Effects of All-Trans Retinoic Acid on Lipopolysaccharide-Induced Synovial Explant

Kuiqing Lu1, Qilin Bao1, Dan Wang1, Yanhua NING2, Xuejian Xie3, Mingming Zhou1, Linhua Zhou1, Xiang ZENG1, Jingyan SHAN1 and Yun LI1,4,*

1 Department of Nutrition, Food Hygiene and Toxicology, West China School of Public Health, Sichuan University, 17 Renmin South Road, Wuhou District, Chengdu, Sichuan 610041, China
2 Department of Community Care, Nursing School, Ningxia Medical University, 1160 Shengli Street, Xingqing District, Yinchuan, Ningxia 750004, China
3 People’s Hospital of Deyang City, 173 North Taishan Road, Deyang, Sichuan 618000, China
4 West China School of Public Health and Healthy Food Evaluation Research Center, Sichuan University, 24 South Section One, First Ring Road, Chengdu, Sichuan 610065, China
(Received July 3, 2018)

Summary  The present study was conducted to assess the effect of all-trans retinoic acid (ATRA) on synovial explants from rats with rheumatoid arthritis (RA) induced by lipopolysaccharides (LPS). In our study, synovial membranes were excised from the knees of healthy adult Wistar female rats under sterile conditions. We first investigated the synoviums incubated in a control medium or in a medium containing 10 μg/mL LPS, each for 24, 48, and 72 h (LPS-experiment). The changes in inflammatory response from the synoviums were observed at different culture times. Then, we assessed the synoviums exposed to different ATRA concentrations for 24 h (ATRA-experiment). The controls (blank, model group, and solvent groups) were set up. The effects of ATRA on synovitis were evaluated by measuring the production of cytokines, nitric oxide (NO) and the expression of cartilage damage related proteases. In the LPS-experiment, LPS contributed to the release of interleukin-6 (IL-6), tumor necrosis factor alpha (TNF-α), and matrix metalloproteinase-9 (MMP-9) in synovial explants. Importantly, LPS did not cause a significant pathological damage. The inflammatory response observed in this model was significant for 24 h, suggesting that LPS-induced synovial explants were successfully established. In the ATRA-experiment, ATRA suppressed the expression of IL-6, TNF-α, NO, a disintegrin and metalloprotease with thrombospondin motifs-4 (ADAMTS-4), MMP-3, and MMP-9. Taken together, ATRA exhibited inhibitory effects on LPS-induced synovial immune inflammatory response stimulated by the regulation of inflammatory mediators and cartilage damage related proteases in synovial explants, demonstrating a potential protective effect on synovitis and joint destruction in the patients with RA.

Key Words  all-trans retinoic acid, synovial explant, rheumatoid arthritis, lipopolysaccharide, immune inflammatory response

Rheumatoid arthritis (RA) is a disease of unknown etiology characterized by arthrosis pain, hyperplasia (swelling), and skeletal disorders. Synovitis is a typical pathological alteration observed in patients with RA, accounting for the main symptoms of RA.

Despite the uncertain etiology of RA, many hypotheses have been presented to elucidate its pathogenesis. After an undesirable activation of the immune system, the autoimmune response of the synovium results in monocytes infiltration in the synovium, predominantly CD4+T lymphocytes, which induces an inflammatory response (1). Fibroblast-like synoviocytes (FLS) and macrophage (MΦ) secrete large amounts of interleukin-1 (IL-1), tumor necrosis factor alpha (TNF-α), IL-6, IL-8, granulocyte-macrophage colony-stimulating factor (GM-CSF), and matrix metalloproteinases (MMPs), thereby promoting synovial inflammation and joint destruction (2, 3). Inflammatory cytokines activate IκB kinase 2 (IKK2) of FLS, resulting in the phosphorylation of inhibitor κB (IκB), and the subsequent activation of nuclear factor-κB (NF-κB) as well as the expression of TNF-α, IL-1, IL-6, IL-8, cyclooxygenase (COX-2), inducible nitric oxide synthase (iNOS), and intercellular cell adhesion molecule-1 (ICAM-1) (4). Simultaneously, cytokines activate mitogen-activated protein (MAP) kinases, leading to the activation of activating protein-1 (AP-1), which further induces cytokine and MMP releases (5). These vicious cycles contribute to cartilage and bone destruction. MMPs play a role in the impaired degradation of extracellular matrix (ECM) on progression of joint damage progression. The increase in MMPs results in an imbalance between MMPs and tissue inhibitor of matrix metalloproteinases-1 (TIMP-1), thereby

*To whom correspondence should be addressed.
E-mail: liyun_611@163.com
exerting a considerable effect on joint destruction (6).

