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NF-κB Phosphorylation Inhibition Prevents Articular Cartilage Degradation in Osteoarthritis Rats via 2-Aminoquinoline

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Funds Collection G

BCDE Jinlong He
AEF Shicheng Zheng

Department of Integrated Traditional Chinese Medicine (TCM) and Western Medicine Orthopedics, Honghui Hospital, Xi'an Jiaotong University, Xi'an, Shaanxi, P.R. China

Corresponding Author: Shicheng Zheng, e-mail: zhengshicheng535@sina.com
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Background: Osteoarthritis is a chronic degenerative disease of the joints that is common in older people worldwide. The characteristic features of osteoarthritis include cartilage degradation, synovitis, and remodelling of subchondral bone. The present study investigated the effect of 2-aminoquinoline on knee articular cartilage degradation in an osteoarthritis rat model.

Material/Methods: The rat model of osteoarthritis was established in Wistar rats by intra-articular injection of monosodium iodoacetate. The rats were randomly divided into 6 groups of 10 rats each: a normal control group, an untreated group, and 4 (5, 10, 15 and 20 mg/kg) treatment groups. The rats in treatment groups received 5, 10, 15, or 20 mg/kg doses of 2-aminoquinoline on day 2 of monosodium iodoacetate injection.

Results: The 2-aminoquinoline treatment of monosodium iodoacetate-injected rats markedly decreased weight-bearing asymmetry, inhibited edema formation, and improved paw withdrawal thresholds. The expression of inflammatory cytokines was markedly higher in the osteoarthritis rats. Treatment with 2-aminoquinoline led to a significant reduction in inflammatory cytokine expression in osteoarthritis rats in a dose-dependent manner. In osteoarthritis rats, the expressions of prostaglandin E2 (PGE2), matrix metalloproteinase-13 (MMP-13), and substance P were also higher in comparison to the control group. The 2-aminoquinoline treatment supressed PGE2, MMP-13, and substance P levels in osteoarthritis rats. Moreover, the expression of phosphorylated nuclear factor kappaB (p-NF-κB) was markedly higher in the untreated rats. However, activation of NF-κB was downregulated in the osteoarthritis rats by treatment with 2-aminoquinoline.

Conclusions: The present study demonstrated that 2-aminoquinoline prevents articular cartilage damage in osteoarthritis rats through inhibition of inflammatory factors and downregulation of NF-κB activation, suggesting that 2-aminoquinoline would be effective in treatment of osteoarthritis.

MeSH Keywords: Angioedema • Cytokines • Synovitis

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Background

Osteoarthritis is a chronic degenerative disease of the joints, commonly detected in older people worldwide [1]. The characteristic features of osteoarthritis include cartilage degradation, synovitis, and remodelling of subchondral bone [1]. Osteoarthritis is second to vascular diseases among long-term disabilities and accounts for 2–6% of arm disabilities in the people above 50 years of age [2]. There are many factors associated with osteoarthritis in humans, including trauma, age, and increased body weight [3]. The symptoms of mild osteoarthritis are joint inflammation, stiffness, pain, and edema, while as at the secondary stage patients suffer deformity of the joints and inability to move [3]. Due to its high disability rate, osteoarthritis is considered to be the most common health problem in humans [4]. Although the mechanism underlying osteoarthritis has not been explored fully, it has been found that inflammation plays a major role in its development and progression [5].

