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Original article

The pro-inflammatory cytokine IL-Iβ alteration by deer (*Rusa unicolor*) antler extract on osteoarthritis rat model

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ABSTRACT

Osteoarthritis is a disease associated with articular cartilage degradation, intra-articular area inflammation, and subchondral bone replacement. Cytokine IL-1β has a prominent function in the inflammations process that passes in the joints. The 70% ethanol extracts of deer antler (250 and 500 mg/kg BW) and glucosamine sulfate (250 kg/BW) were evaluated for four weeks in reducing cytokine IL-1β to rat model OA-induced Monosodium iodoacetate. Measurements of joint diameter in rat's knee and hyperalgesia were performed on weeks 0, 1, 2, 3, 4, 5, 6, and 7. The presence of a significant difference in the stimulation thermal latency ($p = 0.00$) and the resulting increase in swelling of joint diameter ($p = 0.00$) are evidence that MIA has successfully induced the rat modeling of OA. A significant decrease in cytokine IL-Iβ levels was shown on week 3 after MIA injection ($p = 0.00$). Both concentrations of deer extracts significantly reduced knee joint diameter ($p = 0.00$), latency thermal stimulation ($p = 0.00$), and cytokine IL-1β levels ($p = 0.00$). Based on the results, it can be concluded that the 70% ethanol extract of deer antler is a potential medicine for OA therapy.

1. Introduction

Osteoarthritis (OA) is a universal type of arthritis and is known as a degenerative joint disorder (Parker & Parker, 2003). OA is a disease with unknown pathophysiology that includes degeneration of the articular cartilage, inflammation in the intra-articular range, and replacement of the subchondral bone (Kean et al., 2004). OA is a complex disease whose etiology and pathology are not completely understood. Modeling of OA prompted by way of monosodium iodoacetate (MIA) is extensively utilized in animal models to determine the success rate of OA in experimental animals (Bendele, 2001).

Intra-articular injection of MIA triggers articular cartilage injury through inhibition of glyceraldehyde-3-phosphate dehydrogenase (G3PDH) activity in chondrocytes, leading to impaired cell demise and glycolysis. These chondrocytes have relations in histopathol-

Abbreviations: OA, Osteoarthritis; MIA, Monosodium Iodoacetate; G3PDH, Glyceraldehyde-3-Phosphate Dehydrogenase; IL-Iβ, Interleukin- 1 beta; PEG, Polyethylene Glycol; ELISA, Enzyme-Linked Immunosorbent Assay; COX-2, Cyclooxygenase-2; TNF-α, Tumor Necrosis Factor Alpha; MMPs, Matrix Metalloproteinases; iNOS, Inducible Nitric Oxide Synthase; IL-6, Interlekuin-6.

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ogy with humans and OA. MIA-induced animal models also show a connection between pain and structural changes in the joints (Bar-Yehuda et al., 2009; Grossin et al., 2006).

Previously, it was believed that the damaged articular cartilage of the joints could not be healed. Therefore, the therapy was only to relieve symptoms with analgesic, anti-inflammatory, and lubricant drugs. However, in recent years several researchers have suggested that some supplements may be able to slow down the progressive degradation of articular cartilage damage and even repair this damage. It can be seen from the decrease in levels of IL-Iβ as an inflammatory agent in bone (Maldonado & Nam, 2013). Some of the recommended supplements are chondroitin sulfate, glucosamine, and collagen hydrolysate (Bello & Oesser, 2006).

Deer antler is one of the animals with high medicinal value. It is a successful folk remedy for strengthening tendons and bones. According to the researchers, there is an imbalance between cartilage erosion and regeneration in patients with osteoarthritis caused by a deficiency of glycosaminoglycan. This substance has an important role in the structural integrity of cartilage. Through the use of cellulose acetate electrophoresis, enzymatic digestion, and chromatography techniques, glycosaminoglycan is isolated from the four different regions of deer antler, including the tip, upper, middle, and base (Sunwoo et al., 1998).