Neither conventional treatments nor biologic disease-modifying therapies can exert definite curative effects on RA. All-trans retinoic acid (ATRA) has received widespread attention as an active vitamin A derivative and an immune inflammation inhibitor (7), in addition to its ability to cure acute promyelocytic leukemia (APL) (8) and acne (9). With regard to the role of ATRA in immune inflammatory responses, ATRA reportedly participates in ameliorating inflammatory response in the epididymis by downregulating pro-inflammatory cytokines and upregulating anti-inflammatory factors (10). Other studies have also found that ATRA has immunomodulatory actions (11–13). Concerning the effect of ATRA on RA, ATRA has been reported in 1980 to inhibit collagenase production in rheumatoid synovial cells, suggesting the role of ATRA in the protection of articular cartilage (14). ATRA can also downregulate IL-1-mediated IL-6 expression in rat synovial fibroblasts (15) and significantly inhibit inflammatory cytokines after being exposed to rats with collagen induced arthritis (CIA) (16, 17), consistent with the findings from our previous study (18). ATRA has also been demonstrated to decrease humoral immune response in CIA rats and attenuate cartilage damage related proteases, which inhibits osteoclastogenesis in humans (19).

Furthermore, ATRA inhibited nitric oxide (NO), a radical gas produced during several autoimmune diseases, which is induced by action of IL-6 on peripheral blood mononuclear cells (PBMCs) from Algerian patients with RA (20). In addition, a recent study also showed that ATRA ameliorated joint damage and loss of function in patients with RA by reducing the proliferative, migratory and invasive capacity of RA FLS (21). Our preliminary study also found that prophylactic administration of ATRA reduced the expression of TNF-α and IL-17A and increased that of IL-10 in CIA, and thus delayed the progression of RA by correcting an imbalance between Th1/Th2 and Th17/Treg (22). However, the possible mechanism of the effect induced by ATRA during RA inflammation still remains clear.

Therefore, we hypothesized that ATRA attenuates immune inflammatory response in the culture model of synovitis in vitro, i.e., the synovial explant. Studies on synovitis were usually performed using animal and cell models. A more evolved synovitis model employed in vitro using the organ culture method has not yet been used. The organ culture is a method that provides a closed system, excluding the influence by internal environmental factors on an organ, in which the interaction of cells could be studied in detail (23). Currently, organ culture has been used to establish in vitro models of intervertebral disc, cornea, blood vessels and so on (24–27). Some studies have reported that synovial membrane from rats, stimulated with lipopolysaccharide (LPS), produced IL-6, TNF-α, IL-1α, and MMPs (28, 29), which is similar to the characteristics of synovitis in patients with RA. Referring to the methods employed in the aforementioned studies, we examined the effect of ATRA on the basis of LPS-induced synovial explant.

**Materials and Methods**

**Animals.** 36 Wistar female rats, weighing 230±10 g and aged 7–8 wk, were obtained from Chengdu Dossy Experimental Animals Co., Ltd. [License No. SCXK (Sichuan) 2014-028, China] and kept in a specific pathogen free (SPF)-grade lab with a 12:12 h light/dark cycle. Food and water were provided ad libitum. All rats were given humane care based on the guidelines in the Guidebook for the Care and Use of Laboratory Animals. The protocol of animal experiments was performed in accordance with the requirements of the National Act on the Use of Experimental Animals (China). The study and methods were approved by the Animal Ethics Committee of Sichuan University West China Medical School. This study was funded by the National Natural Science Foundation of China (NSFC) [grant number 81372983].

**Preparation of rat synovial membrane.** Rats were given 10% chloral hydrate (Sinopharm Chemical Reagent Co., Ltd., China) at a dose of 0.5 mL/100 g body weights via intraperitoneal injection and were euthanized using the cervical vertebra dislocation method. The separated rat hind limb was immersed into 75% ethyl alcohol (Sinopharm Chemical Reagent Co., Ltd.) for 4 min and then inoculated in phosphate-buffered saline (PBS) solution supplemented with penicillin and streptomycin (Chengdu Hali Biotech. Co., Ltd., China). The synovium was dissected from the rat hind limb under sterile conditions (Fig. 1). A total of 72 synoviums were obtained from rats.

**Incubation of rat synovial explants.** After being washed three times in PBS solution supplemented with streptomycin and penicillin, dissected synovial explants were place in each well of 24-well plates (NEST Biotech Co., Ltd., China) and subsequently inoculated in phosphate-buffered saline (PBS) supplemented with streptomycin (100 U/mL) and penicillin (100 µg/mL) and incubated in a 5% CO₂ incubator with 100% relative humidity atmosphere (Thermo Scientific, USA) at 37°C. The medium was replaced every 24 h.

**Establishment of synovial explants.** In LPS-experiment, the synovial membranes were treated with or without 10 µg/mL LPS (Sigma), each for 24, 48, and 72 h. In other words, membranes were randomly divided into the following six groups: Control group 1 (incubated in DMEM/F12 medium for 24 h, C1), Control group 2 (incubated in DMEM/F12 medium for 48 h, C2), Control group 3 (incubated in DMEM/F12 medium for 72 h, C3) and LPS-challenged group 1 (exposed to 10 µg/mL LPS for 24 h, LPS-1), LPS-challenged group 2 (exposed to 10 µg/mL LPS for 48 h, LPS-2), and LPS-challenged group 3 (exposed to 10 µg/mL LPS for 72 h, LPS-3). No serum was added because endotoxin in serum may mask LPS effects, but the lack of serum may result in an insufficient supplies. Therefore, instead of RPMI which was used in the study conducted by Hyc, DMEM/F12 was used owing to its higher nutrient content. To assess the quality of dissection and preparation of synovial explants, synoviums dissected from
rats were dehydrated and weighed after incubation. The media were collected before the end of the incubation period and stored at $220^\circ$C. An additional 36 synovial explants were used in this experiment.