The main inducer of osteoarthritis is believed to be the cytokine interleukin (IL)-1β, which catalyses release of catabolic enzymes; in excess, this leads to chondrocyte metabolic imbalance [6–8]. The overproduction of catabolic enzymes breaks down the matrix and ultimately results in loss of articular cartilage function [6–8]. In osteoarthritis patients there is higher production of prostaglandins and cytokines by chondrocytes [9]. The inflammatory cytokines generated by synovial membranes alter the cartilage integrity and lead to osteoarthritis development and progression [10]. During inflammation of joints, cartilage tissues are degraded by the matrix metalloproteinases activated by prostaglandin E2 (PGE2) [11]. The expression of matrix metalloproteinase-13 (MMP-13) is specific to the cartilage of osteoarthritis patients and is not found in normal cartilage [12]. In osteoarthritis, joint inflammation and pain is regulated by the expression of substance P (SP) [13]. The superfamily of purinergic membrane receptors is comprised of 2 receptor families: P1 and P2 [14,15]. The P2 family has 2 subtypes – P2X and P2Y – which are involved in osteoarthritis-induced pain [14,15]. The expression of various members of P2X, such as P2X7 and P2X2, has been detected in the cartilage tissues [16,17]. The expression of P2X7 receptor (P2X7R) has been revealed to be linked with arthritis pain and inflammation regulation [18]. It has been shown that P2X7R is a vital target for rheumatoid arthritis treatment [19,20]. Compounds based on 2-aminopyrimidine and 4-aminoquinoline have been synthesized and evaluated for anti-plasmodial and anti-leishmanial activities [21,22]. These compounds encompass a heterocyclic scaffold – quinoline – which exhibits anti-inflammatory activity [23,24]. The present study investigated the effect of 2-aminoquinoline synthesized using a previously described procedure [21] on inflammation and articular cartilage degradation in an osteoarthritis rat model.

Material and Methods

Animals

Sixty male Wistar rats (10 to 14 weeks old) weighing 310–390 g were obtained from the Shanghai SLAC Laboratory, Shanghai, China. The rats were housed individually in plastic cages under a 12/12 h light/dark cycle. The temperature and humidity in the animal center were maintained at 24°C and 65%, respectively. The rats were provided free access to laboratory food. The rats were acclimatized to the laboratory environment for 5 days before starting the experiment. The study was approved (approval number SU/MC/16/007) by the Animal Ethics Committee of the Medical School of Shandong University (Jinan, China). All experimental protocols involving rats were approved by the by the Institutional Animal Care Committee of Kunming Medical University (Kunming, China).

Establishment of osteoarthritis rat model and treatment strategy

Osteoarthritis in the rats was induced by intra-articular injection of 5 mg/kg of monosodium iodoacetate [25] in saline after isoflurane (2% in oxygen) anesthetization. The normal control group of rats were injected with an equal volume of saline without monosodium iodoacetate. The rats were divided randomly into 6 groups of 10 each: a monosodium iodoacetate group, a normal control group, and four 2-aminoquinoline treatment groups. The rats in the treatment groups were injected with single doses of 5, 10, 15, or 20 mg/kg of 2-aminoquinoline in saline through the intra-articular route on the second day of monosodium iodoacetate injection. The monosodium iodoacetate and normal control groups were given normal saline alone.

Paw withdrawal threshold

The paw withdrawal threshold test was conducted on days 4, 8, 12, 16, 20, 24, 28, 32, 36, and 40 of monosodium iodoacetate injection. The equipment consisted of a transparent glass box measuring 20×20×30 cm^3 in which a steel wire mesh measuring 1×1 cm^2 was placed. The rats were put in the box 30 min prior to the test, and Von Frey monofilaments were used to determine the paw withdrawal threshold. After the rats settled on the mesh, an increasing bending force in the range of 0.6–26 g was applied using Von Frey monofilaments. The monofilament used to contact and push the rat left hind limb plantar surface was bent at an appropriate angle. The minimum weight required from the monofilament to elicit the withdrawal reflex was considered as the threshold for paw withdrawal. The reading response of the paw withdrawal frequency to the filament stimulus was measured 3 times for each animal using a previously reported protocol [26].

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Asymmetry in bearing weight

The LE7900-Incapacitance Tester (Nature Gene Corp., Medford, NJ, USA) was employed for determination of weight-bearing asymmetry of the rat hind limbs. The weight-bearing asymmetry was measured on days 3, 6, 9, 12, 15, 18, 21, 24, 27, and 30 of monosodium iodoacetate injection. Briefly, the 2 hind limb paws of the rats were put separately on the electronic balance while the animals were held upright. Rats were allowed to settle so that weight distribution on both the hind limbs could be determined. The measurement of the weight on right and left hind limbs was performed 3 times for 3 s each using a previously reported protocol [27].

Measurement of knee edema

The size of edema was measured on days 4, 8, 12, 16, 20, 24, 28, 32, 36, and 40 of monosodium iodoacetate injection using a previously reported methodology [28]. Briefly, the diameter of the right and left knee joints was measured using commercially available calibrated digital callipers. The diameter of the left knee joint in which monosodium iodoacetate was injected was compared with that of the right knee joint. The difference in diameter between the left and right knee joint was taken as the edema size.

