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Intra-Articular Adhesion Reduction after Knee Surgery in Rabbits by Calcium Channel Blockers

Authors' Contribution:
Study Design A
Data Collection B
Statistical Analysis C
Data Interpretation D
Manuscript Preparation E
Literature Search F
Funds Collection G

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Background: Intra-articular adhesion after knee surgery is a common and serious complication that presents a challenging problem for orthopedic surgeons. Verapamil (VP), a widely used calcium channel blocker, has been shown to prevent synthesis/secretion of extracellular matrix molecules. The object of this study was to investigate the effects of VP on the prevention of joint adhesion in post-surgery rabbits.

Material/Methods: A controlled double-blinded study was conducted in 40 healthy New Zealand white rabbits divided randomly into 4 groups according to the treatment method, with 10 in each group: 1) 1 mg/ml VP treatment group; 2) 2.5 mg/ml VP treatment group; 3) 5 mg/ml VP treatment group; 4) control group. Rabbits underwent surgery through the medial parapatellar approach and both lateral sides and the medial of the femoral condyle were surgically exposed. After treatment, the surgical limbs were subjected to extra-articular knee-joint immobilization in the full flexed position employing Kirschner wires for 4 weeks.

Results: The knee surgery was successfully performed on all rabbits. The rabbits were killed 4 weeks post-operatively. The histological evaluation, hydroxyproline content, visual score, fibroblasts density, and vimentin expressional levels were conducted to assess the effect of VP on preventing joint adhesion.

Conclusions: In our rabbit model of knee surgery, intra-articular application of VP was able to decrease intra-articular adhesion formation after surgery. VP could prevent rabbit intra-articular adhesion in a dose-dependent manner and the highest concentration used in the study (5 mg/ml) proved to be the most effective.

MeSH Keywords: Knee Joint • Rabbits • Tissue Adhesions • Verapamil

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Background

Intra-articular adhesion after knee surgery is a common and serious complication and is a challenge problem for orthopedic surgeons. The formation of intra-articular adhesions can lead to severe knee biomechanical change and dysfunction such as arthralgia, stiffness, cartilage degeneration, and subsequent pain, and thus leads to the failure of the surgery [1,2].

No effective treatments are widely accepted [3]. A variety of methods have been developed to prevent intra-articular adhesions in animal models and humans, including arthroscopic lysis of adhesions, intra-articular and/or mitomycin C application, continuous corticosteroid administration, and hyaluronan derivative gel [4–8]. However, none of these methods have achieved complete success.

Verapamil (VP), a widely applied calcium channel blocker, can prevent synthesis/secretion of extracellular matrix molecules, including collagen, fibronectin, and glycosaminoglycans [9]. Recently, based on clinical and experimental results, VP is reported to be an excellent choice as a scar modulator [9–11]. Also, some studies have indicated VP's multiple effects, such as anti-inflammation, anti-fibrosis, anti-scar, anti-cancer, and neuro-protection [11–15]. Intra-articular joint adhesion formation, being closely related to inflammatory response and scar formation, has a similar formation mechanism with keloid [11–13]. Thus, the present research was designed.

In the present study with knee operative rabbits, VP was used to investigate its efficacy on the prevention of intra-articular adhesion. Specifically, we performed macroscopic assessment, analysis of histology, and hydroxyproline content. We hypothesized that topically applied VP could be a useful preventive approach for reducing intra-articular adhesion, which could be tested in future clinical trails.

Material and Methods

Animals and experiment design

A total of 40 healthy adult New Zealand white rabbits (mean weight 4.0 kg) were purchased from the Radiation Study Institute Animal Center, Tianjin, China. The present research was approved by the Tianjin Medical University Medical Ethics Committee. According to the principles of both the European Communities Council Directive (86/809 /EEC) and International Laboratory Animal Care (ILAC), animals were housed in the laboratory equipped with full-time staff, constant 20 to 25°C room temperature, a 12-hour light-dark cycle, and accessible clean food and water ad libitum. Before the experiment, the rabbits were housed for 10 days to adjust them to the environment. Animals were randomly divided into 4 groups (10 rabbits in per group) according to the different treatments: 1) 1mg/ml VP treatment group; 2) 2.5 mg/ml VP treatment group; 3) 5 mg/ml VP treatment group; 4) control group (vehicle treatment group, vehicle composition: 5% propylene glycol, 50% alcohol, and 45% distilled water).