Previous studies have shown that LPS-stimulated RAW 264.7 macrophages can be inhibited (40% and 80%) by 70% ethanol and an aqueous extract of deer antlers from East Kalimantan (Widyowati et al., 2020). This extract also can successfully reduce the pro-inflammatory impact of TNF-stimulated MH7A RA-FLS cells. In *in vivo* studies triggered by zymosan, the injection of deer antler extract treatment significantly reduced clinical arthritis scores and protected synovial and cartilage damage caused by cytokine-mediated immune cells (Cheng et al., 2022). The primary components of deer antler extract are protein, lipid, ash, calcium, collagen, chondroitin sulfate, and glucosamine (Kawtikwar et al., 2010). Hyaluronic acid, keratan sulfate, and dermatan sulfate are also present, but in fewer amounts (Sui et al., 2014). Then, some research indicates that deer antlers can reduce or completely remove osteoarthritis symptoms.

Cytokine IL-1 is one of the important factors for pain response and is the focus of current research. Pronociceptive mediator nerve growth factor (NGF) is critical to pain processing mechanisms and can be regulated more effectively by IL-1. Prostaglandins, IL-6, substance P, and matrix metalloproteinase-9 (MMP-9) are released and activated due to the IL-1 signaling cascade (Moilanen et al., 2015). Therefore, this study was conducted on the efficacy test related to osteoarthritis in rats induced by MIA and treated with deer antler extract. It aimed to analyze changes in the pro-inflammatory cytokine IL-1β in an osteoarthritis rat model in the use of deer antler extract as a therapy for OA.

2. Materials and methods

2.1. Materials used

The solvent extracts were a combination of ethanol p.a (Merck) and aquadest. Other materials were 0.9% physiological salts solution, carboxy methyl cellulose (CMC), monosodium iodoacetate (MIA) (Sigma-Aldrich, Darmstadt, Germany), 10% ketamine (Agrovet, Nicaragua), IL-Iβ ELISA kit (Bioenzy, Germany), glucosamine sulfate (Lipa Pharmaceuticals Ltd, NSW, 2566, Australia), and deer antler powder of *Rusa unicolor* was collected in the middle of August 2020 in UPTD (Technical Implementation Service Unit) of East Kalimantan, Indonesia, and voucher specimens were deposited at UPTD East Kalimantan, Indonesia.

2.2. Extraction of Rusa unicolor antlers

Deer antler powder of *Rusa unicolor* was received from UPTD of East Kalimantan, Indonesia. The 500 g of deer antler powder was extracted with 70% ethanol (2.0 L × 3) by maceration method at room temperature for 24 h. Then it filtered with Whatman paper no. 41 and evaporated by a BUCHI rotary evaporator at 40 °C and 40 rpm to create a thick extract with a constant weight of 16.8 g (Hariyadi et al., 2019; Widyowati et al., 2021, 2020).

2.3. Ethical considerations

Twenty-five male Wistar rats (*Rattus norvegicus*) purchased from the Faculty of Pharmacy, Airlangga University, Surabaya, Indonesia, were in good health. Rats aged 3–4 months (200–300 g) were acclimatized for seven days at the Animal Laboratory of the Faculty of Pharmacy, Airlangga University. Rats were maintained in a room at a temperature of 22 ± 3 °C, with a relative humidity of 30–70% and 12 h of light and 12 h of darkness. Rats were kept in cages with dimensions of 45 cm(L) × 30 cm(D) × 20 cm(H), with each cage consisting of one rat. The cage is waterproof, robust, easy to clean, and free from noise. This research has followed the Guidelines for the Care and Use of Laboratory Animals issued by the National Institutes of Health was revised in 1985 and has been approved by the ethical committee of Faculty of Veterinary Medicine, Universitas Airlangga. (No.2.KE.176.09.2019).

2.4. Osteoarthritis model

Rats in healthy conditions were injected with MIA at a dose of 4 mg that was dissolved in saline (50 μl) by a 27-G needle intra-articularly (Nagy et al., 2017). It was done below anesthesia with the usage of 10% ketamine (Agrovet, Nicaragua). Then, the swelling condition and joint damage in rats were observed daily (Khotib et al., 2020).