Measurement of cytokine levels

The cytokine level in rat blood was determined on day 45 of monosodium iodoacetate injection. The rats treated with 5, 10, 15, or 20 mg/kg of 2-aminoquinoline were sacrificed after euthanasia with pressurised CO_2 followed by cervical dislocation. A blood sample from each rat was collected from the carotid artery. The samples were centrifuged for 20 min at 4000 g to obtain supernatant, which was stored at –78°C for determination of cytokine levels. Commercially available ELISA kits (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) were used for determination of inflammatory cytokine levels in rat serum.

Western blot analysis

The rats were sacrificed on day 45 of monosodium iodoacetate injection using euthanasia with pressurised CO_2 followed by cervical dislocation. We incubated 2-mm-thick cartilage tissue slices in microcentrifuge tubes for 15 min with 1 mL hyaluronidase at 37°C followed by centrifugation for 10 min at 1000 g. The samples were then centrifuged with trypsin-EDTA and collagenase type II to collect the supernatant, which was subsequently treated with zirconium oxide beads. Homogenization buffer (0.4 mL) was added to the tubes, which were placed in a Bullet Blender™. The bicinchoninic assay (BCA) protein assay kit (Beyotime Institute of Biotechnology, Nanjing, China) was used for determination of concentration of proteins in the lysates. The protein samples (20 µg) were loaded on 12% SDS-PAGE for resolution by electrophoresis. The proteins were then transferred to PVDF membranes in which non-specific sites were blocked by incubation for 2 h with 3% non-fat milk at 37°C. The incubation of membranes with primary antibodies was performed overnight at 4°C. The antibodies used were against: IL-1β, IL-6, P2X7R, MMP-13, SP, IKKα, IKKβ, IκBα, NF-κB p65, PGE2, and TNF-α. The membrane incubation was followed by washing 3 times with ice-cold PBS. Then, membranes were incubated for 2 h with HRP-conjugated secondary antibody. The protein bands were visualised by an enhanced chemiluminescence system, and densitometry was performed using Image-Pro Plus 6.0, with GAPDH as an internal control [28].

Statistical analysis

The expressed data are the average±standard deviation (SD). The data analysis was performed s using one-way analysis of variance (ANOVA) in combination with Tukey's post hoc test. Statistical data were analysed using SPSS 13.0 software (SPSS, Inc., Chicago, IL, USA). $P<0.05$ was regarded as a statistically significant difference.

Results

Effect of 2-aminoquinoline on paw withdrawal threshold in OA rats

The 2-aminoquinoline treatment improved paw withdrawal thresholds in the OA rats (Figure 1). The paw withdrawal threshold was measured on days 4, 8, 12, 16, 20, 24, 28, 32, 36, and 40 of monosodium iodoacetate administration. A significant ($P<0.05$) decrease in paw withdrawal threshold was observed in OA rats from day 8 of monosodium iodoacetate administration. Treatment of OA rats with 2-aminoquinoline prevented monosodium iodoacetate-induced decrease in paw withdrawal threshold in a dose-dependent manner. At the 20 mg/kg dose, 2-aminoquinoline almost completely prevented monosodium iodoacetate-induced decrease in paw withdrawal threshold.

Effect of 2-aminoquinoline on weight-bearing asymmetry in OA rats

The weight-bearing asymmetry was measured on the days 3, 6, 9, 12, 15, 18, 21, 24, 27, and 30 of monosodium iodoacetate injection. Treatment of OA rats with 2-aminoquinoline markedly decreased weight-bearing asymmetry in comparison to the untreated group (Figure 2). The OA-induced increase in weight-bearing asymmetry was reduced to a minimum in the rats treated with 20 mg/kg doses of 2-aminoquinoline.

