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Effect of topical application of mitomycin-C on wound healing in a postlaminectomy rat model: An experimental study

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ABSTRACT

The aim of this study was to investigate the effects of topical application mitomycin-C (MMC) on wound healing after laminectomy. 60 adult male SD rats were equally and randomly divided into five groups. Laminectomy was performed at the level of L1 in all rats. After hemostasis was achieved, cotton pads soaked with saline and MMC (0.1 mg/ml, 0.3 mg/ml, 0.5 mg/ml and 0.7 mg/ml) were directly subjected to the exposed dura for 5 min in each group. Two weeks after laminectomy all the rats were killed. The vertebral column including the back scar tissue and muscles was obtained to make paraffin sections. The hematoxylin–eosin staining and Masson staining were performed with the obtained paraffin sections. The number of the fibroblast and the capillary density were counted by the hematoxylin–eosin staining slice. The extent of epidural fibrosis and the expression of vascular endothelial growth factor (VEGF) were evaluated by the immunohistochemical slice through a computer image analysis system. Our data showed that the number of fibroblast, capillary density and fibrotic tissue in the 0.5 and 0.7 mg/ml MMC groups was significantly lower than the control, 0.1 and 0.3 mg/ml MMC groups; while the expression of VEGF in control and 0.1 mg/ml MMC groups was notably higher than 0.3, 0.5 and 0.7 mg/ml MMC groups. Topical application of MMC above the concentration of 0.3 mg/ml could affect all steps of the wound healing process via inhibiting the angiogenesis and fibroblast proliferation, thus delayed the wound healing after laminectomy.

1. Introduction

Mitomycin-C (MMC) is a potent a chemotherapeutic agent by virtue of its anti-tumor antibiotic activity. Recently, MMC has been successfully used as an adjuvant treatment in preventing adhesion because of its effect on the prevention of scar formation by inhibiting fibroblast proliferation (Cincik et al., 2005; Ilbay et al., 2005). MMC was also used to prevent epidural fibrosis after laminectomy via reducing scar formation in the last few years (Lee et al., 2006a, 2006b; Liu et al., 2010; Yildiz et al., 2007). The expression of extracellular matrix (ECM) proteins which plays an important role in wound repair and the scar formation was decreased because MMC inhibited the proliferative fibroblasts (Ferguson et al., 2005). Previous studies have shown that orthodox anticancer chemotherapeutic agent such as cyclophosphamide and Vinblastine has shown an antiangiogenic component (Vacca et al., 1999; Browder et al., 2000). Lots of studies have reported that MMC prevented the scar formation after ophthalmologic surgery (Chung et al., 2002; Majmudar et al., 2000; Schipper et al., 1997) as well as decreased wound strength (Porter et al., 2006) and delayed the wound healing (Ando et al., 1992; Demir et al., 2002). However, whether MMC could influence the wound healing after laminectomy is still not clearly defined.

Vascular endothelial growth factor (VEGF) is a potent endothelial-cell-specific mitogen and angiogenic factor that has been shown to play a central role in neovascular responses. The ability of VEGF is to induce endothelial cells to proliferate and organize into capillary-like sprouts that both directly and indirectly stimulate angiogenesis (Ferrara, 1996). VEGF is required for normal wound healing (Brown et al., 2002).

In this study we established a rat laminectomy model, and attempted to investigate the effects of MMC on wound healing and sought to determine possible mechanisms.

2. Materials and methods

2.1. Animals

Sixty adult male Sprague–Dawley rats weighing 170–200 g were used for this study. Animals were housed in a conventional animal facility that was kept at a 12:12 h light–dark cycle with water and food provided ad libitum. Rats were randomly divided into five experimental groups: (1) 0.1 mg/ml group; (2) 0.3 mg/ml group; (3) 0.5 mg/ml group; (4) 0.7 mg/ml group; and (5) injured-control group. Each group consisted of 12 rats. The experiment was performed according

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to the National institutes of Health Guide for the Care and Use of Laboratory and protocols were approved by the animal ethics committee of Nanjing Medical University.

