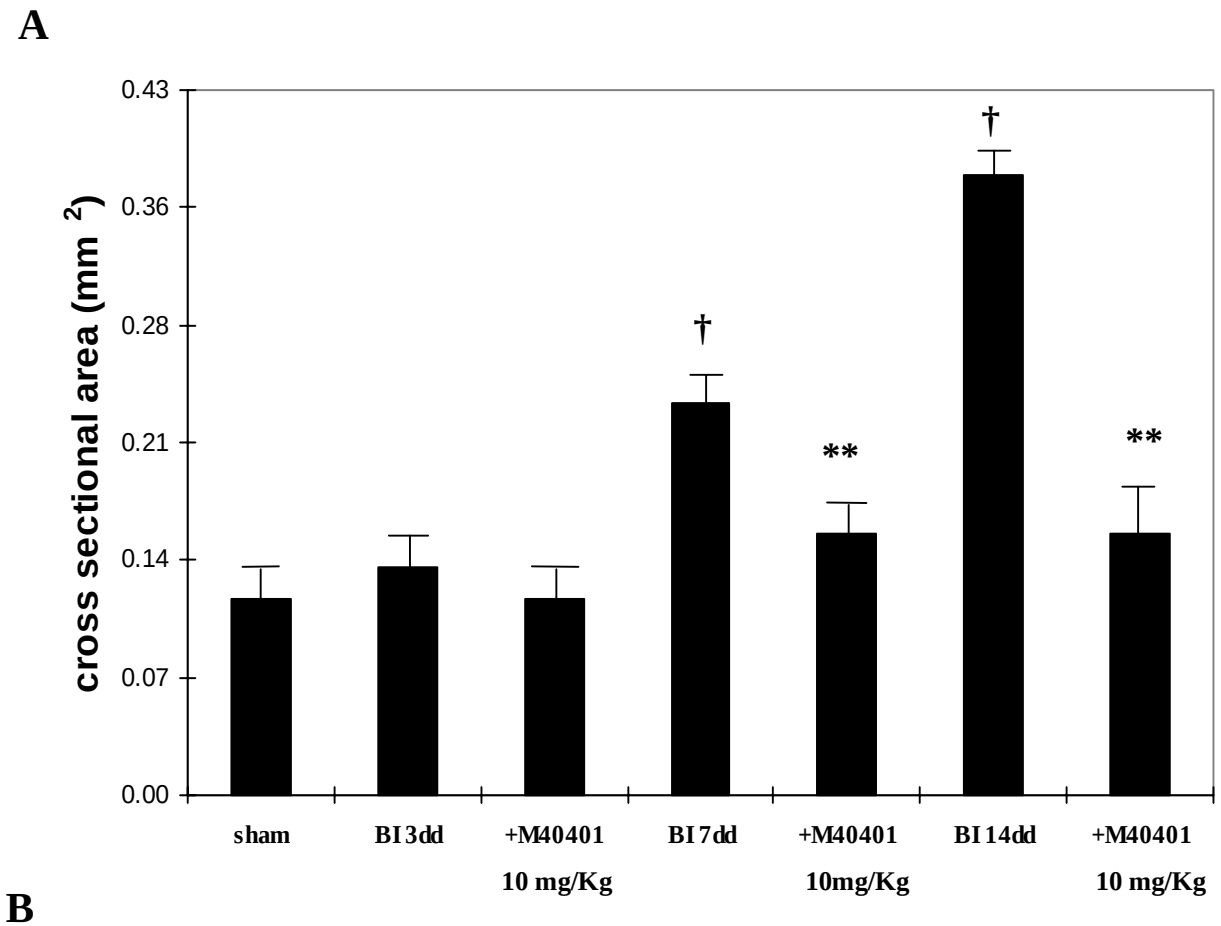

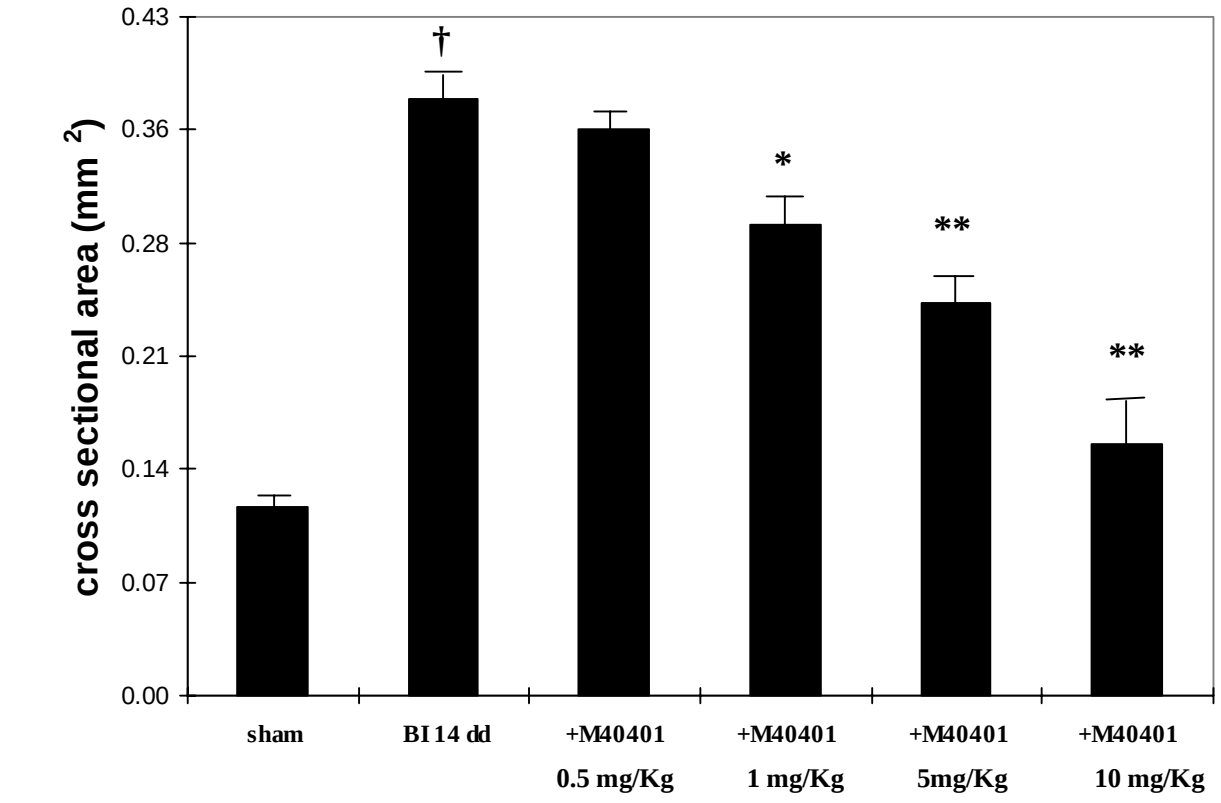

Figure 3