Reagents and antibodies

Verapamil and β-dimethylaminobenzaldehyde were bought from Sigma-Aldrich Corporation. Cal-EX II solution for decalcification and dehydration was purchased from Thermo Fisher Scientific (Waltham, MA, USA). Primary antibody (ab92547) was purchased from Abcam (Cambridge, UK). Secondary antibodies were bought from Santa Cruz Biotechnology (USA).

Surgical procedure and topical treatment

The rabbit knee intra-articular adhesion model was performed according to the previous protocol [3]. Sterile conditions were prepared beforehand. After induction of anesthesia by urethane (20%, intravenous injection), the fur around the left knee joint was shaved. Iodophor was used to sterilize the exposed skin. With a medial parapatellar approach, the knee was opened, and the lateral and the medial sides of the femoral condyle were exposed. With a dental burr operated on both sides of the femoral condyle without destroying the articular cartilage, the cortical bone (approximately 10×10 mm squares) was removed until the exposure of the cancellous bone underneath.

Verapamil (1 mg/ml, 2.5 mg/ml, and 5 mg/ml separately diluted in vehicle) or vehicle was administered to the operative sites with cotton for 5 min separately [14,16], then the cotton was removed from the surgical field. Immediately, the operative site was irrigated with saline to eliminate surplus VP. Then the site was sutured surgically. To reduce the risk of infection, the animals were postoperatively given antibiotic (Baytril; Bayer AG Leverkusen) for 5 days. Employing Kirschner wires, the surgical limbs were subjected to extra-articular knee-joint immobilization in the full flexed position for 4 weeks. All the animals were individually housed in the aforementioned condition.

Macroscopic evaluation of joint adhesion

Selecting 3 rabbits randomly per group, macroscopic evaluation was performed 4 weeks post-surgery. The surgical knee was reopened with re-anesthesia, and the presence and severity of intra-articular adhesions was evaluated, with the results based on the visual scoring system (Table 1) [1–3].

Hydroxyproline content determination

We measured the hydroxyproline content (HPC) in the rabbits used for macroscopic assessment. Approximately 20 mg

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Table 1. Visual score system.

Grade 1	No adhesions
Grade 2	Weak, mild, filmy adhesions that can be easily dissected by minimal manual traction
Grade 3	Moderate adhesions that can be dissected by manual traction
Grade 4	Dense and firmly fibrous adhesions that must be surgically removed

wet-weight adhesion tissue was obtained from the decorticated area. The HPC was examined using a previously described method [17]. With 6 mol/l HCl, the samples were lyophilized, ground, and hydrolyzed at 130°C for 12 hours.

Using methyl red as the indicator, the samples were then neutralized with NaOH. One milliliter of chloramine T solution was added to the both standards and samples. After incubation for 20 minutes at room temperature, 1 ml of β-dimethylaminobenzaldehyde was added to the standards and samples. The absorbance was determined at 550 nm using a spectrophotometer. Finally, based on a standard curve constructed with serial concentrations of commercial hydroxyproline, the hydroxyproline content of scar tissue was obtained.

Histological analysis

Three rabbits were randomly selected per group at 4 weeks post-operatively. After euthanizing with an overdose of urethane, the entire knee joints, including all fibrotic adhesive scar tissue and connective tissue, were removed. All samples were fixed in 10% phosphate-buffered formaldehyde solution. After decalcification and dehydration with Cal-Ex II solution for 1 week, samples were paraffin-embedded, and 5-μm axial sections of the sample were made.

Six odd-numbered sections from each sample were stained in Masson's trichrome staining. Fibrous adhesion was determined under the help of a light microscope (Leica CM3050S, Germany). Presence of fibrous adhesions was accepted as positive if the fibers were observed at the surface of the knee articular cartilage.

Six even-numbered sections from each sample were stained with hematoxylin-eosin (H&E). Fibroblast count in every section was defined as the average count from 3 different fields randomly selected at the margins and in the middle of the decorticated sites within every section.

To further quantify the density of fibroblasts, the vimentin immunohistochemistry was performed. The number of vimentin was evaluated. Three areas were selected. The positive vimentins were counted and mean was calculated.

