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All-Trans Retinoic Acid Reduces Joint Adhesion Formation: An Experimental Study in Rats

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Statistical Analysis C
Data Interpretation D
Manuscript Preparation E
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Background: Intra-articular adhesion is a common complication in post-surgical knees. The formation of post-surgical joint adhesion could lead to serious conditions. All-trans retinoic acid (ATRA) is a physiological metabolite of vitamin A that has a wide range of biological activities. The aim of the study was to verify the effects of (ATRA) in preventing adhesions in the post-operative rat knee.

Material/Methods: Eighty healthy adult male Wistar rats underwent femoral condyle-exposing surgery. After surgery, cotton pads soaked with the vehicle or various concentrations of ATRA (0.1%, 0.05%, 0.025%) were applied to the surgery site for 5 min. The post-surgical knee joints were fixed with micro-Kirschner wires in a flexed position for 4 weeks. The rats were killed 4 weeks after surgery. The effect of ATRA on the prevention of intra-articular adhesion was evaluated using histological analyses, hydroxyproline content, visual score, and inflammatory factor activity evaluation.

Results: No obvious postoperative complications or signs of infection in the rats were observed. None of the rats died before the scheduled time. The rats in the 0.1% ATRA group showed better outcomes, as suggested by the visual scores, hydroxyproline contents, and inflammatory factors expressional levels, than the other 2 groups. The local application of 0.1% ATRA was able to suppress adhesions, collagen expression, and inflammatory activity in the post-surgical rat knees.

Conclusions: In the rat knee surgery model, the application of intra-articular ATRA was able to decrease intra-articular scar adhesion formation, collagen expression, and inflammatory activities. ATRA was found to work in a dose-dependent manner, with 0.1% being possible optimal concentration.

MeSH Keywords: Knee Joint • Tissue Adhesions • Tretinoin

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Background

Intra-articular adhesion is a common complication in post-surgical knees. The formation of joint adhesions in the post-surgical knee could interfere with knee biomechanics, leading to severe dysfunctions such as knee stiffness, cartilage degeneration, and arthralgia [1–3].

No effective treatments exist for patients with established intra-articular adhesion, and most reoperations for intra-articular adhesion are unsuccessful [4]. A variety of agents and mechanical barriers have been studied to prevent joint adhesions both in animal models and humans, including intra-articular chitosan injection, intra-articular mitomycin C injection, calcium channel blockers, hyaluronan derivative gel, and topical application of colchicine [5–8]. Although some of these interventions can confer a certain level of satisfactory range of joint motion, it is common for the adhesion to reappear after the treatment. Thus, we sought an approach with long-term effect.

The ideal treatment for intra-articular adhesion is to prevent the scar formation. Recently, reports of the anti-fibrotic and anti-inflammatory effects of all-trans retinoic acid (ATRA) inspired us to investigate its effect in preventing intra-articular adhesion [9–13]. ATRA is a physiological metabolite of vitamin A that has a wide range of biological activities: it can affect cell differentiation, embryogenesis, proliferation, apoptosis, and inflammation [9,10]. ATRA might ease bleomycin-induced pulmonary fibrosis via inhibition of expressions of interleukin (IL)-6 and transforming growth factor (TGF)-β [9]. It has been reported that joint adhesion formation is closely related to inflammatory response and can be regarded as tissue fibrosis [11–13].

The purpose of the present study was to determine the effect of ATRA on fibrotic adhesion reduction after knee surgery.

Material and Methods

Animals

Eighty healthy adult male Wistar rats, weight 400±20 g, were used in this study. Experiments were carried out in compliance with the EU Directive 2010/63/EU for animal experiments and were approved by the Animal Research Committee of Tianjin Medical University. All rats were randomly divided into 4 groups, with 20 rats in each group: 0.1% ATRA, 0.05% ATRA, 0.025% ATRA, and vehicle (composition: propylene glycol – 5%, alcohol – 50%, and distilled water – 45%). The animals were given 7 days to acclimate to the surroundings.

Drugs and antibodies

ATRA and β-dimethylaminobenzaldehyde were bought from the Sigma Corporation (St Louis, USA). Cal-EX II solution for both dehydration and decalcification was bought from Thermo Fisher Scientific (Waltham, USA), Orangeburg. Reverse transcriptase was bought from Promega (Madison, USA).