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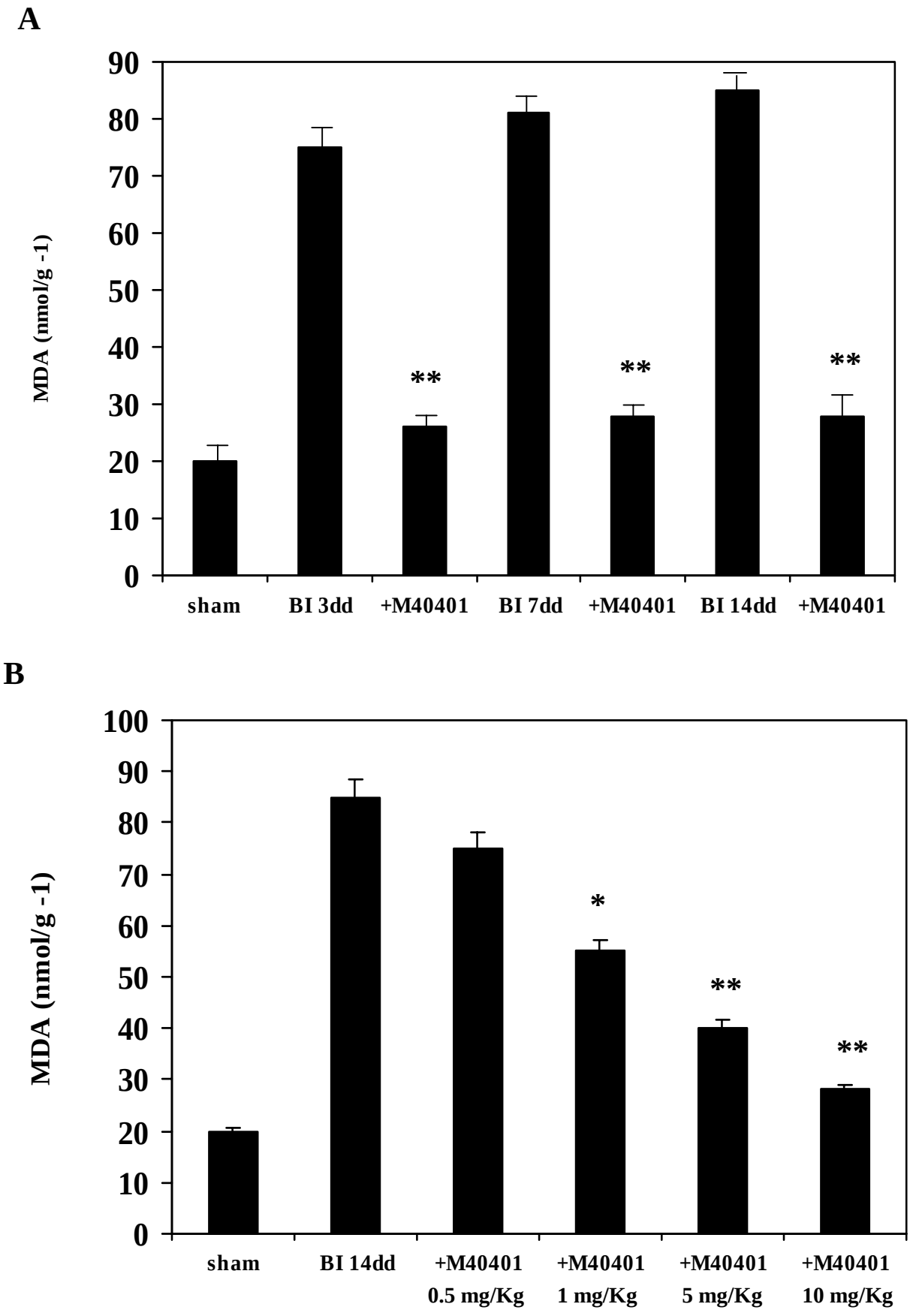

Figure 4

