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The Topical Application of Rosuvastatin in Preventing Knee Intra-Articular Adhesion in Rats

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Background: Intra-articular adhesion is one of the common complications of post knee surgery and injury. The formation of joint adhesion can lead to serious dysfunction. Rosuvastatin (ROS) is a new 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitor, with multiple biological effects. In our study, the object was to evaluate the effectiveness of ROS in the prevention of post-operative knee adhesion in rats.

Material/Methods: Femoral condyle exposing surgery was performed on 45 healthy Sprague Dawley rats. Gelatin sponges soaked with 20 mg/kg of ROS, 10 mg/kg of ROS, or saline were used to cover the surgical site. The post-operative knee joints were fixed in a flexed position with micro Kirschner wires for four weeks. ROS effectiveness for treating intra-articular adhesion was determined with visual score evaluation, hydroxyproline content, histological analyses, immunohistochemistry, and inflammatory and vascular endothelial growth factors expression.

Results: The animals' recovery was stable after surgery. The hydroxyproline content, visual score, and inflammatory vascular growth factors expression levels suggested that, compared with the control group, the ROS treatment groups showed better outcomes. ROS prevented joint adhesion formation, collagen deposition, and vascularization at the surgical site, and also inhibited inflammatory activity post-operatively. Compared with the 10 mg/kg ROS group, the 20 mg/kg ROS group showed significantly better outcomes.

Conclusions: The local application of ROS reduced intra-articular adhesion formation, collagen deposition, and vascularization at the surgical site, and inhibited inflammatory activity post-operatively. These results suggested optimal concentration of ROS to be 20 mg/kg.

MeSH Keywords: **Hydroxymethylglutaryl-CoA Reductase Inhibitors • Knee Joint • Tissue Adhesions • Vascular Endothelial Growth Factors**

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Background

Intra-articular adhesion is accepted as one of the common complications post knee surgery and injury. Intra-articular adhesion can lead to the failure of surgery and severe functional impairments, including knee stiffness, persistent arthralgia, and cartilage degeneration [1,2]. Until recently, no effective treatment has been development to overcome intra-articular adhesion.

A number of methods have been studied to overcome joint adhesions both in experimental models and patient studies and some method have achieved a certain level of success [3–5]. Wound healing post-surgery involves multiple events, including inflammation, fibroblasts proliferation, matrix synthesis, vascularization, and epithelialization [6–8]. It is widely accepted that the optimal treatment should include anti-fibrotic, anti-inflammatory, and vascularization promoting effects [9–11].

Rosuvastatin (ROS), a new 3-hydroxy-3-methylglutaryl co-enzyme A (HMG-CoA) reductase inhibitor, has been proven to have potent affinity to HMG-CoA reductase [12]. It is currently used for treating hyperlipidemia and preventing cardiovascular disease [13,14]. Aside from this current application, ROS has also been reported to have multiple biological activities, including anti-fibrotic, anti-inflammatory, and vascularization promoting effects. ROS was recently reported to enhance anti-inflammatory activities and inhibit pro-inflammatory functions in cultured microglial cells [15]. In addition, ROS was shown to prevent the epidural fibrosis post spine surgery and attenuate fibrosis in a rat model of cyclosporine-induced nephropathy [16–18]. The latest literature suggests ROS can improve hepatopulmonary syndrome through inhibition of inflammatory angiogenesis [19]. ROS was reported in one study to lead to myocardial revascularization on angiogenesis in a clinic setting [20]. Based on previous reports of ROS effectiveness, we performed the present research.

The object of our study was to evaluate the effect of ROS on preventing fibrotic adhesion in a rat model.