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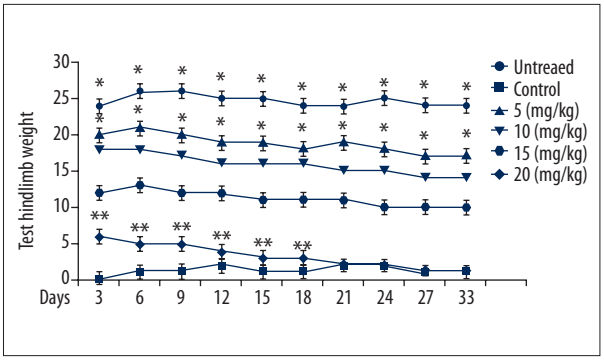

Figure 1. Effect of 2-aminoquinoline on paw withdrawal thresholds in rats with osteoarthritis. The osteoarthritis rat model was prepared by injecting monosodium iodoacetate through the intra-articular route. The rats were injected with 5, 10, 15, or 20 mg/kg doses of 2-aminoquinoline after monosodium iodoacetate injection. * P<0.05, ** P<0.02, and ** P<0.001 *vs.* untreated group.

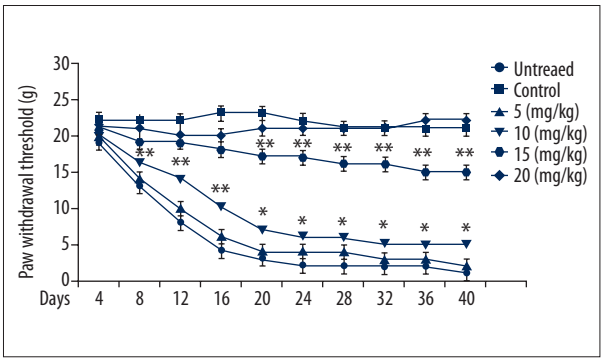

Figure 2. Effect of 2-aminoquinoline on weight-bearing asymmetry in rats with osteoarthritis. The osteoarthritis rat model was prepared by injecting monosodium iodoacetate through the intra-articular route. The rats were injected with 5, 10, 15, or 20 mg/kg doses of 2-aminoquinoline after monosodium iodoacetate injection. * P<0.05, ** P<0.02 and ** P<0.001 *vs.* untreated group.

Effect of 2-aminoquinoline on knee edema in OA rats

The size of edema in the knee joint of rats was measured on days 4, 8, 12, 16, 20, 24, 28, 32, 36, and 40 of monosodium iodoacetate injection. In the untreated OA rats, the knee edema was markedly larger in size compared to the untreated group. However, treatment with 2-aminoquinoline markedly suppressed edema formation in comparison to the untreated group (Figure 3). Edema formation in OA rats was almost completely inhibited by treatment with 20 mg/kg doses of 2-aminoquinoline.

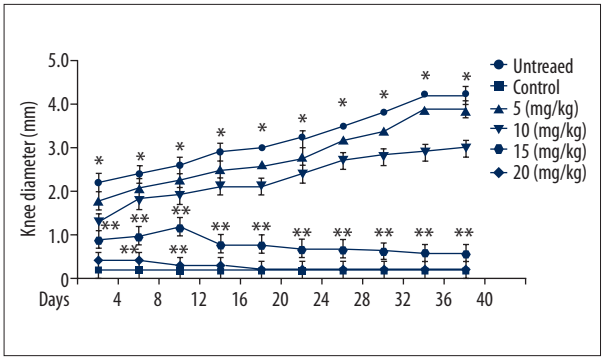

Figure 3. Effect of 2-aminoquinolineon edema formation in rats with osteoarthritis. The osteoarthritis rat model was prepared by injecting monosodium iodoacetate through the intra-articular route. The rats were injected with 5, 10, 15, or 20 mg/kg doses of 2-aminoquinoline after monosodium iodoacetate injection. * P<0.05, ** P<0.02, and ** P<0.001 *vs.* untreated group.

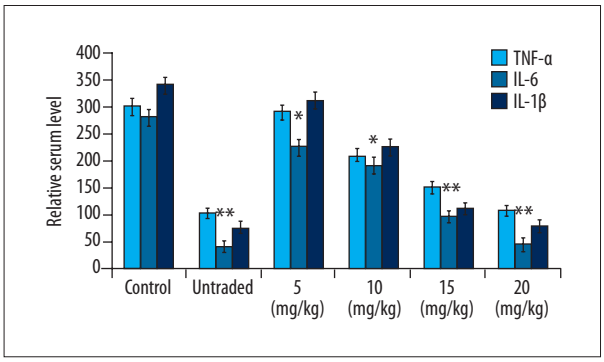

Figure 4. Effect of 2-aminoquinoline on cytokine production in OA rat serum. The rats were treated with 5, 10, 15, or 20 mg/kg doses of 2-aminoquinoline after monosodium iodoacetate injection. The levels of cytokines were measured in rat serum using ELISA. * P<0.05 and ** P<0.02 *vs.* untreated group.