**Fig. 1.** Preparation of rat synovial explant. A: Knee joint is cut off together with fragments of the femur and tibia. Opening up of the knee joint started from the femoral end. B: The articular capsule is cut off together with the adjoining structures. C: The patella ligament and collateral ligaments are cut off to expose the synovium. D: The cruciate ligament and meniscus are cut off. E: The synovium connected with the two sides of the knee joint is exposed. F: The synovium is separated from the patella and patellar ligament. G: The dissected synovium is inoculated into PBS solution supplemented with penicillin and streptomycin.

**Fig. 2.** The mean weight of synovial explants in the controls and LPS-challenged groups after incubation. Data were presented as mean $\pm$SD; n=6 in each group. Inter-group difference was significant as per ANOVA and LSD at $p<0.05$. *$p<0.05$ vs. control.

** Intervention with ATRA (Sigma) in synovial explants.**
In the ATRA-experiment, synovial explants were allocated randomly into six groups: Blank control group (incubated with DMEM/F12 medium, BC), Model control group [exposed to 10 $\mu$g/mL LPS and 2% dimethyl sulphoxide (DMSO), SC], ATRA-challenged group 1 (exposed to 1 $\mu$M ATRA, ATRA-1), ATRA-challenged group 2 (exposed to 10 $\mu$M ATRA, ATRA-2), and ATRA-challenged group 3 (exposed to 100 $\mu$M ATRA, ATRA-3); 5% fetal bovine serum (Chengdu Hali Biotech. Co., Ltd.) was added to medium according to the study conducted by Moses et al. (30) to maintain the viability of synovial explants. All groups were incubated for 24 h. After incubation, synovial explants were dehydrated and collected. Media were collected before the end of the incubation period and stored at $-20^\circ$C. An additional 36 synovial explants were used in this experiment.

**Morphological variation of synovial explants demonstrated by HE staining.** Morphological variation in a synovial explant was evaluated by HE staining in accordance with a previous study. Briefly, fixed synovium tissues were embedded in paraffin and serially sectioned (3–6 $\mu$m). The sections were stained with haematoxylin (Beyotime Institute of Biotechnology, China) for 5 min and eosin (Beyotime Institute of Biotechnology) for 2 min and then observed under an optical microscope (Nikon Eclipse CI). The morphology and structure of the synovial tissue were observed at low magnification. The category, morphology, cells quantity and morphology, location, and the proportion of non cellular components were observed; further, central necrosis and structural disorder were detected at high magnification. HE staining was used in both the experiments.

**Cell viability of synovial explants detected fluorescent staining.** The cell viability of synovial explant was checked by fluorescent staining using Normal/Apoptotic/Necrotic Cell Detection Kit (Nanjing KeyGen Biotech. Co., Ltd., China) according to the manufacturer’s protocol. Synovial explants from one group were mixed and minced (about 1 mm$^3$); 4 mL of 4 mg/mL Type
II Collagenase (Invitrogen) was added to the minced synovial explants and the cell suspension of synovial explants were prepared. The mixed dyes reagent from the kit was added to this cell suspension. Cell viability of synovial explants was observed at low magnification using inverted epifluorescence microscopy (TE2000-U, Nikon). A green, uniform, single round cell was regarded as a live cell. An orange and oval cell was considered as a necrotic cell. A green and irregular shaped cell was perceived as an early-stage apoptotic cell. An orange and anomalous cell was deemed a late-stage apoptotic cell. Fluorescent staining was performed in the LPS-experiment.

Cytokines and MMP-9 by enzyme-linked immuno sorbent assay (ELISA). IL-6, TNF-α, and MMP-9 released into the culture medium were measured using ELISA (Beijing 4A Biotech Co., Ltd., China) according to the manufacturer’s protocol. The optical density (OD) of each sample was read by a microplate reader (Thermo Fisher Scientific, USA) and expressed as picograms per milliliter. ELISA was used in both the experiments.

Nitric oxide (NO) and NO synthase measured by a chemical method. NO concentration and NO synthase activity were evaluated using Nitric Oxide Assay Kit (Nanjing KeyGen Biotech. Co., Ltd.) and Nitric Oxide Synthase Assay Kit (Nanjing KeyGen Biotech. Co., Ltd.) respectively according to the manufacturer’s protocols. The optical density (OD) of each sample was read by a microplate reader (Thermo Fisher Scientific). NO concentration and NO synthase activity were calculated by the formulas giving in the manufacturer’s manual. This chemical method was used only in the ATRA-experiment.