Material and Methods

Forty-five healthy adult male Sprague Dawley rats (average weighted 400 ± 20 g) were involved in the present research. The experiment was conducted based on the EU Directive 2010/63/EU for animal experiments and the Ethics Committee of the Russian National Research Medical University. Gelatin sponges (SPONGOSTAN, Ethicon Endo-Surgery, Inc., USA) were soaked with ROS 10 mg/kg, ROS 20 mg/kg, or saline. Rats were randomly divided into 3 treatment groups: ROS 20 mg/kg group ($n=15$); ROS 10 mg/kg group ($n=15$); and saline control group ($n=15$). The animals were kept at room temperature (20°C

to 25°C) with a 12-hour light cycle, and free access to clean food and water. They were acclimated 10 days before surgery.

Surgery procedures

Sterile conditions were created before surgery. The study model for intra-articular adhesion was based on previously published rat model protocols [4,7,8]. The animals were anesthetized using ketamine (50 mg/kg) and xylazine (10 mg/kg) via intra-peritoneal injection. The body temperature was intra-operatively maintained at around 35°C with heating pads.

After anesthetizing, the rats were placed in the supine position. Iodine (etodolac) was used to sterilize the exposed skin after shaving the fur. Approximately 4×4 mm² of cortical bone was removed from the femoral condyle lateral and medial sides. The underlying cancellous bone surface was exposed and the articular cartilage was left intact. Careful hemostasis was performed.

Sterile wet gauze was used to protect the surrounding tissues from the reagents; the surgical field was covered with the prepared gelatin sponges soaked with ROS or saline. The gelatin sponges were removed after 10 minutes. Silk sutures were used to close the wounds. Cefazolin sodium was post-operatively given for 3-continuous-day to reduce the risk of infection. The post-surgical knee was immobilized with micro Kirschner wire for four weeks.

Assessment

Assessment was based on a previously established visual score system [4,5]. Four weeks post-surgery, five rats were randomly selected from each group. After re-anesthetizing and reopening the surgical sites, the intra-articular adhesions were evaluated (double-blind evaluation) based on the visual score system: 0, no adhesion; 1, weak, mild, filmy adhesions that can be easily eliminated by manual traction; 2, moderate adhesions that were able to be eliminated by manual traction; 3, dense and firm adhesions that had to be surgically removed [6].

Intra-cardial perfusion with 4°C saline and 4% paraformaldehyde was performed in the aforementioned selected rats. The scar tissue generated around the surgical sites was collected. Five samples randomly selected from each group were analyzed by hydroxyproline content (HPC) evaluation and 5 mg of wet weight scar tissue was separated. HPC evaluation was conducted as previously reported [6,9]. Collected samples were lyophilized, ground, and hydrolyzed using 6 mol/L HCl at 110°C for 24 hours. Then 1 mL hydroxyproline developer (β -dimethylaminobenzaldehyde) was added to the samples and standards. The absorbance of each sample was evaluated at 550 nm using the spectrophotometer. The HPC/mg of samples was generated according to the standard curve.

Table 1. Macroscopic determination.

Group	Grade			
	0	1	2	3
ROS (20 mg/kg, n=5)	2	3	0	0
ROS (10 mg/kg, n=5)	0	2	3	0
Control (saline, n=5)	0	0	0	5

Another five rats were randomly selected from each group. After re-anesthetizing, intra-cardial perfusion was performed and scar samples collected. After fixing in 4% paraformaldehyde for 24 hours, the samples were embedded in paraffin. Then 15 successive sections (4 μ m sections) were cut from each sample, and of which five were stained with Masson's trichrome. Intra-articular fibrotic density, and collagen deposition were observed using a light microscope (400 \times magnification).

To determine fibroblast proliferation and vascularization, another five sections were stained with H&E staining, and the number of fibroblast and blood vessels were counted at 200 \times magnification using a light microscope. To further evaluate vascularization status, another five sections were evaluated using immunohistochemistry with primary antibodies to vascular endothelial growth factor (VEGF, 1:400, Biosynthesis, Beijing, China) and secondary antibodies (1:200, Biosynthesis, Beijing, China). The section slices were evaluated by light microscopy at 400 \times magnification and the VEGF expression positive area rate was calculated.