2.5. Animals grouping

This research was conducted using 25 rats divided into five groups (n = 5 rats). They were (1) the healthy group (S, was given food and water *ad libitum* but not induced by MIA), (2) the negative group (N, rats injected 4 mg MIA dissolved in 50 μl saline and treated 0,5% Carboxy Methyl Cellulose (CMC), (3) the positive group (P, rats injected with MIA 4 mg dissolved in 50 μl saline and treated with glucosamine sulfate (250 mg/kg BW) (Lipa Pharmaceuticals Ltd, NSW, 2566, Australia), (4) low dose group (L, rats injected with 4 mg MIA dissolved in 50 μl saline and treated deer antler extract 250 mg/kg BW), and (5) the high dose group (H, rats injected with 4 mg MIA dissolved in 50 μl saline and treated deer antler extract 500 mg/kg BW). Intra-articular MIA injection (4 mg MIA dissolved in 50 μl saline) (Sigma-Aldrich, Darmstadt, Germany) was performed in all groups except the healthy group to obtain the OA rat model. After 3 weeks MIA induction, all rats were blood drawn to see IL-1β levels were measured (pre-test), followed by administration of deer antler extract orally once a day and carried out every day for 28 days based on the group. Furthermore, swelling in the rat joint diameter and hyperalgesia were measured with a calibrated micrometer screw and hot plate for seven weeks (at weeks 0, 1, 2, 3, 4, 5, 6, and 7). The latency time in hyperalgesia was measured with a stopwatch. At week 7, blood was taken to measure IL-1β levels in blood serum (as a post-test). Physically, there was an expansion in the diameter of the rat's ipsilateral joint where the injection had been done, and functionally it reduced the latency time (hyperalgesia) to thermal stimulation using the hot plate method (Khotib et al., 2020).

2.6. Hyperalgesia experiment

Hyperalgesia experiments were carried out in each group at weeks 0, 1, 2, 3, 4, 5, 6, and 7 using the hot plate method (Ugo Basile Hot/Cold Plate 35100, Gemonio, Italy). The hot plate method is a well-established technique that relies on rats' visual cues to communicate their thermal pain and uses uncontrolled rats. The rats were placed one by one on a hot plate at 55 ± 0.5 °C for the hyperalgesia test. Heat exposure was carried out until a nociceptive response in rats occurred. The response latency and the time documented using video to observe the nociceptive response has occurred. Nociceptive responses were shown as rats licking their hind legs, rubbing their front legs, or jumping. The rats were taken off the hot plate immediately after the response was observed. Latency response is evaluated manually with a stopwatch (second).

2.7. Joint swelling measurement

The measurement of joint swelling was carried out on the right knee of rats after injection of MIA intra-articular in negative, positive, and treatment groups. Measurements had taken on weeks 0, 1, 2, 3, 4, 5, 6, and 7. The diameter of the rat's joint was measured using a screw micrometer that was calibrated in mm (millimeter) to determine swelling that occurs during the development stage of osteoarthritis with a time interval of several days.

2.8. ELISA analysis of pro-inflammatory cytokine IL-1β levels

The rat blood was taken 1–2 mL in the tail at weeks 3 and 7 and centrifuged at 3000 rpm for 10 min to get some of the serum. Proinflammatory cytokine IL-IB levels were measured using a commercial rat IL-1β ELISA kit (Bioenzy, Germany), following the manufacturer's instructions. Then, it analyzed the results using the ELISA reader instrument (EZ-2000).

2.9. Statistical analysis

The results were performed as means ± standard deviation of 5 rats in each group. All statistical tests were performed using SPSS 23 one-way ANOVA and two-way ANOVA with 95% confidence intervals (p-value < 0.05). Then, followed by LSD Post Hoc tests to establish significant differences between groups.

3. Results

3.1. Osteoarthritis rat models induced by monosodium iodoacetate (MIA)

Osteoarthritis progress in rat models OA induced by intra-articular MIA was observed for 3 weeks and evaluated by way of several parameters, namely, measurement of the joint diameter of rats (physical parameters), observation of the resistance of rat's movement to temperature stimulus by the hot plate method (functional parameters), and measurement of the pro-inflammatory cytokine IL-1β levels (biochemical parameters). The physical parameter utilized to characterize the accomplishment of an osteoarthritis model is estimating the measurement diameter of the joint of rats induced by MIA using a micrometer screw. The results showed that the negative group has a larger joint diameter than the healthy group that was not induced by MIA (p < 0.05) are shown in Fig. 1. It indicates that the MIA induction was successful to make the OA model in rats.

Determination behavior of osteoarthritis can be evaluated from functional parameters or behavior changes in rats through the pain level in an inflammatory state by measuring resistance (hyperalgesia) to responses in the thermal stimulation of the hot plate method application (see Fig. 2.). It showed that the negative group has hyperalgesia time in thermal stimulation faster than the healthy group with a significant difference of p < 0.05.