Rat model

Sterile conditions for surgery were prepared. The rat model of intra-articular adhesion was created based on the previously established approach [2,4,11]. Anesthetization was initiated by the intra-peritoneal injection of chloral hydrate. After successful administration of anesthesia, the left knee joint fur was shaved. Iodine was used to sterilize the exposed skin. A medial parapatellar approach was used to open the sterilized knee. After exposing the lateral and medial sides of the femoral condyle, approximately 4×4 mm^2 of cortical bone of the femoral condyle was removed with an electrical dental burr. The cancellous bone surface was exposed and the articular cartilage was left intact.

The application of ATRA

A total of 4 solutions were prepared (0.1%, 0.05%, and 0.025%; propylene glycol-5%, distilled water 45%, and alcohol 50%) [15].

The cotton pads with a volume of 4×4 mm^2 were made, which can absorb a volume of 0.8 ml liquid. One of 3 concentrations of ATRA (0.1%, 0.05%, or 0.025%) or vehicle was applied to the surgery sites for 5 min. Wet gauze pads were used to protect the surrounding tissues. After removing the cotton pads from the surgical field, saline was used to immediately irrigate the decorticated areas of the femoral condyle to remove the remaining ATRA. The surgical site was then sutured to close. The rats were postoperatively given antibiotic to reduce the risk of infection (Baytril; Bayer AG Leverkusen) for 7 days. The post-surgical knee joints were fixed with micro-Kirschner wires in a flexed position for 28 days. Rats were kept in individual cages with food and clean water ad libitum.

Macroscopic assessment of joint adhesion

Five rats were randomly selected from each group 4 weeks after the surgical procedure for macroscopic assessment. The surgical sites were reopened, and the intra-articular adhesions were evaluated in a double-blind fashion, with the results according to the visual score (Table 1) [14].

Determination of hydroxyproline content in scar tissue

Hydroxyproline content (HPC) determination was conducted after the rats were killed. A total of 5 mg of wet-weight scar

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Table 1. Visual score.

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

tissue was collected from the surgical site from each rat. HPC determination was examined according to the protocol of our previous study [16]. The collected samples were then lyophilized, ground, and hydrolyzed with 6 mol/l HCl at 110°C for 24 h. After that, 1 ml hydroxyproline developer (β-dimethylaminobenzaldehyde) was added to the processed samples and standards. Absorbance was evaluated at 550 nm using a spectrophotometer. Finally, the HPC/mg of collected sample was calculated based on the standard curve constructed with serial concentrations of hydroxyproline.

Histological analysis

Intracardial perfusion with saline and then 4% paraformaldehyde was performed. The knee joint capsules were resected en bloc, preserving both soft tissues and adhesive scar, and fixed in 10% phosphate-buffered formaldehyde solution. After 5 days of decalcification and dehydration with Cal-Ex II solution, the samples were embedded in paraffin. Then 5-μm axial sections of the samples were stained with hematoxylin and eosin (H&E). The specimens were observed using a light microscope (Leica CM3050S, Germany) for scar adhesions. The presence of fibrous adhesions was accepted as positive if the fibers were seen on the surface of the articular cartilage [1].

Analysis of concentrations of inflammatory factors

We analyzed mRNA levels of IL-6 and TGF-β1. The scar tissue around the knee joint was collected. Then total RNA was extracted using TRIzol reagent. The RNA (2 μg) was transcribed into cDNA. Quantitative real-time PCR (RT-PCR) was conducted by Bio-Rad MYIQ2 (Hercules, USA) [16,17].

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 1-way analysis of variance (ANOVA) using SPSS 19.0 software. Bonferroni correction was performed as a post hoc test. Differences were considered statistically significant when $p<0.05$.