2.2. Surgical procedures

Rat laminectomy model were established according to the protocol as described previously (Lee et al., 2004) with a slight modification. In brief, animals were anesthetized with an intraperitoneal application of pentobarbital (50 mg/kg body weight). The hair around L1 was shaved and the naked skin was sterilized with povidone–iodine solution. A dorsal skin incision was made with a number-15 blade and continued down to the spinous process. The paraspinal muscles were stripped away from the lamina and spinous process. Laminectomies at L1were carried out by rongeurs, and then a laminectomy defect was created, leaving the dura mater clean and fully exposed. All these procedures were performed carefully to keep the neural tissues intact.

2.3. Topical application of MMC

After hemostasis was achieved, cotton pads ($4\times4\ mm^2$) were soaked with saline and MMC (0.1 mg/ml, 0.3 mg/ml, 0.5 mg/ml and 0.7 mg/ml) (Kyowa Hakko Kogoyo Co., Ltd, Tokyo, Japan). The cotton pads were picked up by forceps and after not dripping covered the surgical area for 5 min. The volume of MMC administrated to each group was about 3 ml. Then, the soaked cotton pads were removed and the laminectomy area was rinsed with physiological saline to wash off any leftover agent. The wound was closed in layers using the same suture material in each animal. The fascia at the laminectomy site was marked with 3–0 silk to facilitate the harvest of pathological specimens. There were no complications or adverse effects from the surgery. No prophylactic antibiotics were used.

2.4. Histological staining

Histological staining was performed in all groups two weeks postoperatively. All rats were killed by means of an overdose of pentobarbital (60 mg/kg). The whole spine columns including surrounding muscle tissues were removed at L1 level. The specimens were decalcified by ethylenediamine tetraacetic acid (EDTA) (R&D Systems Inc., Minneapolis, MN, USA). After complete decalcification, they were dehydrated and embedded in the paraffin.

Twenty successive transversal sections of 4 μm were obtained through the L1 vertebra from the top to the bottom in all groups. Ten odd sections of each group were stained with hematoxylin–eosin. The blood vessel density and the number of fibroblasts were counted under the light microscope (Nikon FDX-35, Japan); ten even sections stained with Masson's trichrome of each group were selected and the histological images at 40× magnification were obtained. Sections for an individual case were counted blinded to the group assignment.

2.5. Immunohistochemical analysis

Ten sections of each group were selected to be deparaffinized, rehydrated and treated with hydrogen peroxide to block endogenous peroxidase. They were also treated with Ultra V Block (Lab Vision; Fremont, CA, USA) to reduce non-specific binding. The sections were incubated with a rabbit polyclonal anti-VEGF antibody (R&D Systems Inc., Minneapolis, MN, USA) for 30 min at room temperature. After that, they were incubated with biotinylated secondary antibodies and subsequently peroxidase-conjugated streptavidin (Lab Vision; Fremont, CA, USA). To reveal the immunostaining, the sections were incubated in darkness for 10 min with 3-3′ diaminobenzidine tetra hydrochloride (Sigma Chemical Co., St. Louis, MO, USA), in the amount of 100 mg in 200 ml 0.03% hydrogen peroxide in phosphate buffered saline solution (PBS). Counterstaining was performed with hematoxylin.

The expression of VEGF was evaluated under the light microscope at 400× magnification.

2.6. Histological image analysis

The blood vessel density was assessed in the selected fields with the three most vascularized areas at 40× magnification. Then, the numbers of blood vessels with a visible lumen were counted in each of these areas at 400× magnification. Only these kinds of blood vessels with a clearly defined lumen were counted. Vessel density was then expressed as the number of counted micro vessels per mm^2.

To quantify the number of epidural fibroblast in the scar tissue, the sections stained with hematoxylin–eosin of each rat were selected, and the histological images at 400× magnification were also obtained. The counting area was selected at the middle and at the margins of the laminectomy sites, and each was about 100 μm × 100 μm from three fields on each section. The number of fibroblasts was calculated, and the number of fibroblasts for each section was defined as the average of three fields.

The areas of collagen tissue were delineated from the paraspinal muscles to the laminectomy margins and the dura mater in the sections stained with Masson's trichrome, which were automatically calculated by Leica Owin plus image analysis system.