Expression of cartilage damage related proteases detected using immunohistochemistry. Expression of a disintegrin and metalloprotease with thrombospondin motifs-4 (ADAMTS-4), MMP-1, MMP-3 and TIMP-1 were detected using PV two-step immunohistochemical detection kit (Wuhan Boster Biological Technology Co., Ltd., China) according to the manufacturer’s protocol. The average optical density (AOD) was analyzed using Image-pro Plus 6.0. Such immunohistochemical analysis was used in the ATRA-experiment alone.

Statistical analyses. Values were expressed as mean ± standard deviation (SD). Data analysis was performed using IBM SPSS statistics 20 software. One-way analysis of variance (ANOVA) and Least-Significant Difference (LSD) test were used for comparing between different groups. Statistical significance was set at α=0.05 and was acknowledged with a probability of <5%.

RESULTS

Effect of LPS on synovial explant weight

We first compared the weight of rat synovial explants in controls and LPS-challenged groups. The method of dissecting synovial explants emerged from the study conducted by Hyc, and some details were modified based on our practice. Compared with the method proposed by Hyc, synovial explants were first separated from the
joint capsule and the popliteal fossa was opened up for exposing the synovium in a better manner; hence, a larger synovial explant was obtained. The structure was well maintained, except for the fragments connected with the two sides of the knee joint.

Thirty-six dissected rat synovial explants were weighed after incubation. The mean weight of all synovial explants was 16.79 (15.80, 17.77) mg. There were no significant differences between the mean weight of the two groups (Fig. 2). These results indicated that dissection and preparation of synovial explants had no significant effect on the present study.

Effect of LPS and ATRA on the morphological variation of the synovium

To investigate morphological variation in synovial explants, HE staining was used. Both results of the LPS- and ATRA-experiments showed that the synovial lining and synovial subintima were clearly visible and a demarcation line between them was obvious. There were two or three layers of synoviocytes in the synovial lining. Large amounts of vacuole-shaped adipocytes were seen in the synovial subintima, accompanied by some fibroblasts, macrophages, mastocytes, collagen fibers, and blood vessels (Fig. 3).

In LPS-experiment, there were no significant morphological differences between the LPS-challenged groups and controls incubated for the same times. In the ATRA-experiment, there were also no significant morphological differences between the ATRA-challenged groups and the three controls. No significant central necrosis and histological structural disorder was observed in any of the two experimental groups. These observations

Fig. 4. Fluorescent staining in the LPS-experiment. Images represent arbitrarily selected areas (40× magnification) of the stained sections.

Fig. 5. IL-6 and TNF-α concentrations (pg/mL) in LPS-experiment (A) and ATRA-experiment (B). IL-6 and TNF-α concentration was measured using ELISA. Data were presented as mean ± SD (n=6 in each group). The inter-group difference was significant according to ANOVA and LSD at p<0.05. (A) *) p<0.05 vs. control incubated for the same time; **) p<0.05 vs. C1; **p<0.05 vs. LPS-1. (B) *) p<0.05 vs. BC; **p<0.05 vs. MC and SC; **p<0.05 vs. ATRA-1; **p<0.05 vs. ATRA-2.
The Effect of ATRA on Synovitis in RA

suggested that LPS at a concentration of 10 μg/mL and ATRA ranging between 1 μM and 100 μM had no detrimental effect on synovial explants.

Effect of LPS on cell viability of synovial explants

In an attempt to assess the cell viability of synovial explants, fluorescent staining was used. After incubation, four types of cells could be differentiated using epifluorescence microscopy. In the LPS-experiment, most of these cells in all groups were live, especially in C1 and LPS-1. A small fraction of apoptotic cells at early and late stage accompanied with a predominant population of live cells was observed in all groups except C1 and

Fig. 6. NO concentration (μmol/L) (A) and NO synthase activity (U/mL) (B) in ATRA-experiment. NO concentration and NO synthase activity were evaluated using a Nitric Oxide Assay Kit and Nitric Oxide Synthase Assay Kit, respectively. Data were presented as mean±SD (n=6 in each group). Inter-group differences were significant according to ANOVA and LSD at p<0.05. * p<0.05 vs. BC; △ p<0.05 vs. MC and SC; ◊ p<0.05 vs. ATRA-1; † p<0.05 vs. ATRA-2.

Fig. 7. MMP-9 concentration (pg/mL) in LPS-experiment (A) and ATRA-experiment (B); AOD (U/mL) of ADAMTS-4, MMP-3, MMP-1, and TIMP-1 in ATRA-experiment (C, D). MMP-9 concentration was measured using ELISA. AODs of ADAMTS-4, MMP-3, MMP-1, and TIMP-1 were evaluated by immunohistochemistry. Data were presented as mean±SD (n=6 in each group). The difference between groups was significant according to ANOVA and LSD at p<0.05. (A) * p<0.05 vs. control incubated for the same time; △ p<0.05 vs. C1; ◊ p<0.05 vs. LPS-1. (B) * p<0.05 vs. BC; △ p<0.05 vs. MC and SC. (C) * p<0.05 vs. BC; △ p<0.05 vs. MC and SC; † p<0.05 vs. ATRA-1 or ATRA-2.
LPS-1 (Fig. 4).