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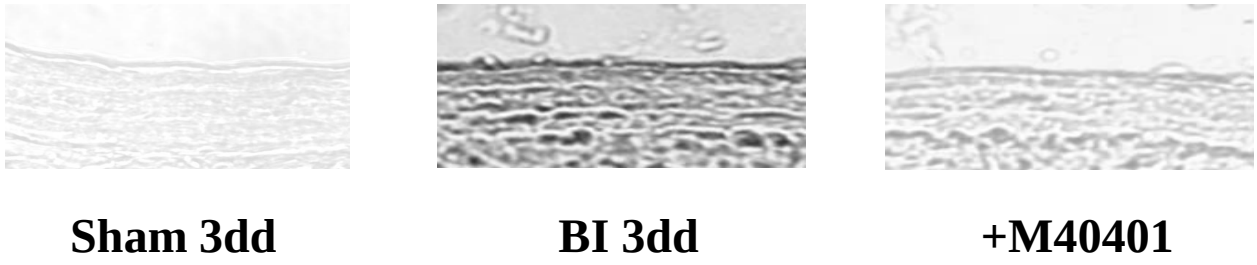
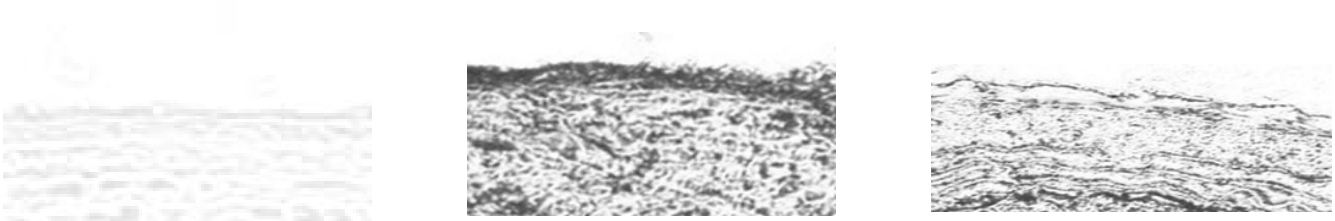