The fields to be counted for VEGF expression was chosen at 400× magnification from well-labeled areas. Staining of VEGF was assessed using a semiquantitative grading system that reflected the intensity of staining present within 20 randomly chosen blood vessels in each scar: (0) no staining; (1) weak staining; (2) moderate staining; and (3) strong staining. Each scar was then given an overall modified H-score (number of vessels that scored 0×0) + (number of vessels that scored 1×1) + (number of vessels that scored 2×2) + (number of vessels that scored 3×3) (Kumar et al., 2009).

All analysis was carried out by a histopathologist who was blinded to the treatment.

2.7. Statistical analysis

Data are expressed as the median and statistical range of the H-score for each group. All other results were expressed as mean ± S.D., The statistical analysis was performed using SPSS software (version 13.0, SPSS, Inc.). The single factor analysis of variance and q-test were applied to evaluate five independent samples. Statistical significance was assumed at $P<0.05$.

3. Results

None of the animals in either group had a full-thickness wound dehiscence.

3.1. Blood vessel density

Representative sections from control and MMC-treated groups are shown in Fig. 1B. The density of blood vessel in the 0.5 mg/ml and 0.7 mg/ml MMC groups was obviously lower than the control group, 0.1 mg/ml and 0.3 mg/ml MMC groups. The moderate number of vessel was observed in 0.3 mg/ml MMC group (Fig. 1A). MMC is an effective inhibitor of blood vessel density in vivo and this inhibitory effect is dose-dependent.

3.2. Fibroblast counting

Among the five different groups, statistically lower fibroblast counts were found in the MMC-treated groups. The count was significantly