Effect of LPS and ATRA on cytokines production

In the LPS-experiment, we noticed that after LPS treatment, a significant high level of IL-6 and TNF-α in LPS-1 were reported in comparison with that in C1 ($p<0.05$). However, when the culture time is prolonged, a significant reduction in the concentrations of IL-6 and TNF-α occurred ($p<0.05$), and no difference between LPS-2 and C2 as well as LPS-3 and C3 was reported (Fig. 5A). These observations indicated that LPS-induced synovial explants showed a significant inflammatory response only during the first 24 h.

In the ATRA-experiment, we noticed that IL-6 and TNF-α levels in MC did not differ from those in SC, which indicated that DMSO had no effect on cytokines production. IL-6 and TNF-α levels were reported to be a significantly increased in SC and MC compared with BC ($p<0.05$), suggesting an inflammatory effect of LPS. Concerning IL-6, we noticed a significant reduction in IL-6 production with 100 μM ATRA in comparison with SC ($p<0.05$), but both ATRA concentrations of 1 μM and 10 μM did not produce a significant decrease in IL-6 production. For TNF-α, despite no noticeable reduction in ATRA-1 in comparison with SC, we observed that ATRA treatment at concentrations of 10 μM and 100 μM reduced TNF-α production opposed to SC ($p<0.05$), and our results indicated a significant decrease in TNF-α production in a dose-dependent manner when treated with increasing concentrations of ATRA (Fig. 5B).

Effect of ATRA on NO and NO synthase expression

In the ATRA-experiment, NO production significantly increased in MC and SC in comparison with BC ($p<0.05$). Similarly, no differences were observed between MC and SC. In contrast, treatment in ATRA-2 and ATRA-3 induced a considerable reduction in NO production compared with SC ($p<0.05$). Simultaneously, ATRA exerted a dose-dependent effect on NO production (Fig. 6A).

To further investigate whether ATRA regulated NO production by controlling the expression of NO synthase, a Nitric Oxide Synthase Assay Kit was used. However, there was no significant difference in NO synthase expression between controls and of ATRA-treated samples (Fig. 6B).

Effect of LPS and ATRA on cartilage damage related proteases expression

In the LPS-experiment, MMP-9 expression had a significant increase in LPS-1 as compared with C1 ($p<0.05$), consistent with the increase in LPS-2 as compared with C2 ($p<0.05$). However, no difference was observed between LPS-3 and C3 (Fig. 7A). These observations indicated that LPS-induced synovial explants showed a diminishing inflammatory response with time elapsed.

In the ATRA-experiment, ADAMTS-4, MMP-3, and MMP-9 expressions were reported to be significantly increased in MC and SC compared with those in BC ($p<0.05$), and no difference between MC and SC was observed. These observations were consistent with those of the LPS-experiment. Moreover, treatment in ATRA-1, 2, 3 significantly suppressed ADAMTS-4 expression in comparison with MC and SC ($p<0.05$), and ADAMTS-4 expression in ATRA-3 was lower than that in ATRA-1 and ATRA-2 ($p<0.05$) (Fig. 7C). Regarding MMP-9, only 100 μM ATRA treatment reduced MMP-9 expression as opposed to MC and SC ($p<0.05$) (Fig. 7B). For MMP-3, 100 μM and 10 μM ATRA significantly decreased MMP-3 expression as opposed to MC and SC ($p<0.05$) (Fig. 7C). However, MMP-1 and TIMP-1 expressions in the ATRA-challenged groups did not differ from those in controls (Fig. 7D).

The immunohistochemical analysis revealed that the synovial lining was stained brown, indicating a positive reaction in the synovial lining (Fig. 8).

Total Effects of ATRA on synovial explants

Although some insignificant difference between the untreated and treated groups exists, ATRA exerted a dose-dependent effect on cytokines, cartilage damage...
related proteases, and NO production (Fig. 9).

DISCUSSION

In the LPS-experiment, the results of HE staining showed that LPS neither had detrimental effects on the histomorphological characteristics of synovial explants, consistent with the finding of a previous study conducted by Moses et al. (30), nor did it initiate an inflammatory response. For further research, we also investigated LPS effects on synovial explants and observed change in inflammatory response under different culture conditions using fluorescent staining and ELISA.

In the study conducted by Moses et al. (30), synovial explants were digested with type IV collagenase and stained with trypan blue and suggested that mean viability of synovial explants was 90.9%. According to this method, we used type II collagenase and fluorescent staining. The results indicated that synovial explants in the 24 h groups (C1 and LPS-1) were kept well. However, apoptotic cells in synovial explants gradually increased and a small number of necrotic cells appeared with prolonged incubation, suggesting a possible apoptosis-induced effect of LPS on synovial explants. Another explanation may be that the lack of serum resulted in apoptosis owing to insufficient nutrients.

The results of ELISA demonstrated that LPS exerted a positive effect on IL-6, TNF-α, and MMP-9, especially for 24 h, and its effects were significantly suppressed
when incubation time was prolonged. Previous work of Hyc suggested that inflammatory response in synovial explants were significantly decreased when incubated for 48 h, indicating a possible lack of serum.