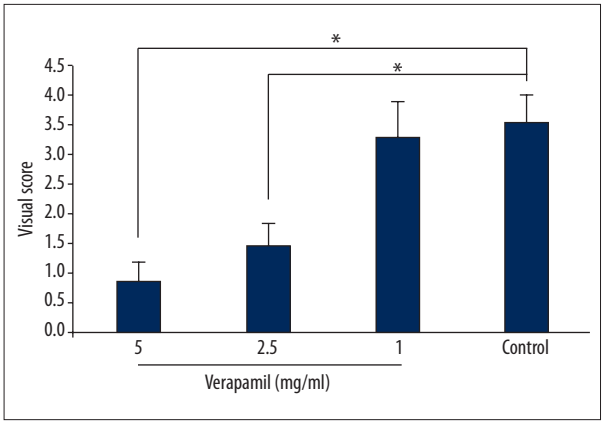

Figure 1. Macroscopic evaluation of intra-articular adhesion among the 4 groups. * P<0.05 compared with the control group.

Statistical analysis

Data are expressed as mean ± standard error of mean (SEM) values of the mean, median, and minimum-maximum. Differences among groups were assessed with one-way analysis of variance (ANOVA) using the SPSS 13.0 statistical package (SPSS Inc., Chicago, IL, USA). Bonferroni correction, as post hoc test, was performed. Differences were considered statistically significant at $p<0.05$.

Results

The knee surgery was successfully performed on all rabbits and none of the rabbits died intra- or post-operatively, and no cutaneous necrosis, obvious adverse effects, or mortality in the rabbits in the post-operative period were observed.

Macroscopic determination of intra-articular adhesion

Macroscopic determination results suggested that weak or soft fibrous adhesion was observed around the surgical sites in the 5 mg/ml VP group (visual score=0.86±0.34). Moderate scar adhesion was seen in the surgical sites in the 2.5 mg/ml VP group (visual score=1.44±0.39. However, tenacious and dense fibrous adhesion was seen around the surgical sites in the 1 mg/ml VP (visual score=3.27±0.61) and control groups (visual score=3.51±0.49). Compared with the control group, the 5 mg/ml and 2.5 mg/ml VP group had significantly better visual score, and the 1 mg/ml VP group showed no significant result (Figure 1).

Hydroxyproline content evaluation

The hydroxyproline contents of the intra-articular scar for the 4 groups are shown in Figure 2. In the 5 mg/ml VP group, the hydroxyproline content was 27.54±5.28 μg/mg. Compared

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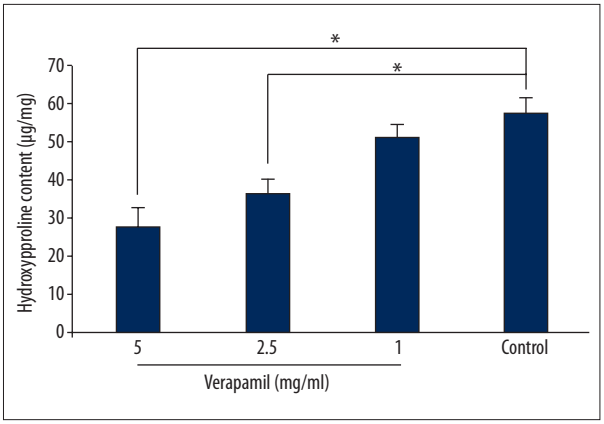

Figure 2. Hydroxyproline content evaluations among the 4 groups. The contents are given as mg/mg. Results are the mean±standard deviation of hygrotissue. * P<0.05 compared with the control group.

with those in the 2.5 mg/ml VP group (36.39±3.89 μg/mg, P<0.001), 1 mg/ml VP group (51.39±3.35 μg/mg, P<0.001), and control group (57.32±4.17 μg/mg, P<0.001), it showed significantly less. The content in the 2.5 mg/ml VP group was lower than those in the 1 mg/ml VP group (P<0.001) and control group (P<0.001). No significant difference in hydroxyproline content was seen between the control group and the 1 mg/ml VP group (P=0.194) (Figure 2).

Histological determination of adhesion

With little or no adhesions, loose and thin scar tissues were found in the 5 mg/ml VP group (Figures 3A, 4A). In the 2.5 mg/ml VP group, moderate adhesion tissues were seen around the operative sites, and the density of fibroblasts significantly decreased compared with those of the 1 mg/ml group and control group (Figures 3B, 4B). In the control group and 1 mg/ml VP group, dense adhesions and markedly fibrous scar tissues were seen around the operative sites (Figure 3C, 3D). Many fibroblasts were seen in the adhesion tissue around the operative sites (Figure 4C, 4D).