Suppression of cytokine production by 2-aminoquinoline in rat serum

The production of cytokines in the OA rat serum was markedly higher in comparison to the normal control group (Figure 4). The 2-aminoquinoline treatment markedly inhibited OA-induced production of TNF-α, IL-6, and IL-1β in rat serum. The suppression of OA-induced production of cytokines in rat serum by 2-aminoquinoline was concentration-dependent. The decrease in OA-induced production of cytokines by 2-aminoquinoline was greatest at 20 mg/kg dose.

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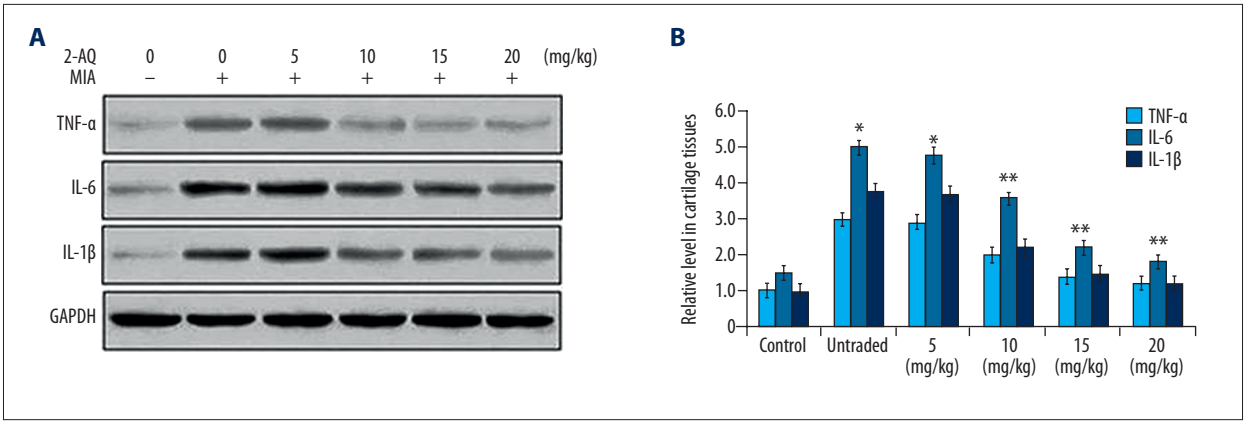

Figure 5. Effect of 2-aminoquinoline on cytokine production in articular cartilage of OA rats. The OA-induced rats were treated with 5, 10, 15, or 20 mg/kg doses of 2-aminoquinoline. (**A**) Western blotting was used for assessment of interleukin-1β, IL-6, and TNF-α levels. (**B**) Densitometric analysis of the data. * P<0.05 and ** P<0.02 *vs.* control group.

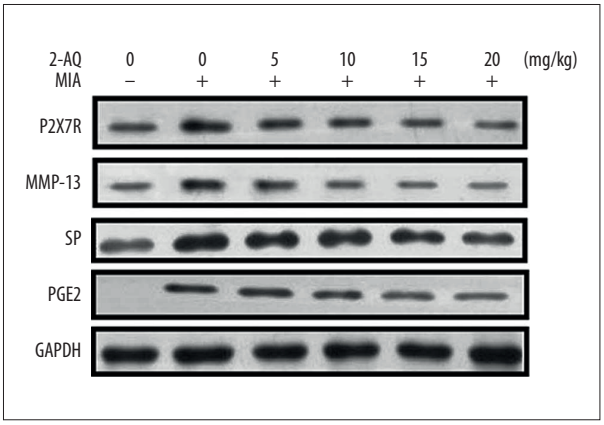

Figure 6. Effect of 2-aminoquinoline on expression of P2X7R, MMP-13, SP, and PGE2 in the articular cartilage tissues. The OA-induced rats were treated with 5, 10, 15, or 20 mg/kg doses of 2-aminoquinoline for 40 days every other day. Western blotting was used for assessment of P2X7R, MMP-13, SP, and PGE2 expression.