Based on the above mentioned results, the synovial explants were successfully established, but inflammatory response of synovial explants does not last for a long time. Therefore, the effect of ATRA was determined when synovial explants were stimulated by LPS for 24 h. The duration of inflammatory response imposed restriction on ATRA intervention. To improve this, we will focus on nutrients during incubation in subsequent studies.

ATRA concentration ranging from 0.01 μM to 10 μM has been commonly employed in previous cell experiments (14, 15, 31, 32). In our study, synovial explants comprised a large quantity of cells and more complex components. Most of all, adipocytes, considered as the storage depot of ATRA, may downregulate ATRA effect. Hence, 1 μM was selected as the lowest concentration for ATRA and 100 μM as the highest concentration. The results of HE stain demonstrated that there were no detrimental effects on the histomorphologic characteristics of synovial explants.

Recent evidences suggested that RA can be controlled by inhibiting synovitis (33). Cytokines were attributed to play a crucial role in synovitis. As the prototypical pro-inflammatory cytokines, IL-6 and TNF-α, contributed to bone metabolism and autoimmunity process in RA (34–36). The results of ELISA showed that 100 μM ATRA suppressed IL-6 and TNF-α and 10 μM ATRA suppressed TNF-α, which indicated that ATRA exhibited an inhibitory effect on the inflammatory response of synovial explants.

IL-6 and TNF-α activated the signal transduction pathway of MAP kinases and NF-κB, thereby inducing MMP and ADAMTS expressions (37, 38). Our study suggested that ATRA inhibited ADAMTS expression induced by LPS to protect articular cartilage from degradation. While one study revealed that pointed out that ATRA can inhibit the gene expression of MMPs (39), reported that ATRA downregulated MMP-9 and upregulated TIMP-1 in human bronchoalveolar lavage cells (40). Our results indicated that ATRA downregulated LPS-induced MMP-9 and MMP-3 expression. However, our study reported no significant change in MMP-1 and TIMP-1 production, which was not in agreement with most of these studies regarding the implication of MMP-1 and TIMP-1 in RA pathogenesis. Wright reported that ATRA upregulated TIMP-1 secretion in human synovial fibroblasts (37). Hyc demonstrated that LPS induced MMP-1, MMP-3, MMP-9, MMP-13, and MMP-14 mRNA production, while it inhibited TIMP-2 and TIMP-4 mRNA expression (29). This inconsistency remains unclear, and the gene expression level of cytokines, MMPs, and cartilage damage related proteases need further detection.

Many reports have highlighted that NO and iNOS responsible for causing joint damage in RA. NF-κB as a transcription factor regulated iNOS production, which was the most important enzyme related to NO production (41). Our findings showed that 100 μM and 10 μM ATRA decreased LPS-induced NO concentration, which was consistent with the study conducted by Balaganur et al. (42). Another study reported that LPS induced iNOS expression by activating NF-κB and AP-1 and subsequently upregulating NO (43). In contrast, our results indicated that both LPS and ATRA had no effect on iNOS. The possible reason is that iNOS in synovial explant was not secreted into the medium. NF-κB expression and gene expression of iNOS should be detected to explain the inconsistency.

In conclusion, the effect of ATRA on histomorphology, inflammatory mediators, inflammatory cytokines and cartilage damage related proteases of RA was investigated based on the successfully established LPS-challenged synovial explants. In light of our results, ATRA inhibited the release of cytokines including IL-6 and TNF-α, suppressed NO concentration, and downregulated the expression of cartilage damage related proteases especially MMPs and ADAMTS in synovial explants, indicating that ATRA has a potential beneficial effect on synovial inflammation and joint destruction in patients with RA. Further studies are warranted to elucidate the definite effects of ATRA on RA.

Acknowledgments
This study was funded by the National Natural Science Foundation of China (NSFC) (No. 81372983). We also appreciate the support of the Public Health and Preventive Medicine Experiment Teaching Center at Sichuan University and Food Safety Monitoring and Risk Assessment Key Laboratory of Sichuan Province.

REFERENCES
1) Mccnnes IB, Schett G. 2011. The pathogenesis of rheumatoid arthritis. N Engl J Med 365: 2205–2219.
2) Sweeney SE, Firestein GS. 2004. Rheumatoid arthritis: regulation of synovial inflammation. Int J Biochem Cell Biol 36: 372–378.
3) Ziołkowska M, Koc A, Luszczykiewicz G, Ksieczopolska-etrzak K, Klimczak E, Chwalinska-Sadowska H, Maslinski W. 2000. High levels of IL-17 in rheumatoid arthritis patients: IL-15 triggers in vitro IL-17 production via cyclosporin A-sensitive mechanism. J Immunol 164: 2832–2838.
4) Han Z, Boyle DL, Manning AM, Firestein GS. 1998. AP-1 and NF-κB regulation in rheumatoid arthritis and murine collagen-induced arthritis. Autoimmunity 28: 197–208.
5) Schett G, Tohidast-Akrad M, Smolen JS, Schmid BJ, Steiner CW, Bitzan P, Zenz P, Redlich K, Xu Q, Steiner G. 2000. Activation, differential localization, and regulation of the stress-activated protein kinases, extracellular signal–regulated kinase, c-Jun N-terminal kinase, and AP-1 and NF-κB regulation in rheumatoid arthritis and murine collagen-induced arthritis. Autoimmunity 28: 197–208.
6) Tchetterikov I, Ronday HK, El BV, Kiers GH, Verzijl N, TeKoppele JM, Huizinga TWJ, DeGroot J. Hanemaaier R. 2004. MMP profile in paired serum and synovial fluid samples of patients with rheumatoid arthritis. Ann
The Effect of ATRA on Synovitis in RA