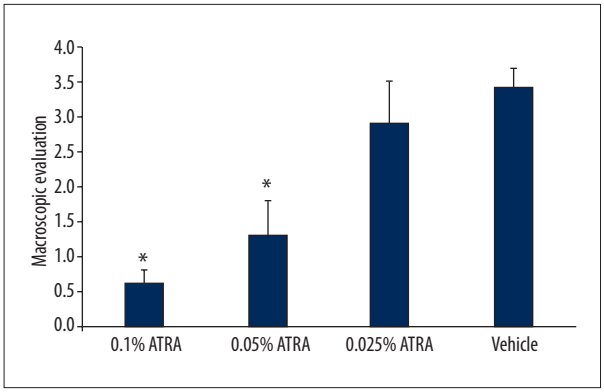

Figure 1. Macroscopic evaluation of intra-articular adhesion among four groups. * P<0.05 compared with vehicle.

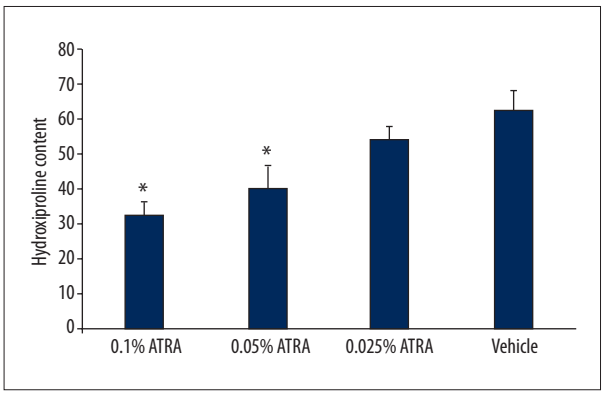

Figure 2. Hydroxyproline content (HPC) among all four groups. HPC expressed as micrograms per milligram (mg/mg). Results are the mean ± standard deviation of hygrotissue. * P<0.05 compared with vehicle.

Results

The knee surgery was well-performed by experienced surgeons and well-tolerated by all rats. None of the rats died intra-operatively or post-operatively, and no obvious adverse effects were observed.

Macroscopic determination of the intra-articular adhesion

Soft or weak fibrous adhesions were observed around the decorticated areas of the femoral condyle in the 0.1% ATRA group (visual score=0.61±0.22). In the 0.05% ATRA group (visual score=1.32±0.51), moderate scar adhesion was observed and it can be dissected with manual traction. Dense and tenacious scar adhesions were seen around the decorticated areas of the femoral condyle in the 0.025% ATRA (visual score=2.92±0.63) and vehicle groups (visual score=3.41±0.32); these adhesions were difficult to dissect. Bleeding could not be avoided in the dissection. The classification of intra-articular adhesion was determined according to the visual score (Figure 1). The 0.1%

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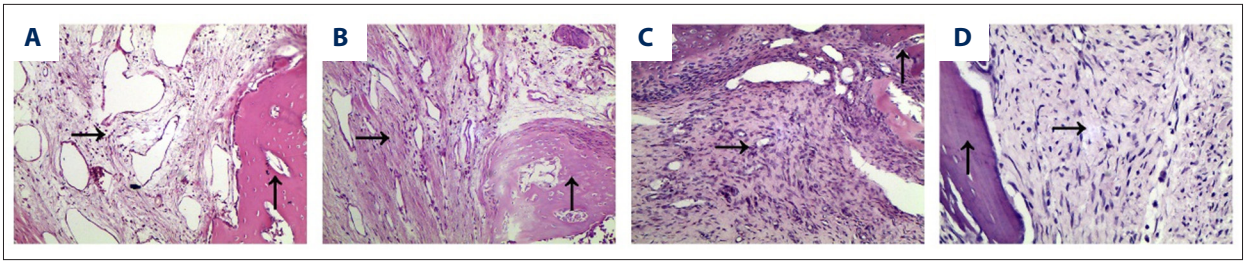

Figure 3. H&E staining for adhesion tissues at operative sites treated with ATRA at 0.1% (**A**), 0.05% (**B**), or 0.025% (**C**) or vehicle (**D**). (Magnification ×100). (**A**) Loose scar tissue without adherence to the knee joint was found in the 0.1% group. (**B**) Moderate scar tissue was observed in the 0.05% group. (**C, D**) Dense scar tissue adherent to the knee joint was noted in the 0.025% ATRA and vehicle groups. Right arrow: joint scar adhesion. Upward arrow: the bony structure of knee.

