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

Yuguang Wang*  
Chao Zhang*  
Huan Cheng  
Patricia Douglas  
Zhiqiang Wang  
Yun Lu

* These authors contributed equally to this work

Corresponding Author: Yun Lu, e-mail: luyuntaida@163.com

Source of support: This research was sponsored by Tianjin Applied Basic and Frontier Technology Research Projects (10CYBJC13900) and (12ZCZDSY02800)

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 expression 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

Full-text PDF: http://www.medscimonit.com/abstract/index/idArt/894086
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 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 mm² 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 mm² 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 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...
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.

**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%

| 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 |

**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.**
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 processes. The development of adhesion is a key factor in the inflammatory response. The inflammatory mediators, cytokines, and growth factors are involved in the adhesion process and influence the formation of scar tissue.

This work is licensed under a Creative Commons Attribution-NonCommercial-NoDerivs 3.0 Unported License.
processes [18]. As a stimulus, surgery will initiate the healing. Healing by fibrosis usually results in unsatisfactory clinical sequelae, including postoperative intra-abdominal adhesions, epidual 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. To effectively prevent scar adhesion, efforts should be made to avoid inflammation activation, fibroblasts accumulation, and collagen deposition.

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.

**Reference:**

1. 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]

2. Li X, Yan L, Wang J et al: Comparison of the effects of mitomycin C and 10-hydroxycamptothecin on an experimental intraarticular adhesion model in rabbits. Eur J Pharmacol, 2013; 703: 42–45

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

![Figure 5](image-url)
3. 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 Arthroscopy, 2004; 12: 545–51
4. Liang Y, Sun Y, Li X et al: The optimal concentration of topical hydrocortisone in preventing intraarticular scar adhesion. Sci Rep, 2014; 4: 4621
5. Liu Y, Skardal A, Shu XZ et al: Prevention of peritendinous adhesions using a hyaluronan-derived hydrogel film following partial-thickness flexor tendon injury. J Orthop Res, 2008; 26: 562–69
6. Sun Y, Liang Y, Hu J et al: Reduction of intraarticular adhesion by topical application of colchicine following knee surgery in rabbits. Sci Rep, 2014; 23:4: 6405
7. Li Y, Ma X, Yu P et al: Intra-articular adhesion reduction after knee surgery in rabbits by calcium channel blockers. Med Sci Monit, 2014; 20: 2466–71
8. Riccio M, Battiston B, Pajardi G et al: Efficiency of Hyaloglide in the prevention of the recurrence of adhesions after tenolysis of flexor tendons in zone II: a randomized, controlled, multicentre clinical trial. J Hand Surg Eur, 2010; 35: 130–38
9. Dong Z, Tai W, Yang Y et al: The role of all-trans retinoic acid in bleomycin-induced pulmonary fibrosis in mice. Exp Lung Res, 2012;38: 82–89
10. Datta PK, Lianos EA: Retinoic acids inhibit inducible nitric oxide synthase expression in mesangial cells. Kidney Int, 1999;56: 486–93
11. Wang J, Yan L, Sun Y et al: A comparative study of the preventive effects of mitomycin C and chitosan on intraarticular adhesion after knee surgery in rabbits. Cell Biochem Biophys, 2012; 62: 101–5
12. Eakin CL: Knee arthrofibrosis: prevention and management of a potential ly devastating condition. Phys Sportsmed, 2001; 29: 31–42
13. Griffith M, Hindocha S, Iordan D et al: An overview of the management of flexor tendon injuries. Open Orthop J, 2012; 6: 28–35
14. Rothkopf DM, Webb S, Szabo RM et al: An experimental model for the study of canine flexor tendon adhesions. J Hand Surg Am, 1991; 16: 694–700
15. Dematte MF, Gempeler R, Salles AG et al: Mechanical evaluation of the resistance and elasitance of post-burn scars after topical treatment with tretinoin. Clinics (Sao Paulo), 2011; 66: 1949–54
16. Zhang C, Kong X, Zhou H et al: An Experimental Novel Study: Angelica sinensis Prevents Epidual Fibrosis in Laminectomy Rats via Downregulation of Hydroxyproline, IL-6, and TGF-B1. Evid Based Complement Alternat Med, 2013; 2013: 291814
17. Zhang C, Kong X, Liu C et al: ERK2 small interfering RNAs prevent epidual fibrosis via the efficient inhibition of collagen expression and inflammation in laminectomy rats. Biochem Biophys Res Commun, 2014; 444(3): 395–400
18. Zielins ER, Atashroo DA, Maan ZN et al: Wound healing: an update. Regen Med, 2014; 9(6): 817–30
19. Inagaki NF, Inagaki FF, Kokudo N, Miyajima A: Use of mouse liver mesothelial cells to prevent postoperative adhesion and promote liver regeneration after hepatectomy. J Hepatol, 2015; 62(5): 1141–47
20. Kuru S, Boktaril OB, Barlas AM et al: The preventive effect of dexmedetomidine against postoperative intra-abdominal adhesions in rats. Int Surg, 2015; 100(1): 87–95
21. Zhang C, Feng S, Hu N et al: Letter to the editor regarding: “Evaluation of topical application and systemic administration of rosuvastatin in preventing epidual fibrosis in rats” by Bora Gürer et al. Spine J, 2015; 15: 1165–66
22. Zhang C, Baklaushev VP, Alexandrovich MP et al: Osteopontin induces the extension of epidual fibrosis into the spinal canal. Pain Physician, 2015; 18: E93–95
23. Reinke JM, Sorg H: Wound repair and regeneration. Eur Surg Res, 2012; 49(1): 35–43
24. Oseto S, Moriyama T, Kawada N et al: Therapeutic effect of all-trans retinoic acid on rats with anti-GBM antibody glomerulonephritis. Kidney Int, 2003; 64: 1241–52
25. Tabata C, Kodokawa Y, Tabata R et al: All-trans-retinoic acid prevents radiation- or bleomycin-induced pulmonary fibrosis. Am J Respir Crit Care Med, 2006; 174: 1352–60
26. Janssen de Limpens AM: The local treatment of hypertrophic scars and keloids with topical retinoic acid. Br J Dermatol, 1980; 103: 319–23
27. Zhang C, Kong X, Ning G et al: All-trans retinoic acid prevents epidual fibrosis through NF-κB signaling pathway in post-laminectomy rats. Neuropharmacology, 2013; 79: 275–81