At the same time, an analysis of mRNA level of VEGF and IL-6 was performed. Five rats were randomly selected from each group. After re-anesthetizing, scar tissue samples from the surgery site were collected. Then total RNA was extracted using TRIzol reagent. Total RNA (2 μ g) was transcribed into cDNA. Quantitative real-time PCR was performed (Biosystems 7300HT, Fermentas, USA). Primer sequences used were as follows [6,7]: VEGF: forward 50-TGCAGATTATGCGGATCAAACC-30 and reverse 50-TGCATTCACATTTGTTGTGCTGTAG-30; IL-6: forward, 50-ACCCCAACTTCCAATGCTCT-30; reverse, 50-TGC CGAGTAGACCTCATAGTACC-30; GAPDH: forward 50-TGC ACCACCAACTGCTTAGC-30 and reverse 50-GGCATGGA CTGTGTCATGAG-30. GAPDH amplification was performed as an internal control.

Statistical analysis

The results were expressed as \pm the standard error of the mean (SEM). One-way analysis of variance (ANOVA) was used to assess the differences among groups using SPSS software (version 19.0). Bonferroni correction, as post hoc test, was performed; $p < 0.05$ was considered to be a statistically significant difference.

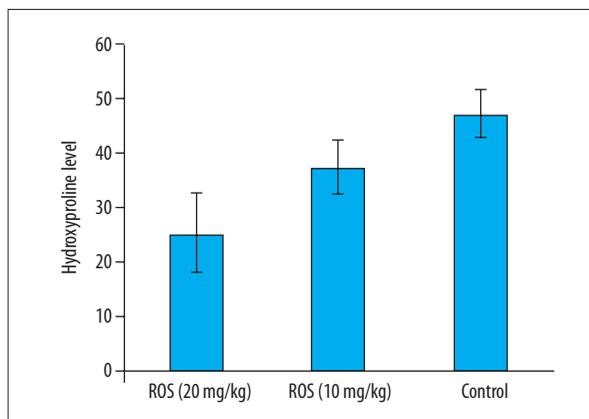


Figure 1. Hydroxyproline concentration in the three groups. The concentrations are given as μ g/mg.

Results

The recovery of all animals was uneventful post operation. None of the rats showed signs of wound infection, cutaneous necrosis, or mortality during the experimental period.

Macroscopic determination

In the 20 mg/kg ROS group, the formation of fibrous adhesions was soft or partial weak between the joint capsule and the femoral condyle's decorticated areas. In the 10 mg/kg ROS group, the surgical area was filled with moderate scar adhesion that could be dissected with manual traction. In the control group, dense scar adhesions were found between the femoral condyle's decorticated areas and the joint capsule (Table 1).

HPC analysis

As shown in Figure 1, compared with the HPC in the control group (47.12 ± 4.45), the HPC in the ROS treated groups were significantly less: ROS 20 mg/kg, 25.36 ± 7.38 ; ROS 10 mg/kg, 37.38 ± 5.02 , ($p = 0.014$, $p = 0.025$, $p < 0.05$, respectively). The HPC in the 20 mg/kg ROS group (25.36 ± 7.38) was less than that in the 10 mg/kg ROS group (37.38 ± 5.02) ($p = 0.019$, $p < 0.05$, respectively).