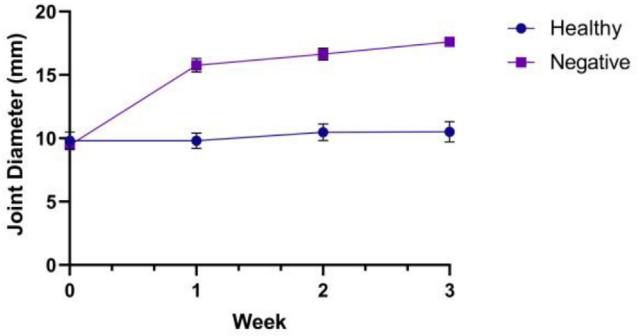

Fig. 1. Rat's joint diameter during OA model development. Data are present as Mean ± SD based on n = 5 for all groups.

On week 3 after MIA injection, rats' blood was taken to measure the levels of pro-inflammatory cytokine IL-1β using ELISA before being treated with deer antler extracts. The result showed a significant difference between the healthy and negative control group (p < 0.05) in the pro-inflammatory cytokine IL-1β levels in the rats' blood serum and negative group has 4 times higher cytokine Il-1β levels compared to the healthy group (Fig. 3).

3.2. Effect of deer antler extract administration on rats with OA

After the osteoarthritis condition, every rat in the extract group (L & H) was administered 250 mg/kg BW (L) and 500 mg/kg BW (H) of deer antler extract, then for the positive group was administered glucosamine sulfate (250 mg/kg BW) orally. Measurements of rat's right joint diameter and rat resistance time to thermal stimulation were carried out at 4, 5, 6, and 7 weeks, and the week-7 determined pro-inflammatory cytokine IL-1β levels. Based on the analysis result during therapy, the negative, the extract (L & H), and the positive groups had significant differences (p < 0.05) in the joint diameter parameter of rats starting from weeks 4 to 7 as shown in Fig. 4. It indicated that deer antler extract and glucosamine sulfate reduce joint inflammation in rats injected with MIA.

The negative, extract, and positive groups also showed significant differences (p < 0.05) from weeks 4 to 7 during therapy in rat heat resistance time (Fig. 5).

In week 7, the rat blood of the extract group was drawn to evaluate altered pro-inflammatory cytokine IL-Iβ levels. The result has shown a significant difference between cytokine IL-Iβ levels before

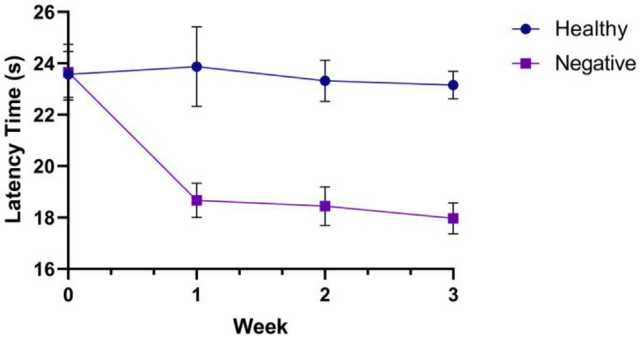

Fig. 2. Hyperalgesia in rats during OA model development. Data are present as Mean ± SD based on n = 5 for all groups.

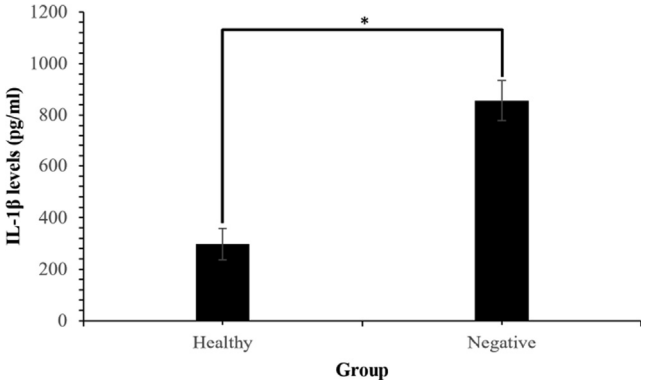

Fig. 3. The pro-inflammatory cytokine IL-Iβ levels in rats' blood serum. The IL-Iβ levels were increased after MIA injection as compared to the healthy group ($p < 0.05$). Data are present as Mean ± SD based on n = 5 for all groups.