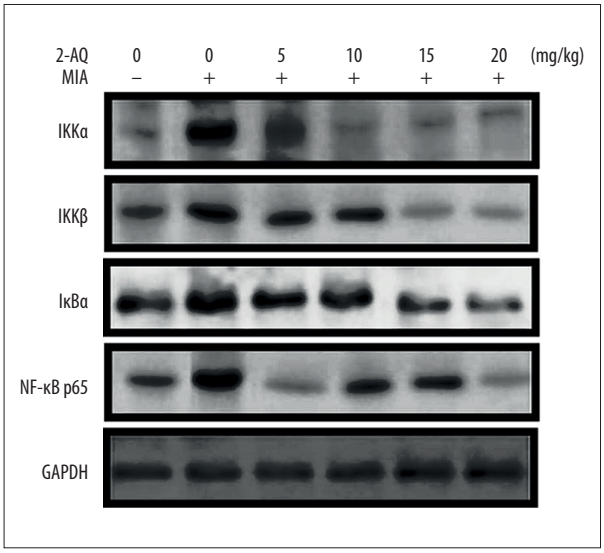

Figure 7. Effect of 2-aminoquinoline on NF-κB activation in OA rats. The OA rats were treated with 5, 10, 15, and 20 mg/kg doses of 2-aminoquinoline every other day for 40 days after OA induction. The expression of IKKα, IKKβ, IκBα, and NF-κB p65 were determined by Western blotting assay.

Suppression of OA-induced cytokine level by 2-aminoquinolinein rat knee joint cartilage

Western blotting showed markedly higher levels of cytokines in the OA rat knee joints in comparison to the normal control group (Figure 5). Treatment of the OA rats with 2-aminoquinoline markedly reduced the levels of interleukin-1β, IL-6, and TNF-α in the knee tissues. The reduction of interleukin-1β, IL-6, and TNF-α in the OA rats by 2-aminoquinoline was greatest at 20 mg/kg doses.

Reduction of P2X7R, MMP-13, SP, and PGE2 expression by 2-aminoquinoline in OA rats

The expressions of P2X7R, MMP-13, SP, and PGE2 were increased in the OA rats in comparison to the normal control group (Figure 6). Treatment of OA rats with 2-aminoquinoline slightly decreased the expressions of P2X7R, MMP-13, SP, and PGE2 in a dose-dependent manner. In the OA rat cartilage tissues, the expression of P2X7R, MMP-13, SP, and PGE2 was reduced to minimum levels by 20 mg/kg 2-aminoquinoline.

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Inhibition of NF-κB signalling pathway by 2-aminoquinoline

The 2-aminoquinoline treatment of OA rats markedly reduced NF-κB signalling factor expression in the articular cartilage tissues (Figure 7). The reduction of NF-κB signalling factor expression by 2-aminoquinoline in the articular cartilage of OA rats was greatest at 20 mg/kg 2-aminoquinoline. Treatment of the OA rats with 2-aminoquinoline also markedly reduced the expression of phosphorylated NF-κB signalling factor in comparison to the untreated group.

Discussion

Osteoarthritis patients generally suffer from joint inflammation, decomposition of articular cartilage, and damage to subchondral bone [29]. Acute pain in osteoarthritis patients is associated with the neuronal activation by chondrocytes after inflammation [7,29]. The compounds with anti-inflammatory activity are reported to inhibit inflammation, prevent cartilage matrix degradation, and protect damage to synovial tissues [30]. A study has revealed that weight-bearing asymmetry is markedly increased in osteoarthritis rats [25]. In the rats with osteoarthritis, large quantities of edema are formed, which increase in size over time [25]. The present study showed that 2-aminoquinoline treatment markedly decreased weight-bearing asymmetry in the osteoarthritis rats in a dose-dependent manner. There was significantly improved paw withdrawal thresholds in osteoarthritis rats treated with 2-aminoquinoline. Moreover, the edema volumes in osteoarthritis rats were very small compared to the untreated group. These findings clearly indicated that 2-aminoquinoline inhibits inflammation induced by osteoarthritis in the rats.