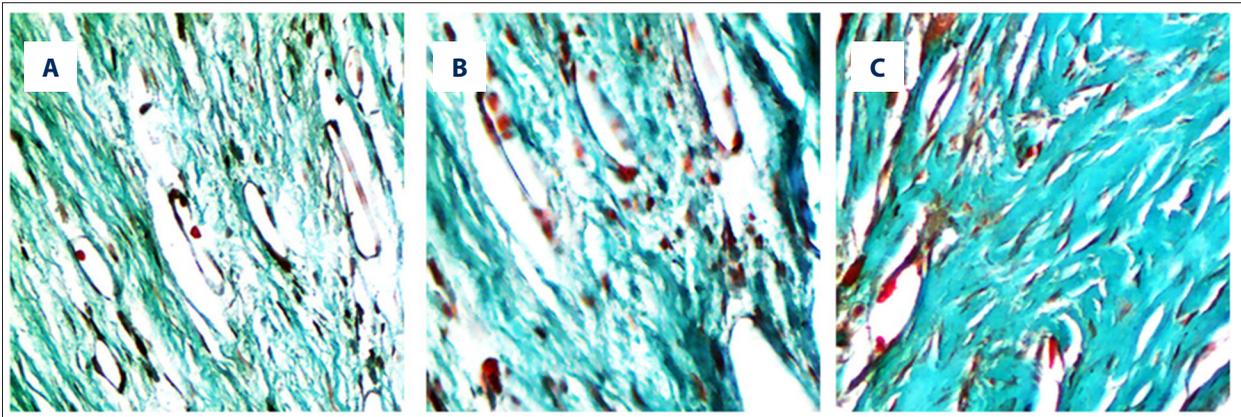


Figure 2. Masson's trichrome staining for scar tissues at the operative sites. (A) 20 mg/kg ROS group, loose collagen deposition was observed in the surgical sites; (B) 10 mg/kg ROS group, moderate collagen deposition was observed; (C) Control group, the rich collagen tissue deposition was found.