7) Djeraba Z, Boumedine K, Arroul-Lammali A, Otmani F, Belguendouz H, Touill-Boukoffa C. 2014. Ex vivo immunomodulatory effect of all-trans-retinoic acid during Behçet’s disease: a study in Algerian patients. *Immunopharmacol Immunotoxicol* **36**: 78–86.

8) Azé L, Guerci A, Rajfoux E, Sazm M, Chevallier P, Lapusun S, Recher C, Thomas X, Rayon C, Castaigne S, Tourmilac O, Botton SD, Ibrah C, Cahn JY, Solary E, Gardin C, Fegaux N, Bordessoule D, Ferrant A, Meyer-Monard S, Vey N, Dombret H, Degos L, Chevret S, Fenaux P. 2009. Very long-term outcome of acute promyelocytic leukemia after treatment with all-trans retinoic acid and chemotherapy: the European APL Group experience. *Blood* **115**: 1690–1696.

9) Castro GA, Ferreira LM, Bueno VTL. 2008. Characterization of a new solid lipid nanoparticle formulation containing retinoic acid for topical treatment of acne. *J Microencapsulation* **23**: S30–S35.

10) Cao W, Chen W, Liang X, Zhou J, Wei C, Cui S, Liu J. 2014. All-trans-retinoic acid ameliorates the inflammation by inducing transforming growth factor beta 1 and interleukin 10 in mouse epidermiditis. *Am J Reprod Immunol* **71**: 312–312.

11) Pino-Lagos K, Benson MJ, Noelle RJ. 2010. Retinoic acid in the immune system. *Ann NY Acad Sci* **1143**: 170–187.

12) Mucida D, Park Y, Kim G, Nowak E, Benson MJ, Kronenberg M, Noelle RJ, Cheroutre H. 2007. Preparation of rat synovial membrane for studies of cytokine secretion. *Science* **317**: 256–260.

13) Mucida D, Pinolagos K, Kim G, Nowak E, Benson MJ, Kronenberg M, Noelle RJ, Cheroutre H. 2009. Retinoic acid can directly promote TGF-beta-mediated Foxp3(+) Treg cell conversion of naïve T cells. *Immunity* **30**: 472–473.

14) Brinkerhoff CE, McMillan RM, Dayer JM, Harris ED Jr. 1980. Inhibition by retinoic acid of collagenase production in rheumatoid synovial cells. *J Exp Med* **163**: 432–436.

15) Kirchmeyer M, Koubany M, Sebillaud S, Netter P, Jouzeau J, Brinckerhoff CE, McMillan RM, Dayer JM, Harris ED Jr. 2001. Preparation of rat synovial membrane for studies of cytokine secretion. *Folia Histochem Cytobiol* **39**: 256–260.

16) Xie XJ, Shen YY, Song J, Zhong LY, Ning YH, Zeng X, Shan JY, Liu Y, Zhu YF, Li Y. 2016. The effects of all-trans retinoic acid on the expression of inflammatory cytokines and cartilage damage related protease in rats with collagen induced arthritis. *J Sichuan Univ Med* **47**: 479.

17) Kwok SK, Park MK, Cho ML, Oh HJ, Park EM, Lee DG, Lee J, Kim HY, Park SH. 2012. Retinoic acid attenuates rheumatoid inflammation in mice. *J Immunol* **189**: 1062.

18) Arroul-Lammali A, Rahal F, Chetouane R, Djeraba Z, Medżeber O, Ladjouze-Rezig A, Touill-Boukoffa C. 2017. Ex vivo all-trans retinoic acid modulates NO production and regulates IL-6 effect during rheumatoid arthritis: a study in Algerian patients. *Immunopharmacol Immunotoxicol* **39**: 87–96.

19) Mosquera N, Rodríguez-Trillo Á, Bravo SB, Mera A, Conde C. 2017. FRK0068 Reduces proliferation, migration and invasion of rheumatoid synoviocytes by all-trans retinoic acid. *Am Rheum Dis* **76(Suppl 2):** 503.

20) Zeng X, Shan J, Liu Y, Ning X, Xie Z, Sheng Y, Song J, Li Y. 2017. Prophylactic administration of all-trans retinoic acid alleviates inflammation in rats with collagen-induced arthritis. *J Southern Med Univ* **37**: 172.

21) Fell HB, Jubb RW. 2014. The effect of synovial tissue on the breakdown of articular cartilage in organ culture. *Arthritis Rheumatol* **20**: 1359–1371.