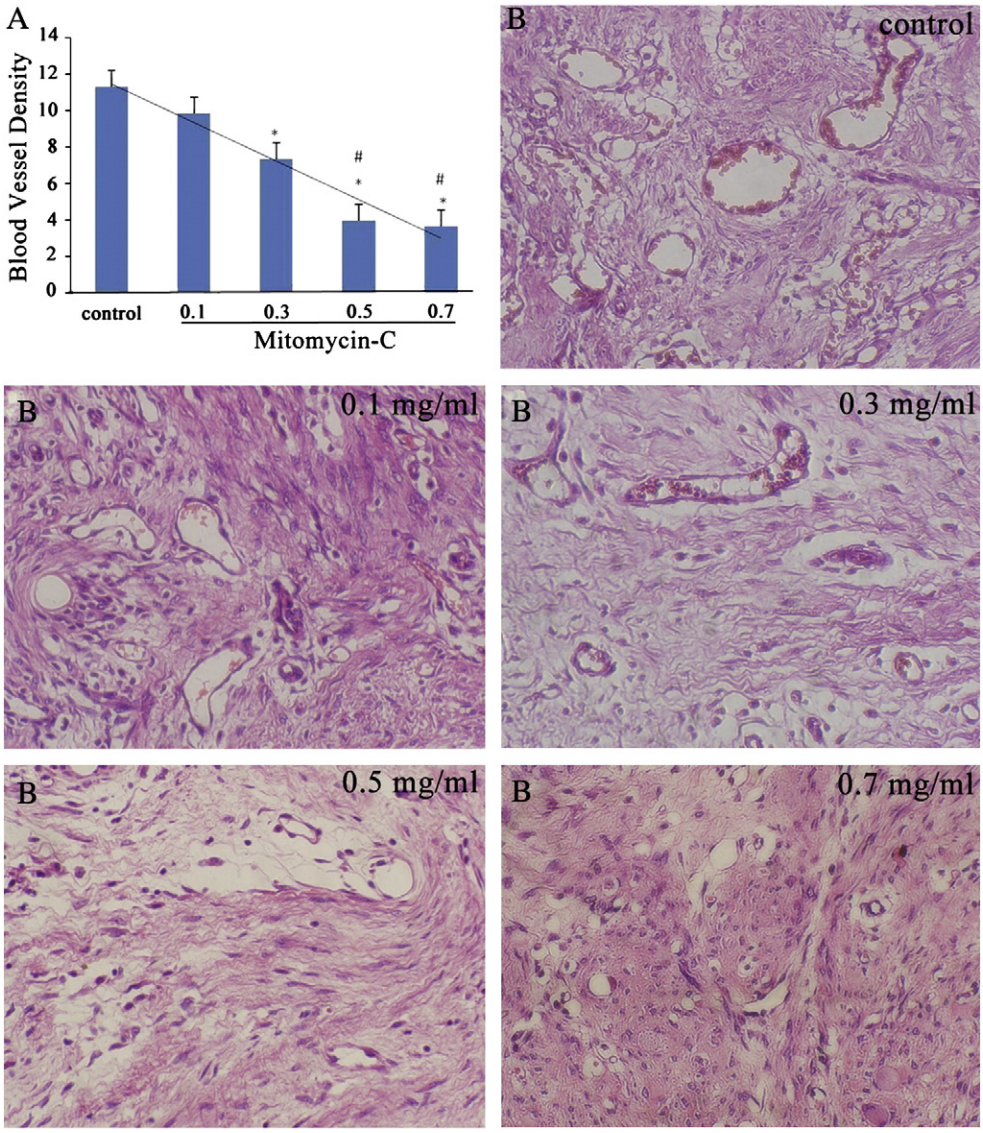

Fig. 1. Capillary density of wound healing. (A) Quantification of capillary number in rats treated with or without MMC. The trend of capillary density decreases following the increasing concentration of MMC. #P<0.05 vs the 0.1 mg/ml group and 0.3 mg/ml MMC group, *P<0.05, vs control and 0.1 mg/ml MMC groups. (B) Representative photomicrographs of capillary density measured by hematoxylin–eosin staining in rats treated with or without MMC, two weeks after cell transplantation (400×).

fewer in the 0.5 mg/ml and 0.7 mg/ml MMC groups than the 0.1 mg/ml and 0.3 mg/ml MMC groups (P<0.05), whereas the fibroblast counting in 0.5 mg/ml MMC group was similar to 0.7 mg/ml MMC group (P>0.05). There was an overall decreasing in fibroblast count in MMC treated groups compared with the control group (Table 1).

Table 1
Quantitative fibroblast analysis after the two weeks. Data are represented as mean ± S.D. (n = 3). The counting of fibroblast decreases with the increasing concentration of MMC.

Group	Fibroblast number
Control	22.0 ± 2.1
0.1 mg/ml	14.9 ± 2.3[a]
0.3 mg/ml	13.3 ± 3.3[a]
0.5 mg/ml	7.1 ± 1.8[a,b]
0.7 mg/ml	6.2 ± 0.9[a,b]

[a] P<0.05 vs the control group.
[b] P<0.05 vs the 0.1 mg/ml group and 0.3 mg/ml MMC group.

3.3. Fibrosis evaluating

At two weeks postoperatively, dense fibrous tissue with rich neovascularization was found to be adhered to the dura as well as the surrounding tissues in the control group. In contrast, the experimental sites consistently exhibited less epidural fibrosis, with the deposition of collagen, appearing smaller and with fewer fibroblasts Table 1. The overall view is shown in Fig. 3. The slightly thickened epidural fibrosis was arranged with moderate neovascularization in 0.3 mg/ml group. Loosely arranged epidural fibrosis with mild neovascularization was found in the 0.5 mg/ml MMC and 0.7 mg/ml MMC groups in reference to the control, 0.1 mg/ml and 0.3 mg/ml MMC groups.

3.4. Vascular endothelial growth factor expression

VEGF is known to play a central role in neovascular responses. Its expression level after MMC treatment for 5 min was detected by immunohistochemistry. As shown in Fig. 2A, the level of VEGF in 0.3 mg/ml, 0.5 mg/ml and 0.7 mg/ml MMC groups is obviously lower than the