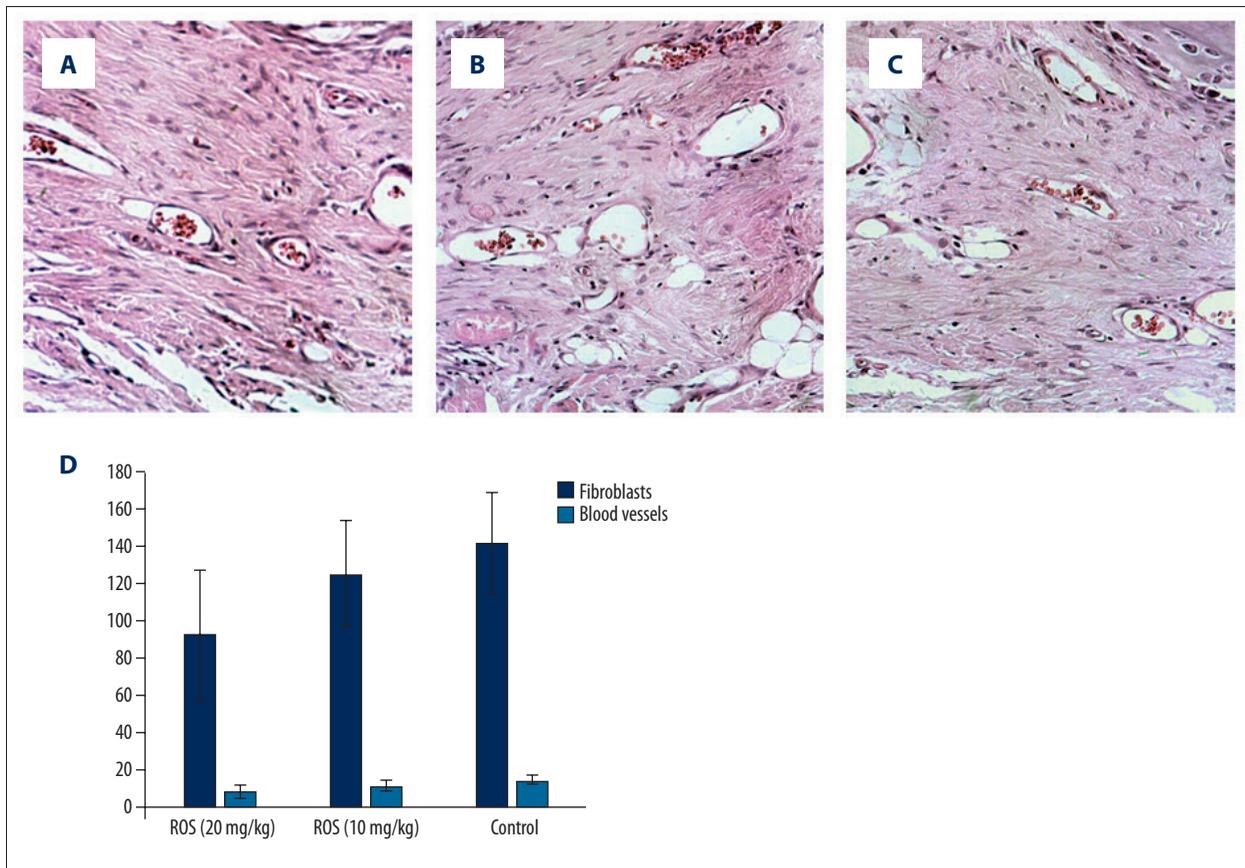


Figure 3. Fibroblasts and blood vessels counting of the surgical sites in the three groups. (A) 20 mg/kg ROS group; (B) 10 mg/kg ROS group; (C) Control group; (D) Quantitative analysis.

Histological analysis

The situation of intra-articular adhesion and collagen deposition was shown in Figure 2 with Masson's trichrome. In the surgical sites of the 20 mg/kg ROS group, loose collagen deposition was observed in the surgical sites (Figure 2A). In the

surgical sites of the 10 mg/kg ROS group, moderate collagen deposition was observed (Figure 2B). However, in the surgical sites of the control group, rich collagen tissue deposition were found (Figure 2C).