To confirm the anti-inflammatory effect of 2-aminoquinoline, the changes in inflammatory cytokine levels in bronchoalveolar lavage fluid (BALF) and articular cartilage tissues of the osteoarthritis were observed. The catabolic effect of IL-1β cytokine on chondrocyte metabolism causes reduction in proteoglycan collagen production and promotes aggrecan secretion via protease blockage [31]. The ECM proteolysis and cleavage are also catabolised by IL-1β though synovial cell activation, leading to over-expression of MMPs [32]. In addition, production of mediators of inflammation, such as NO, is induced in synovial cells by IL-1β [33]. Higher secretion of NO causes damage to tissues by activating an inflammatory response [34]. It is believed that protein kinase C suppression by NO leads to apoptosis of chondrocytes in the articular cartilage [35]. Inflammatory cytokines such as TNF-α, IL-6, PG, IL-1β, NO, and IL-8 are secreted in markedly higher levels by the chondrocytes of osteoarthritis patients [9]. The levels of inflammatory cytokines are also increased after P2X7R activation in osteoarthritis rat mast cells [36]. It is reported that mast cells of rats also produce higher levels of inflammatory cytokines following P2X7R activation [37]. Studies have shown that blocking or silencing TNF-α in animal models inhibits inflammation [38–40]. In the present study, osteoarthritis induction markedly raised the levels of inflammatory cytokines in the articular cartilage tissues and serum of rats. The 2-aminoquinoline treatment of osteoarthritis rats reduced the secretion of these inflammatory cytokines in serum, as well as in articular cartilage tissues, in a dose-dependent manner. The ion channel P2X7R has been found to be associated with the development of osteoarthritis and inflammation [14]. Increased levels of PGE2 promote secretion of matrix metalloproteinases and aggrecanases, which catalyse degeneration of cartilage [11]. The neuropeptide SP has been found to play an important role in suppression of osteoarthritis by an anti-inflammatory effect [41,42]. MMP-13 is the main MMP family member expressed in the cartilage of osteoarthritis patients but not in normal adults [43–45]. The level of MMP-13 in osteoarthritis patients indicates the degree of cartilage degeneration [43–45]. Activated MMPs catalyse break-down of cartilage matrix and induce chondrocyte apoptosis, resulting in damage to the cartilage [40,46]. The present study showed markedly higher secretion of MMP-13 and P2X7R in the articular cartilage of osteoarthritis rats. The production of MMP-13 and P2X7R was markedly suppressed in the osteoarthritis rats receiving treatment with 2-aminoquinoline. There are reports that stimulation of chondrocytes with IL-1β leads to activation of the NF-κB pathway [47–49]. Activation of NF-κB and phosphorylation of IκBα is catalysed by P2X7R through activation of IKKα and IKKβ during arthritis [50]. MMPs consist of extracellular enzymes that contain Zn^{2+} ions as cofactors and are responsible for ECM degradation [51,52]. The resorption of bones and degradation of matrix are also caused by MMPs [53,54]. The main strengthening components for cartilage – type II collagen and proteoglycans – are proteolyzed by MMPs, resulting in osteoarthritis [51,52,55]. The secretion of MMPs is much higher in the synovial fluid of patients with joint pain [56–58]. The imbalance between ECM proteinase secretion and inhibitors also causes cartilage degradation [59]. Therefore, the evidence suggests that 2-aminoquinoline inhibits inflammation in osteoarthritis rats by interfering with the NF-κB signalling pathway. The results from the present study showed that 2-aminoquinoline treatment reduced the levels of IKKα, IKKβ, IκBα, and NF-κB p65 in osteoarthritis rats.

Conclusions

The present study demonstrated that 2-aminoquinoline prevents articular cartilage degradation and inhibits inflammation in osteoarthritis rats. The osteoarthritis inhibition by 2-aminoquinoline involves suppression of inflammatory cytokines and downregulation of the NF-κB signalling pathway. Therefore, further studies need to be performed to investigate 2-aminoquinoline as a potential target for treatment of osteoarthritis.

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