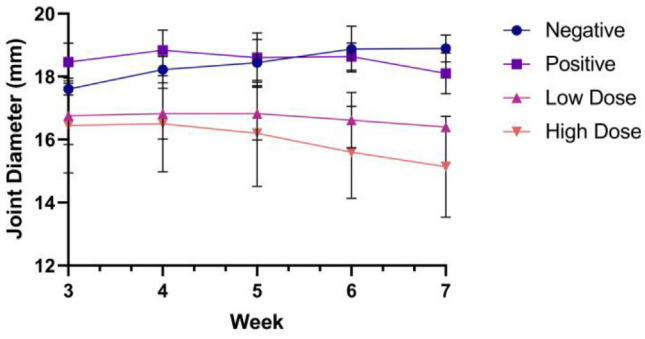

Fig. 4. Rat's joint diameter during therapy was significantly decreased after treatment with deer antler extract as compared to the negative group. Data are present as Mean ± SD based on n = 5 for all groups.

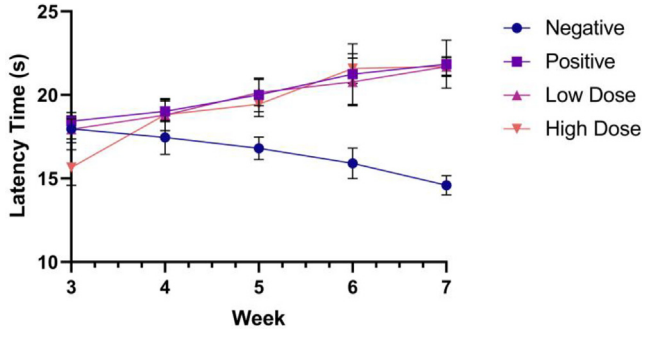

Fig. 5. Rat's latency time during therapy was significantly increased after the treatment with deer antler extract as compared to the negative group. Data are present as Mean ± SD based on n = 5 for all groups.

(pre-test) and after therapy (post-test) ($p < 0.05$) in the extract group (see Fig. 6). Then, it was analyzed using an LSD test that showed the negative and extract groups had a significant decrease in cytokine IL-Iβ levels between the pre-test and post-test.

4. Discussion

In this study, we determined pro-inflammatory cytokine IL-Iβ alteration levels in OA rat models that had received deer antler extract therapy. This parameter is one of the main pro-inflammatory cytokines that have an important role in OA (Mahajan et al., 2005). The increased sensitivity of chondrocytes to the release of IL-Iβ makes the OA inflammatory worse condition

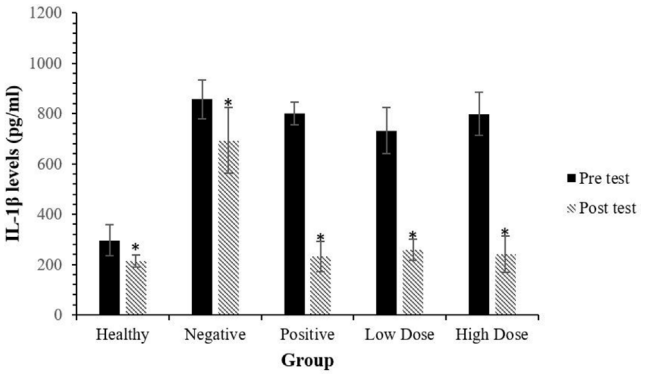

Fig. 6. The pro-inflammatory cytokine IL-Iβ levels in rats before and after therapy. The IL-Iβ levels were decreased after treatment as compared with the negative group ($p < 0.05$). Data are present as Mean ± SD based on n = 5 for all groups. $^{*}p < 0.0$.

(Melo-florián, 2011). Cytokines IL-Iβ can be combined by mononuclear cells in excited joints as a major mediator in cartilage matrix reduction and stimulate secretion and synthesis of various degradative enzymes in cartilage.