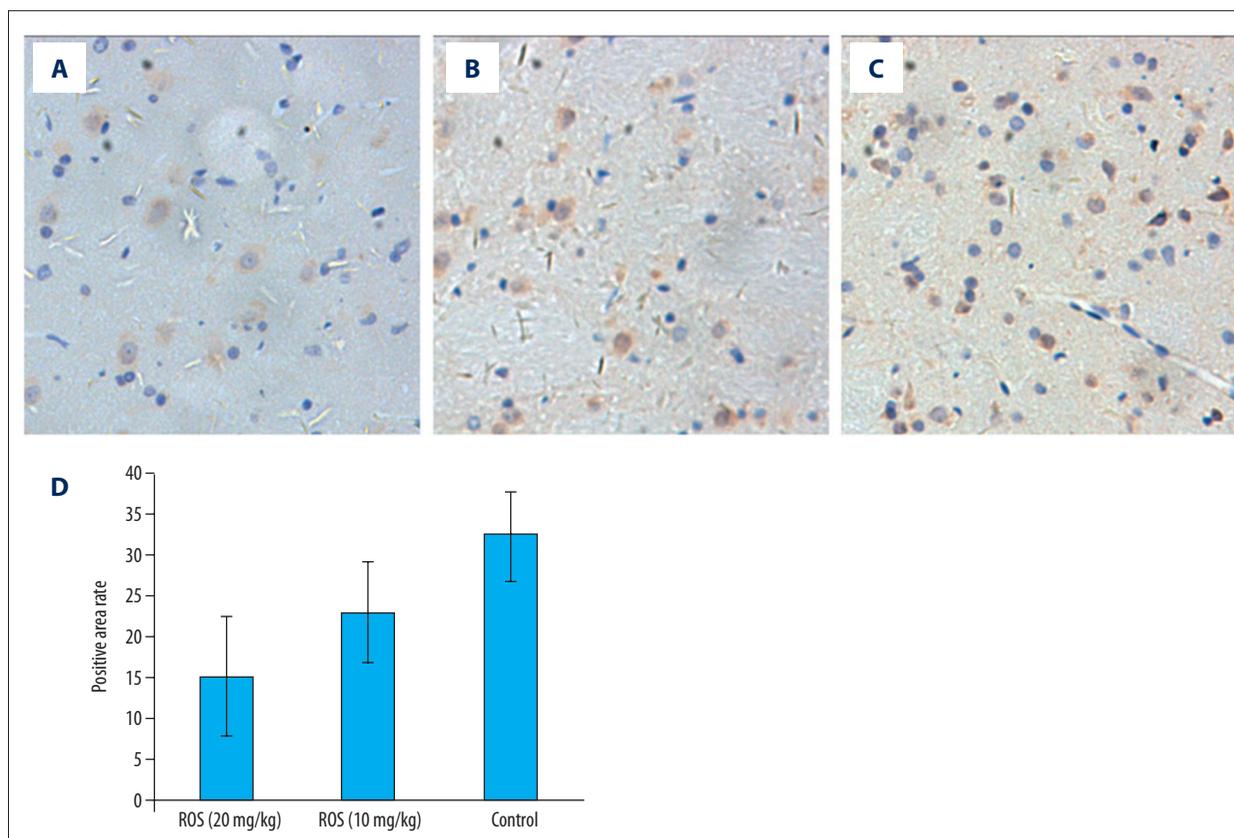


Figure 4. VEGF expression levels in adhesion tissues. (A) 20 mg/kg ROS group; (B) 10 mg/kg ROS group, (C) Control group, (D) Positive area rate analysis.

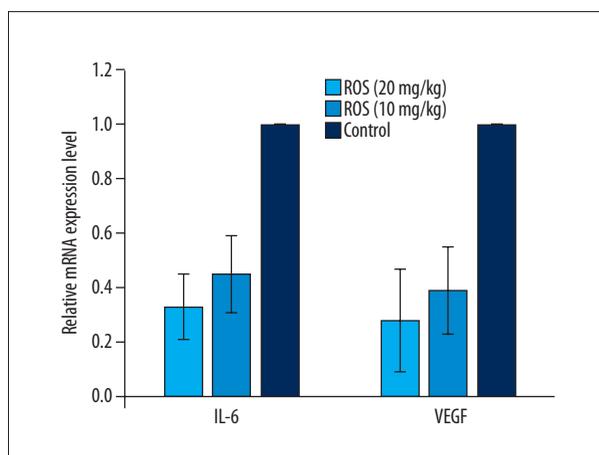


Figure 5. IL-6 and VEGF mRNA expression in the three groups. Compared with control group, the levels of mRNA expression were inhibited in the ROS treated groups; and the levels showed the lowest values in the 20 mg/kg ROS group.

With H&E staining shown in Figure 3, the number of fibroblasts were successfully counted. The number of fibroblasts in the ROS treated groups (20 mg/kg 92.31 ± 35.22 ; 10 mg/kg, 124.72 ± 28.95) were less than that in the control

group (141.22 ± 27.42) ($p=0.009$, $p=0.011$, $p<0.05$, respectively). Compared with 10 mg/kg ROS group (Figure 3B), the number of fibroblast in 20 mg/kg ROS group (Figure 3A) was further significantly inhibited ($p=0.016$, $p<0.05$, respectively). At the same time, the number of blood vessels in the ROS treated groups (20 mg/kg, 8.15 ± 3.51 ; 10 mg/kg, 11.39 ± 2.89) were less than that in control group (Figure 3C) (15.14 ± 2.35) ($p=0.014$, $p=0.017$, $p<0.05$, respectively), Compared with the 10 mg/kg ROS group (Figure 3D), the blood vessels in the 20 mg/kg ROS group was further significantly inhibited ($p=0.021$, $p<0.05$).

As shown in the Figure 4, with the evaluation of VEGF immunohistochemistry, the immune-reactive area rate was calculated (Figure 4D). The area rate in the ROS treated groups (20 mg/kg, 14.98 ± 7.33 ; 10 mg/kg, 22.91 ± 6.15) were less than that in control group (32.33 ± 5.51) ($p=0.018$, $p=0.021$, $p<0.05$), Compared with 10 mg/kg ROS group, the area rate in 20 mg/kg ROS group further significantly decreased ($p=0.011$, $p<0.05$).