Fibroblasts density in surgery sites

Figure 4 shows the fibroblast density situations of the intra-articular scar tissue in the 4 groups. For fibroblast counting in scar tissue, the number in the 5 mg/ml VP group was (35.26±11.71), significantly less than in the 2.5 mg/ml VP group (44.82±20.36, P=0.005), 1 mg/ml VP group (68.32±22.49, P<0.001), and control group (71.92±19.38, P<0.001). There were fewer fibroblasts in the 2.5 mg/ml VP group than in the control group (P=0.001) and 1 mg/ml VP group (P=0.001). The fibroblast number in the 1 mg/ml VP group and control group showed no significant difference (P=0.302).

In order to be more definitive, an additional immunohistochemistry analysis for vimentin was performed. Figure 5 shows the density of vimentin in the intra-articular scar tissue of the 4 groups. In the 5 mg/ml VP group, the positive vimentin number in intra-articular scar tissue was 20.19±8.32, significantly less than those in the 2.5 mg/ml VP group (29.34±11.47, P=0.004), 1 mg/ml VP group (35.49±19.33, P<0.001), and control group (44.27±15.26, P<0.001). The number of vimentin in the 2.5 mg/ml VP group was lower than in the control group

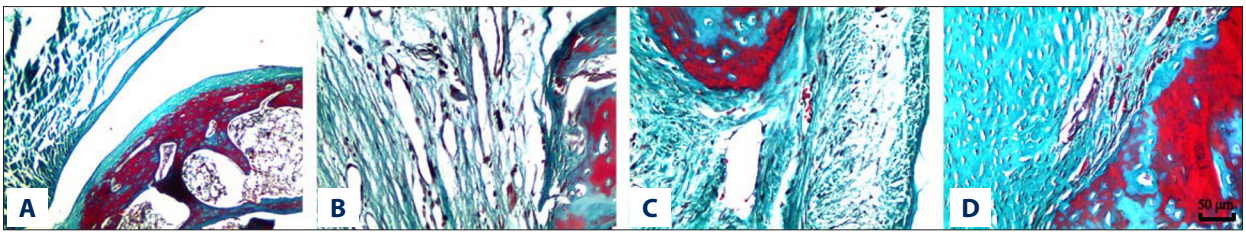

Figure 3. Masson's trichrome staining for adhesion tissues at operative sites treated with VP at 5 mg/ml (**A**), 2.5 mg/ml (**B**), 1 mg/ml (**C**), or vehicle (**D**) (Magnification× 100). (**A**): Loose scar tissue without adherence to the knee joint was found in the 5 mg/ml VP group. (**B**): Moderate scar tissue was observed in the 2.5 mg/ml VP group. (**C, D**): Dense scar tissue adherent to the knee joint was noted in the 1 mg/ml VP and control groups.

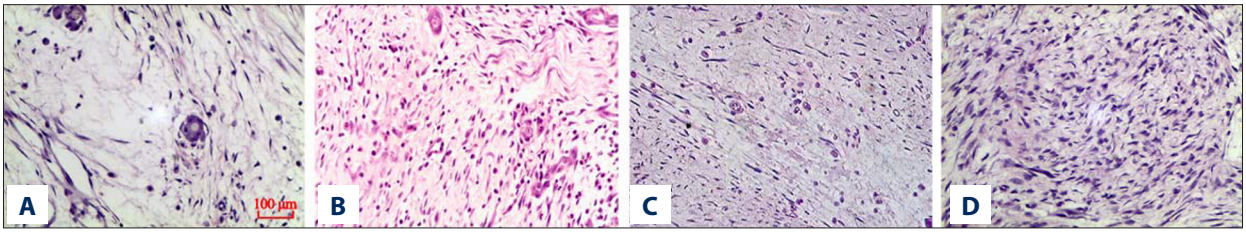

Figure 4. H&E staining analysis of fibroblasts in post-operative scar tissues treated with VP at 5 mg/ml (**A**), 2.5 mg/ml (**B**), 1 mg/ml (**C**), and vehicle (**D**) (Magnification ×400).