22) Illien-Jünger S, Pattappa G, Peroglio M, Benneker LM, Stoddart MJ, Sakui D, Mochida J, Grad S, Alini M. 2012. Homing of mesenchymal stem cells in induced degenerative intervertebral discs in a whole organ culture system. *Spine* **37**: 1865–1873.

23) Thuret G, Manissolle C, Campos-Guyotat L, Guyotat D, Gain P. 2005. Animal compound-free medium and poloxamer for human corneal organ culture and deswelling. *Invest Ophthalmol Vis Sci* **46**: 816–822.

24) Bottini N, Firestein GS. 2013. Duality of fibroblast-like synoviocytes in RA: passive responders and imprinted aggressors. *Nat Rev Rheumatol* **9**: 24–33.

25) Davis NP, Han HC, Wayman B, Vito R. 2005. Sustained axial loading lengthens arteries in organ culture. *Ann Biomed Eng* **33**: 867–877.

26) Hyc A, Osiecka-Iwan A, Dzianycz P, Moskalewski S. 2007. Preparation of rat synovial membrane for studies of cytokine secretion. *Folia Histochem Cytobiol* **45**: 57–60.

27) Hyc A, Osiecka-Iwan A, Niderla-Bielinska J, Moskalewski S. 2011. Influence of LPS, TNF, TGF-β1 and IL-4 on the expression of MMPs, TIMPs and selected cytokines in rat synovial membranes incubated in vitro. *Int J Molec Med* **27**: 127–137.

28) Moses VS, Hardy J, Bertone AL, Weisbrode SE. 2001. Effects of anti-inflammatory drugs on lipopolysaccharide-challenged and -unchallenged equine synovial explants. *Am J Veterinary Res* **62**: 54–60.

29) Wright JK, Clark IM, Cawston TE, Hazleman BL. 1991. The secretion of the tissue inhibitor of metalloproteinases (TIMP) by human synovial fibroblasts is modulated by all-trans-retinoic acid. *Biochim Biophys Acta* **1133**: 25–30.

30) Gütter BD, Kochneke EM. 1991. Retinoic acid potentiates interleukin-1 and fibroblast growth factor-induced human synovial fibroblast proliferation. *Clin Immunol Immunopathol* **61**: 191–201.

31) Ji JD. 2005. Cytokines in rheumatoid arthritis. *Hanyang Med Rev* **25**: 43–52.

32) Srinangan S, Choy EH. 2010. The role of interleukin 6 in the pathophysiology of rheumatoid arthritis. *Ther Adv Musculoskelet Dis* **2**: 247–256.

33) De Benedetti F, Rucci N, Del Fattore A, Pennuzzi B, Paro R, Longo M, Vivarelli M, Muratori F, Berni S, Ballanti P, Ferrari S, Tetti A. 2010. Impaired skeletal development in interleukin-6-transgenic mice: a model for the impact of chronic inflammation on the growing skeletal system. *Arthritis Rheumatol* **54**: 1351–1361.

34) Lei W, Ying S, Kong XF, Chi Z, Tao Y, Qi Z, He DY, Jiang
LD. 2016. The effects of dopamine receptor 2 expression on B cells on bone metabolism and TNF-α levels in rheumatoid arthritis. *BMC Musculoskelet Disord* **17**: 352.

37) McInnes IB, Schett G. 2007. Cytokines in the pathogenesis of rheumatoid arthritis. *Nat Rev Immunol* **7**: 429–442.

38) Murphy G, Nagase H. 2008. Reappraising metalloproteinases in rheumatoid arthritis and osteoarthritis: destruction or repair? *Nat Clin Pract Rheumatol* **4**: 128–135.

39) Burrage PS, Mix KS, Brinckerhoff CE. 2005. Matrix metalloproteinases: role in arthritis. *Front Biosci* **11**: 529–543.

40) Frankenberger M, Hauck RW, Frankenberger B, Häussinger K, Maier KL, Heyder J, Ziegler-Heitbrock HW. 2001. All trans-retinoic acid selectively down-regulates matrix metalloproteinase-9 (MMP-9) and up-regulates tissue inhibitor of metalloproteinase-1 (TIMP-1) in human bronchoalveolar lavage cells. *Molec Med* **7**: 263–270.

41) Pautz A, Art J, Hahn S, Nowag S, Voss C, Kleinert H. 2010. Regulation of the expression of inducible nitric oxide synthase. *Nitric Oxide* **23**: 75–93.

42) Balaganur V, Pathak NN, Lingaraju MC, More AS, Latief N, Kumari RR, Kumar D, Tandan SK. 2014. Chondroprotective and anti-inflammatory effects of S-methylisothiourea, an inducible nitric oxide synthase inhibitor in cartilage and synovial explants model of osteoarthritis. *J Pharmacy Pharmacol* **66**: 1021–1031.

43) Lee JY, Woo ER, Kieng KW. 2005. Inhibition of lipopolysaccharide-inducible nitric oxide synthase expression by acteoside through blocking of AP-1 activation. *J Ethnopharmacol* **97**: 561–566.