ROS's effect on suppressing IL-6 and VEGF expressions

In order to determine the effect of ROS on regulating IL-6 and VEGF expressions in post-operative knees, RT-PCR was conducted to examine the mRNA expression levels. As shown in

Figure 5, compared with the control group, the levels of mRNA expression were inhibited in the ROS treated groups, and the levels showed the lowest values in the 20 mg/kg ROS group.

Discussion

Intra-articular adhesion has drawn the attention of many researchers and joint surgeons for a long time [3–11]. Minimally invasive techniques and intra-operative meticulous hemostasis are generally thought to reduce adverse complications [6,7]. From both experimental and clinical studies, a number of pharmacological agents, synthetic barriers, and biological materials have been applied to prevent intra-articular adhesion [4–7]. However, the results seem to either be conflicting or lead to adverse events. Arthroscopic lysis of adhesions has been reported to relieve the situation. Nevertheless, recent studies suggest a high rate of recurrence of intra-articular adhesion post-surgery [3,7].

It is generally accepted that intra-articular adhesion results from the overexpression of extracellular matrix, proliferation of fibroblasts, activation of inflammation, and transformation of fibroblasts into myoblasts [10–12]. In surgical sites post-surgery, in order to repair the wound, the expressed growth factors and inflammatory cytokines lead to the activation of fibroblasts proliferation and collagen fibers production. The proliferated fibroblasts can then secrete VEGF and promote the healing process [7].

As previously reported, one of the most important mechanisms during the healing process is vascularization, which is regulated by several growth factors and modulators [7,21]. VEGF is one of the widely accepted critical factors. VEGF is thought to lead to the proliferation of endothelial cells, which can promote vascularization after wound healing [21,22]. A previous

study showed that neutralizing VEGF had the effect of reducing fibroblasts proliferation and decreasing blood vessel, while activating the VEGF gene had the opposite effect [23]. Thus, VEGF is a very effective indicator for evaluation of intra-articular adhesion post-surgery.

In the present study, we further proved the hypothesis by H&E staining and VEGF immunohistochemistry. Our results suggest that 20 mg/kg ROS results in the lowest vascularization and the lowest VEGF expression in scar tissue. At the same time, ROS can decrease the expression level of IL-6, which suggested ROS has a role in ameliorating inflammatory activity. The results of Masson's trichrome and HPC evaluation suggested the ROS's ability on improving collagen deposition in the surgical sites. Thus, our findings may explain some but not all the ROS's functional mechanisms.

Based on previously reported study [18], the maximum concentration of topical ROS in our study was 20 mg/kg. No systemic complications or mortality were observed before the rats were killed. However, the safety and toxicity of ROS still needs to be further investigated before use in clinical trials.

Conclusions

Topical application of ROS can reduce knee intra-articular adhesion, and 20 mg/kg is more effective than 10 mg/kg. We propose that ROS topical application may be an easy and low cost method for preventing intra-articular adhesion.

Conflict of Interests

We declare that we have no conflicts of interest in the authorship or publication of this contribution.

Reference:

1. Strum, GM, Friedman MJ, Fox JM et al: Acute anteriorcruciate ligament reconstruction. Analysis of complications. *Clin Orthop Relat Res*, 1990; 253: 184–89
2. Mohammed R, Syed S, Ahmed N: Manipulation under anaesthesia for stiffness following knee arthroplasty. *Ann R Coll Surg Engl*, 2009; 91: 220–23
3. Chen MR, Dragoo JL: Arthroscopic releases for arthrofibrosis of the knee. *J Am Acad Orthop Surg*, 2011; 19: 709–16
4. Li Y, Ma X, Yu P, Wang S: Intra-articular adhesion reduction after knee surgery in rabbits by calcium channel blockers. *Med Sci Monit*, 2014; 20: 2466–71
5. Hayashi M, Sekiya H, Takatoku K et al: Experimental model of knee contracture in extension: its prevention using a sheet made from hyaluronic acid and carboxymethylcellulose. *Knee Surg Sports Traumatol Arthrosc*, 2004; 12: 545–51
6. Zhang C, Kong X, Ning G et al: All-trans retinoic acid prevents epidural fibrosis through NF- κ B signaling pathway in post-laminectomy rats. *Neuropharmacology*, 2013; 79: 275–81
7. Yan L, Sun Y, Li X et al: The effect of hydroxycamptothecin on wound healing following reduction of the knee intra-articular adhesion in rabbits. *Cell Biochem Biophys*, 2015 [Epub ahead of print]
8. Zhang C, Kong X, Liu C et al: ERK2 small interfering RNAs prevent epidural fibrosis via the efficient inhibition of collagen expression and inflammation in laminectomy rats. *Biochem Biophys Res Commun*, 2014; 444(3): 395–400
9. Wang Y, Zhang C, Cheng H et al: All-trans retinoic Acid reduces joint adhesion formation: an experimental study in rats. *Med Sci Monit*, 2015; 21: 1598–603
10. Wang M, Liu C, Xiao W: Intra-articular injection of hyaluronic acid for the reduction in joint adhesion formation in a rabbit model of knee injury. *Knee Surg Sports Traumatol Arthrosc*, 2014; 22(7): 1536–40
11. Kocaoglu B, Akgun U, Nalbantoglu U et al: Adhesion reduction after knee surgery in a rat model by mitomycin C. *Knee Surg Sports Traumatol Arthrosc*, 2011; 19(1): 94–98
12. Khaidakov M, Wang W, Khan JA et al: Statins and angiogenesis: is it about connections? *Biochem Biophys Res Commun*, 2009; 387(3): 543–47
13. Wang C, Tao W, Wang Y et al: Rosuvastatin, identified from a zebrafish chemical genetic screen for antiangiogenic compounds, suppresses the growth of prostate cancer. *Eur Urol*, 2010; 58(3): 418–26
14. Fletcher B, Berra K, Ades P et al: Managing abnormal blood lipids: A collaborative approach. *Circulation*, 2005; 112: 3184–209

15. Kata D, Földesi I, Feher LZ et al: Rosuvastatin enhances anti-inflammatory and inhibits pro-inflammatory functions in cultured microglial cells. *Neuroscience*, 2016; 314: 47–63
16. Nam HK, Lee SJ, Kim MH et al: Rosuvastatin attenuates inflammation, apoptosis and fibrosis in a rat model of cyclosporine-induced nephropathy. *Am J Nephrol*, 2013; 37(1): 7–15
17. Zhang C, Feng S, Hu N et al: Letter to the editor regarding: "Evaluation of topical application and systemic administration of rosuvastatin in preventing epidural fibrosis in rats" by Bora Gürer et al. *Spine J*, 2015; 15(5): 1165–66
18. Gürer B, Kahveci R, Gökçe EC et al: Evaluation of topical application and systemic administration of rosuvastatin in preventing epidural fibrosis in rats. *Spine J*, 2015; 15(3): 522–29
19. Chang CC, Wang SS, Hsieh HG et al: Rosuvastatin improves hepatopulmonary syndrome through inhibition of inflammatory angiogenesis of lung. *Clin Sci (Lond)*, 2015; 129(6): 449–60
20. Semenova AE, Sergienko IV, Masenko VP et al: Effect of rosuvastatin therapy and myocardial revascularization on angiogenesis in coronary artery disease patients. *Kardiologiia*, 2007; 47(11): 4–8
21. Chen F, Zuo Z, Wang K et al: Study on salvianolic acid B in the reduction of epidural fibrosis in laminectomy rats. *BMC Musculoskelet Disord*, 2014; 15: 337
22. Deissler H, Deissler H, Lang S, Lang GE: VEGF induced effects on proliferation, migration and tight junctions are restored by ranibizumab (Lucentis) in microvascular retinal endothelial cells. *Br J Ophthalmol*, 2008; 92(6): 839–43
23. Zhang F, Lei MP, Oswald TM et al: The effect of vascular endothelial growth factor on the healing of ischaemic skin wounds. *Br J Plast Surg*, 2003; 56(4): 334–41