Sham 3dd **BI 3dd** **+M40401**

Sham 7dd **BI 7dd** **+M40401**



Sham 14dd **BI 14dd** **+M40401**

Figure 5

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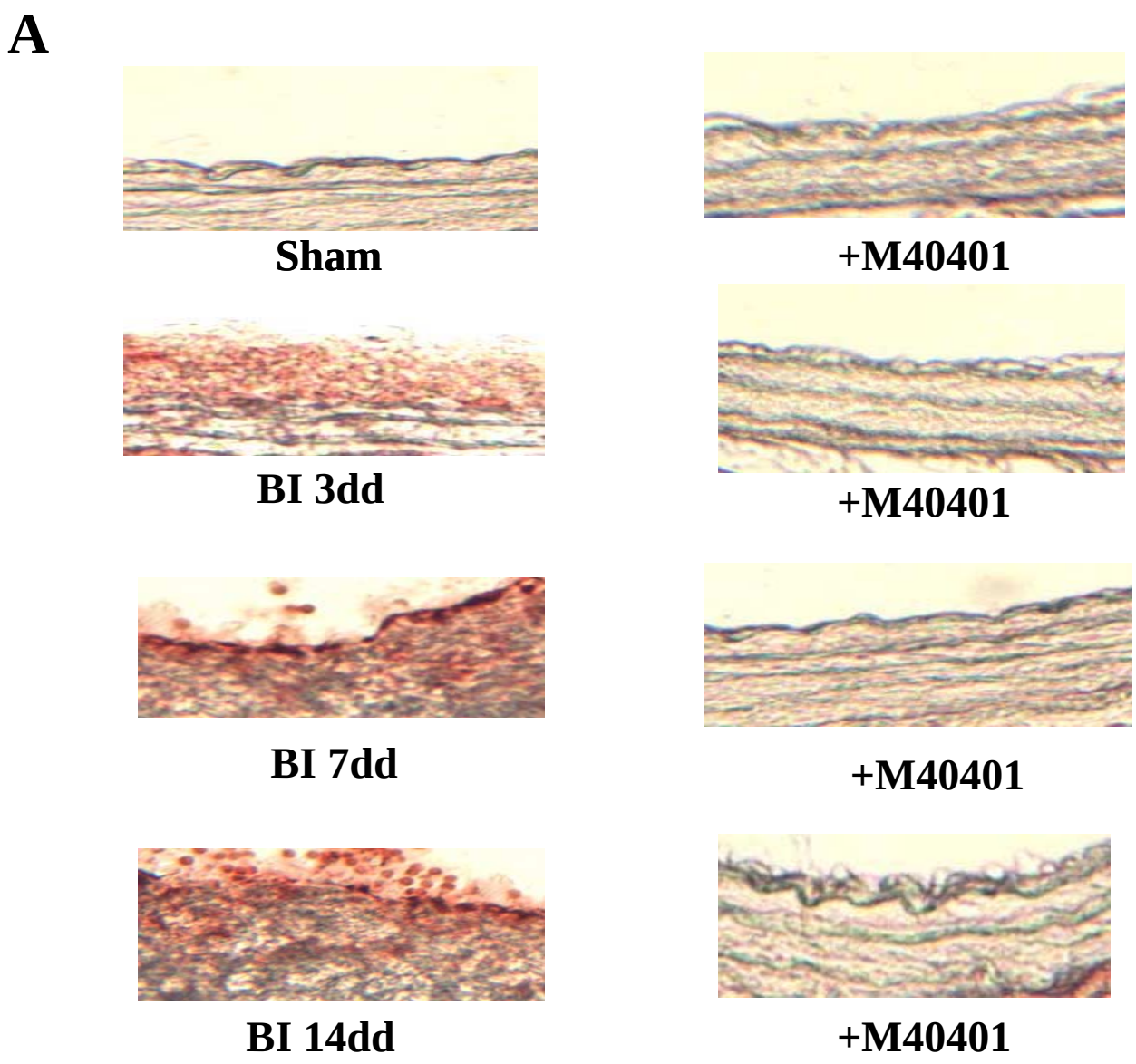