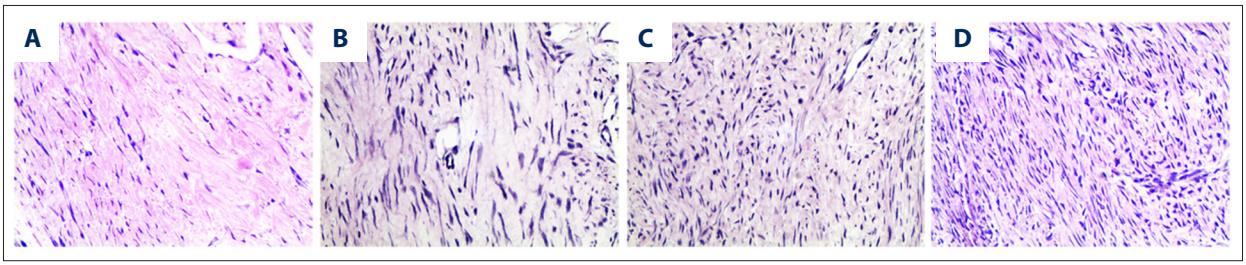

Figure 4. H&E staining analysis of fibroblasts in scar adhesion tissues treated with ATRA at 0.1% (**A**), 0.05% (**B**), or 0.025% (**C**) or vehicle (**D**). Fibroblast density: 0.1% ATRA <0.05% ATRA <0.025% ATRA <vehicle. (Magnification ×400)

ATRA and 0.05% ATRA groups had significantly better visual evaluation results than the vehicle group.

HPC analysis

Figure 2 shows the HPC of the intra-articular adhesion tissue for each treatment group. The HPC in the 0.1% ATRA group was 32.89±3.88 μg/mg, significantly less than those in the 0.05% ATRA group (40.59±6.52 μg/mg, P<0.001), the 0.025% ATRA group (54.33±4.32 μg/mg, P<0.001), and the vehicle group (62.76±5.73 μg/mg, P<0.001). The HPC in the 0.05% ATRA group was less than in the 0.025% ATRA group (P=0.002) and the vehicle group (P<0.001). HPC in the vehicle group was not significantly different from that in the 0.025% ATRA group (P=0.176) (Figure 2).

Histological analysis of the intra-articular adhesion

As shown in Figure 3, in both the 0.025% ATRA group and the vehicle group, notable scar tissues leading to dense adhesions around the surgery site were observed and the dense adhesions tethered the soft tissues to the exposed femur surface. As shown in Figure 4, many visible fibroblasts showed a dense arrangement in the adhesion tissue around the surgical site. The 0.05% ATRA group showed a moderate adhesion around the surgical site and a decrease of fibroblast density. Conversely, the situations in the 0.1% ATRA group suggested a loose and thin adhesion and the least fibroblast density.

ATRA effect on inhibiting fibroblasts proliferation in scar tissue

Fibroblast counting was successfully performed for each group. Figure 4 shows the fibroblast count in the intra-articular scar tissue of each group. In the 0.1% ATRA group, the fibroblast count in the adhesion tissue was 20.12±9.64, significantly less than that of the 0.05% ATRA group (42.37±11.79, P=0.006), the 0.025% ATRA group (62.11±12.31, P<0.001), and the vehicle group (72.34±15.42, P<0.001). The fibroblast count in the 0.05% ATRA group was less than that in the 0.025 ATRA group (P=0.001) and the vehicle group (P=0.001). Conversely, the count in the 0.025% ATRA group did not show a significant change compared with the vehicle group (P=0.288).

ATRA ability to suppress TGF-β1 and IL-6 expression

To determine whether ATRA exerts an effect on TGF-β1 and IL-6 expression in rats after knee surgery, we conducted RT-PCR to examine their mRNA expression levels. The RT-PCR results are shown in Figure 5. The 0.1% group had the lowest values, followed by the 0.05% group, the 0.025% group, and the vehicle group.