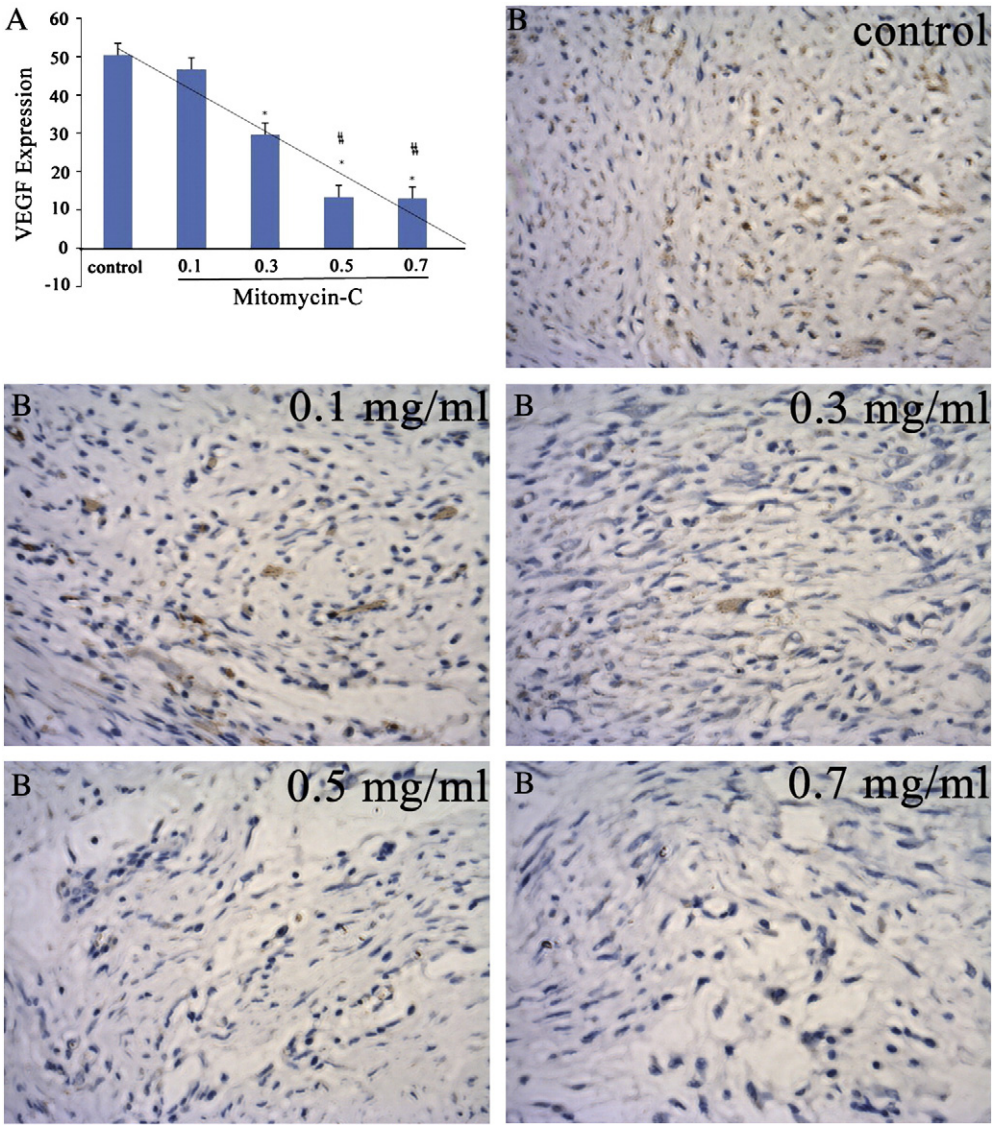

Fig. 2. Effect of MMC on vascular endothelial growth factor (VEGF). (A) Each bar represents mean ± SD of VEGF level in the wound tissue in each treatment group. *P<0.05 vs the control and 0.1 mg/ml groups, #P<0.05vs the 0.1 mg/ml group and 0.3 mg/ml MMC group, and there is no statistically difference between 0.5 and 0.7 mg/ml MMC groups. (B) Immunohistochemistry for VEGF after MMC treatment. The VEGF level decreased in a dose-dependent manner after treated with MMC at increasing concentrations (0.1, 0.3, 0.5 and 0.7 mg/ml). The magnification was 400×.

control group and 0.1 mg/ml MMC group. The immunoreactivity of VEGF tended to be weaker in the wound tissue (Fig. 2B). Decreasing expression of VEGF was observed in wound tissue after MMC treatment at increasing concentrations. This suggests that VEGF can be inhibited by MMC.