Figure 6

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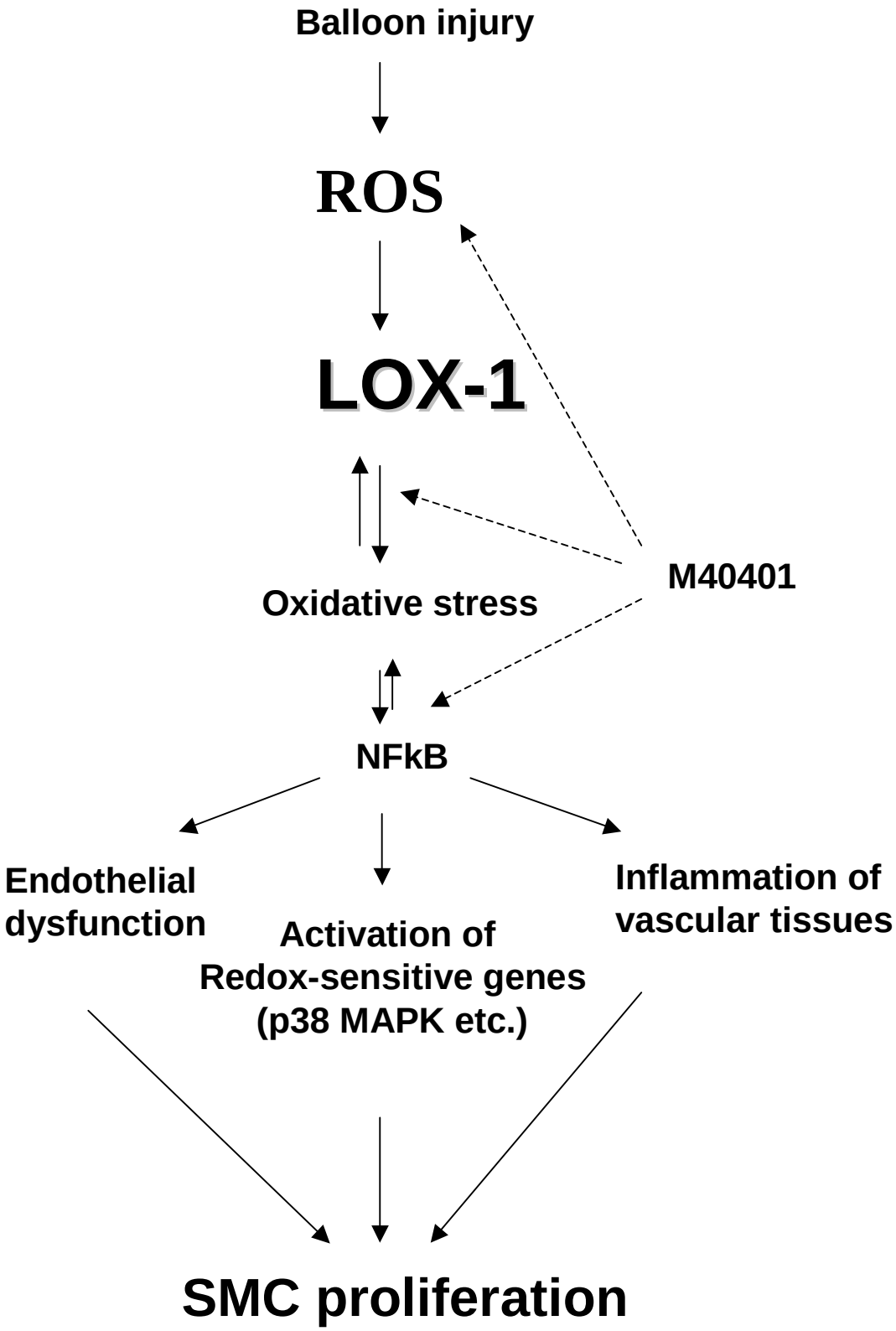

Figure 7