Discussion

The wound repair process occurs in almost all tissues after any destructive stimulus, and is one of the most complex biological

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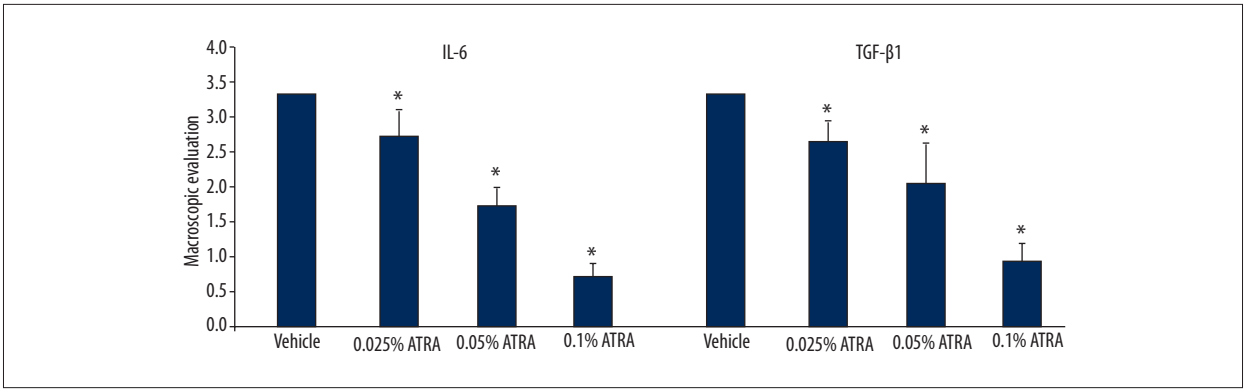

Figure 5. mRNA expression levels of IL-6 and TGF-β1 in scar adhesion tissue in each group. * P<0.05 compared with vehicle.

processes [18]. As a stimulus, surgery will initiate the healing. Healing by fibrosis usually results in unsatisfactory clinical sequelae, including postoperative intra-abdominal adhesions, epidural fibrosis, and postoperative liver adhesions [19–22]. The detailed formation mechanism of intra-articular knee adhesion was not well represented in the literature. Fibroblastic activity exerts a key role in this process [6,7,11]. Fibroblast accumulation in the surgical area is considered to be caused by the activation of inflammatory activity, at which time the fibroblasts synthesize collagen and generate collagenous fibers. To effectively prevent scar adhesion, efforts should be made to avoid inflammation activation, fibroblasts accumulation, and collagen deposition.

The normal tissue response to injury occurs in 3 overlapping but distinct stages [18,23]. The inflammation stage occurs immediately after tissue damage. The immune system and inflammatory pathways are activated to prevent fluid loss, blood loss, and infection. New tissue formation, as the second stage of the wound healing process, occurs 2–10 days after injury. Both kinds of cell proliferation and migration occur during this stage. The third stage, remodeling, occurs 2–3 weeks after injury and lasts for 1 year or more. To overcome the intra-articular adhesion, therapy should be able to affect some, if not all, of the aforementioned stages.

In the present study, 3 different concentrations of ATRA were applied to rat knee joints post-operatively in an attempt to reduce intra-articular adhesion. The data showed ATRA significantly decreased hydroxyproline levels in scar tissues in a dose-dependent manner. In the operative rat model, the number of fibroblast decreased significantly after topical application of ATRA. The scar adhesions showed the best visual scores when treated with the highest concentration ATRA (0.1%). RTPCR analyses also showed that ATRA is able to decrease the expressions of inflammatory factors dose-dependently. All of the above findings support our theory of the effect of ATRA on suppressing the inflammation, fibrotic formation, and fibroblast proliferation. In addition, data in the literature supports our theory by reporting on the multiple suppressing properties of ATRA [24,25]. Pharmacological studies have also shown that ATRA is effective for treating keloid scar tissue [26]. We previously proved that ATRA prevents epidural fibrosis in rats post laminectomy [27]. These studies and our data may explain some possible mechanisms that make ATRA effective in suppressing joint adhesions by inhibiting fibroblast proliferation, collagen deposition, and inflammatory factor expression.

The limitation of the present study is the lack of evaluation toxicity and long-term effects. We plan to focus on determining the toxic dose and side and adverse effects of ATRA in future studies.

Conclusions

In our rat knee surgery model, intra-articular application of ATRA was able to decrease adhesion formation, collagen expression, and inflammatory factor expression. ATRA can prevent adhesion of the rat joint in a dose-dependent manner. The highest concentration used in this study (0.1%) was the most effective.

Conflict of Interests

None.

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