4. Discussion

Since the first study on preventing postlaminectomy epidural fibrosis treated with MMC (Dogulu et al., 2003), multiple studies have shown its ability to decrease fibroblast activity and scar formation (Lee et al., 2006a, 2006b; Su et al., 2010; Sun et al., 2007). Nevertheless with the increasing experience, complications related to the effect of MMC on wound healing have begun to appear in the literature (Khaw et al., 1993; Murayama et al., 1996). However, the effect of topical application of MMC on wound healing after laminectomy is still unclear. In the present study, we present a laminectomy animal model to investigate the effect of MMC on wound healing and we also detected the expression of VEGF to show whether MMC could affect angiogenesis via the inhibition of VEGF level.

Wound healing consists of three phases: (1) the inflammatory phase, which consists of inflammatory cell migration to the healing wound; (2) the proliferative phase, which consists of new tissue formation such as angiogenesis, fibroplasia, epithelialization, and extracellular matrix accumulation; and (3) the tissue remodeling, which is the maturational phase. In this process, the angiogenic activation of endothelial cells plays an important role in promoting and regulating other biological events, such as inflammation, fibroblast proliferation, extracellular matrix synthesis, and epithelialization in wound healing (Zhang et al., 2003). Angiogenesis existed/stimulated by two weeks after surgery and that vessel densities remain increased in fully healed scars more than two years later (Brown et al., 2002). Results from the present study show that with the increasing concentration of MMC the levels of the fibrosis and the number of fibroblast were inhibited. We observed that the vascularization and collagen synthesis in wound was decreased both in 0.5 mg/ml and 0.7 mg/ml MMC groups. We confirmed that MMC had the anti-proliferative effect during wound healing process. Our results supported this previous result (Leccisotti, 2008): fibroblast proliferation was significantly lower in the MMC compared with