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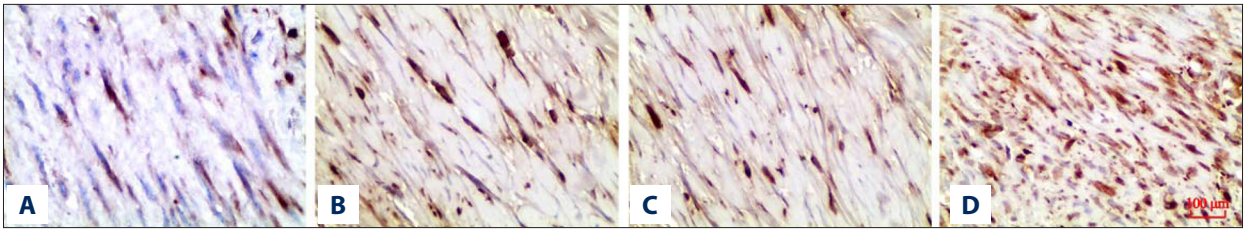

Figure 5. Vimentin expressional levels in post-operative scar tissue: 5 mg/ml VP (**A**), 2.5 mg/ml VP (**B**), 1 mg/ml VP (**C**), and vehicle (**D**) (Magnification ×400).

(P=0.001) and 1 mg/ml VP group (P=0.001). Moreover, compared with that of the control group, no significant result was found in the 1 mg/ml VP group (P=0.418).

Discussion

Until now, the etiology of knee intra-articular adhesion has been unclear. Gradually, it has become widely accepted that the hyperplasia of fibrous tissue plays a clear role during the formation of intra-articular scar adhesion. After extensive literature reviews, among various agents studied to prevent intra-articular adhesion, mitomycin C (MMC) seems to be able to reduce the condition of fibrosis by inhibiting collagen synthesis in fibroblasts in both rats and rabbits [2,6,18]. Previous research has shown that MMC prevents scar tissue formation by inhibiting fibroblasts proliferation, but due to its toxicity it is not widely used clinically. Some research attention has focussed on determining if topical application of MMC would increase the rate of infection [19]. There is a need for an effective therapeutic agent with little or no toxicity.

Potential abilities of VP that could reduce epidural scar adhesion and inhibit fibrotic cells proliferation of surgery sites were suggested in the present study. Multiple evaluations, including the hydroxyproline content evaluation, the histological analysis, the visual score system, and the density grade of fibrotic cells, suggested a good efficacy of VP in inhibiting intra-articular adhesion in rabbits. Previous studies reported the anti-fibrotic, anti-inflammatory, and anti-proliferative properties of VP employed in different fields [11–15]. In the present study, its properties were proven again in knee post-surgery rabbits. VP showed its superior effect in the aforementioned multiple-parameter analyses. Accordingly, both the depositing collagen fibrosis and joint adhesion decreased. Previous research and the present results may indicate some possible mechanisms that make VP effective in preventing knee joint adhesion [14,20]. We hypothesize that the major mechanisms of VP in preventing intra-articular adhesion are its role on reducing fibrotic cells proliferation and down-regulating inflammatory activity [21]. Undoubtedly, this needs further research.

Despite recent advances in the understanding of wound healing and scar formation, the treatment of intra-articular adhesion is still controversial. For the first time, in 1990 by Lee and Ping, VP clinically used collagen matrix in connective tissue remodeling [22]. Subsequently, the literature reported VP to be a promising treatment for earlobe keloids, with a 55% cure rate [23]. A recent orthopedics report suggests that VP could be a good choice for inhibiting epidural fibrosis [14]. A recent hypothesis suggests that the use of platelet-rich plasma can promote the clinical healing of meniscal tears [24]. Thus, the combination of VP and platelet-rich plasma is a potential strategy that needs to be tested. To the best of our knowledge, the present study is the first to investigate the suppressive effects of VP on intra-articular adhesion in rabbits. Further research on drug safety, safe and effective concentration, long-term effects, and possible adverse effects of VP are all needed before clinical trials can be performed.

Conclusions

In our rabbit model of knee surgery, intra-articular application of VP was able to decrease intra-articular adhesion formation after surgery. We showed that VP could prevent rabbit intra-articular adhesion in a dose-dependent manner, and the highest concentration used in the study (5 mg/ml) proved to be the most effective.

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