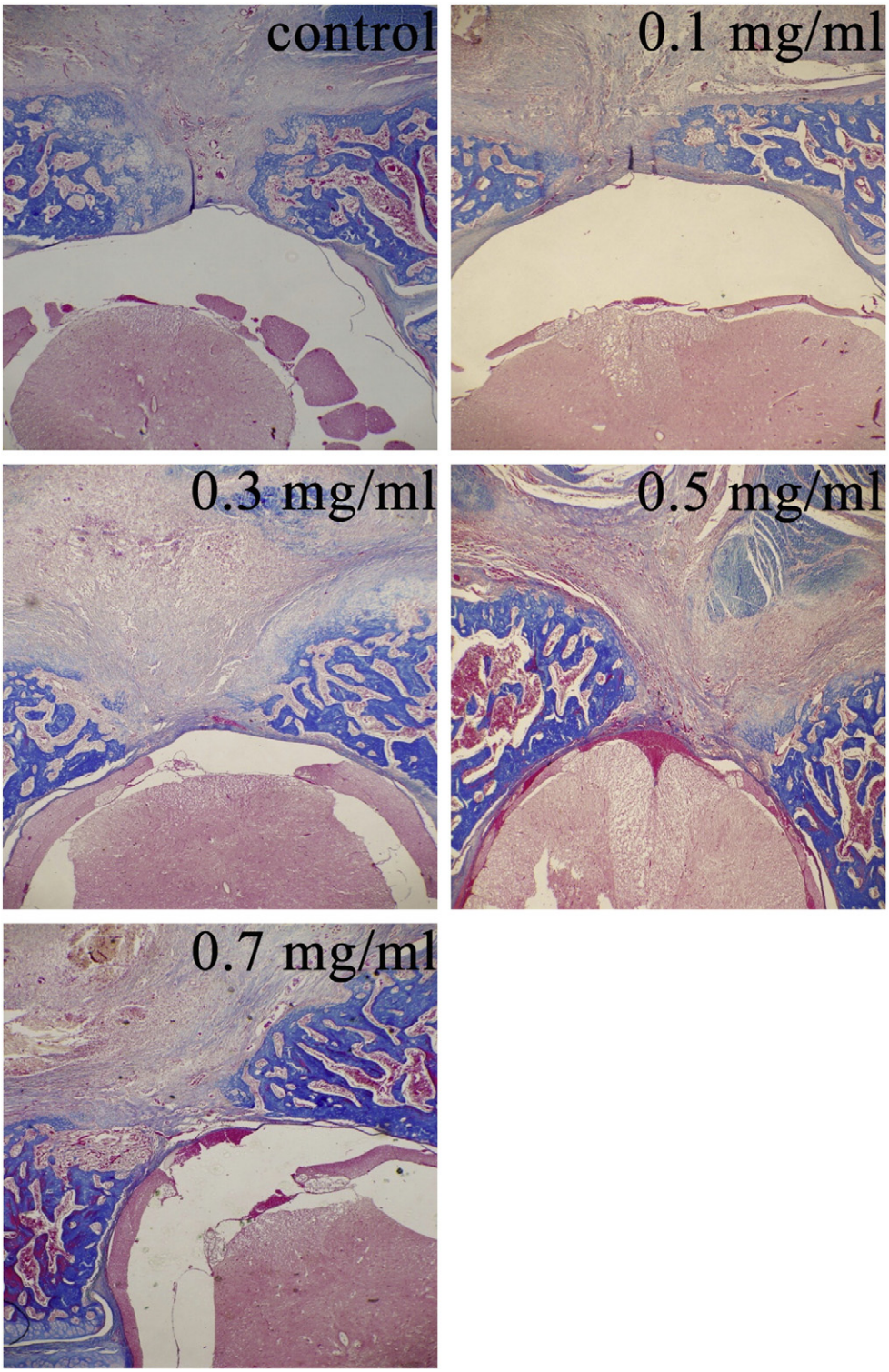

Fig. 3. Photomicroscopic images of fibrosis. The collagen tissues show blue in the section with Masson's trichrome staining under the light microscope. MMC showed potent inhibition effect of fibrosis and collagen synthesis. The density of collagen tissue in the section of the 0.5 and 0.7 mg/ml groups was obviously less than the other concentration groups and control group. The magnification was 40×.

the control group. This low proliferation rate may be attributed to the anti-proliferative effect of MMC.

Revascularization of injured tissue is necessary for wound healing. Following unregulated or insufficient blood vessel growth, wound healing is delayed or pathological. Angiogenesis is regulated by expression of various vascular growth factors and modulators, the most widely expressed and critical of which is VEGF. This protein is secreted by tissues in response to ischemic and inflammatory stimuli and it is a potent endothelial-cell-specific mitogen results in endothelial migration, proliferation (Ferrara, 2002; Werner and Grose, 2003). In our study, the mean blood vessel density was significant lower in the 0.5 mg/ml and 0.7 mg/ml MMC group, which might be related to the directly anti-cell proliferation effect of MMC. Decreasing expression of VEGF was observed in wound tissues after MMC

treatment at increasing concentrations; this expression reached bottom at the concentration of 0.5 mg/ml. This suggests that VEGF can be inhibited by MMC. The kinetics and the trend of VEGF and mean blood vessel density had a similar pattern decrease resulted from MMC treatment, suggesting that the inhibition of the VEGF level by MMC may be another mechanism of the decreasing blood vessel density.

Our results demonstrate that the number of fibroblast and the mean blood vessel density were significantly lower in the 0.5 mg/ml and 0.7 mg/ml MMC groups. These low score may be attributed to the anti-cell proliferation effect of MMC and the MMC treatment with high concentration inhibits the VEGF level of the wound tissues. At last MMC would delay the wound healing process by decreasing angiogenesis and fibroblast proliferation.

CD34 and CD 31 exhibit high specificity for endothelial cells and are widely used to evaluate the degree of angiogenesis. In our future study, CD34 and CD 31 will be used as markers to detect the angiogenesis in wound healing.

In conclusion, topical application of MMC is promising as an alternative anti-fibrotic agent. Nevertheless, it should be emphasized that local application of MMC over 0.3 mg/ml can delay the wound healing via the anti-cell proliferation effect and the inhibition of VEGF. Thus safety measures should be performed to decrease the adverse effect during clinical MMC treatment.

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