OA induction was carried out by MIA injection intra-articularly to the right knee of rats. MIA is one of the metabolic inhibitors that damage the vigorous glycolysis pathway to incite cell death and also repress glyceraldehyde-3-phosphate dehydrogenase causing disruption of chondrocyte digestion consequently influencing the cartilage matrix catabolism and responsive oxygen species (ROS) production (Moilanen et al., 2015). Giving MIA injection causes a decrease in proteoglycans, and osteophyte formation, increases cartilage degradation (Nagy et al., 2017), and induces an increase of pro-inflammatory cytokines as well as TNF-α, IL-1β, and matrix metalloproteinases (MMPs) have an active role in the inflammatory process (Moilanen et al., 2015; Pitcher et al., 2016). Osteoarthritis generally occurs within 2–8 weeks after being induced by MIA and it depends on the dose of MIA (1–4 mg). The condition occurs progressively and is similar to lesion osteoarthritis in humans.

The measurement of the right rat joint aims to determine the occurrence of swelling in the tissue as an indicator of inflammation that occurs due to injection by MIA. Based on the analysis result (see Fig. 1), it was shown that there was an increase in the joint diameter of rats injected with MIA which was marked by swelling ($p < 0.05$). Swelling can occur due to the mechanism of MIA that can cause an inflammatory process in the form of cell migration, vascular permeability, and capillary extravasation. The appearance of swelling also indicates that the occurrence of inflammation in the synovial is correlated with the occurrence of osteoarthritis and can reduce joint movement in rats (Yamada et al., 2019). The time resistance parameter of the negative has been shown at weeks 1 to 3 after induced MIA. It indicates a sign of soreness development in rats induced by osteoarthritis.

There is a significant difference between the healthy and negative control group ($p < 0.05$) in the pro-inflammatory cytokine IL-1β levels in the rats' blood serum (see Fig. 3). It happens because inducing MIA has a mechanism of action in synovial cells and chondrocytes to respond to intracellular signals and produce pro-inflammatory mediators as well as proteinases and cytokines (Orita et al., 2011).

Deer antlers are assessed as a valuable traditional Chinese restorative material and have been popular to reinforce the kidney's yang, provide a gist, and fortify the bone capacity (Chen et al., 2015; Widyowati et al., 2020). The leading bioactive constituents in deer antlers are water-soluble proteins that present

potency roles in repair and bone formation (REN et al., 2019; Yu et al., 2017). Deer antler extract also has a function as a nominee supplement to inhibit cartilage inflammation and degeneration, as well as repair cartilage homeostasis. This effect can be accomplished by stimulating the useful gene expression included in the arrangement, development, and repair of cartilage, including suppressing the expression of helplessness qualities involved within the osteoarthritis pathophysiology (Yao et al., 2021). Glucosamine has the function to restore components that produce the extracellular matrix of cartilage and prevent further cartilage degradation along with activating IL-1β and chondrocytes activation (Fajardo & Di Cesare 2005).

According to Lee et al., (2014), the administration of deer bone extract effectively protects against bone damage and reduces the number of void erosion. Then Choi et al., (2016) concluded that the administration of deer bone oil extract in the treatment of OA rats has a good effect and recommend it for osteoarthritis repair. Widyowati et al., (2020) reported that 70% ethanol extract of deer antlers showed higher NO inhibitory activity than aqueous extract with a 40% inhibition value. However, both had no cytotoxic effect on LPS 264.7 stimulated RAW. The 70% ethanol extract of deer antlers can stop NO production up to 40% in a concentration of 10 μg/mL. Likewise, aqueous extract of deer antlers can inhibit up to 80% in the same concentration. Therefore, the extract can be considered successful in reducing the expression of inflammatory markers in osteoblasts and maintain osteoblast function. In addition, deer antler extract *in vitro* reduced the pro-inflammatory impact of TNF-stimulated MH7A RA-FLS cells and *in vivo* treatment with deer antler extract reduced clinical arthritis scores and offered protection against synovial and cartilage damage induced by cells. Cytokine-mediated immunity in mice induced by zymosan (Cheng et al., 2022).

Based on these results, there are cytokine IL-Iβ levels decreation after therapy due to the mechanism of deer antler extract and glucosamine sulfate by inhibiting pro-inflammatory cytokine IL- Iβ release. The IL-1β is a major pro-inflammatory cytokine that is yielded in large quantities in osteoarthritis that can cause the expression of factors as well as iNOS, IL-6, and COX-2 (Kucharz et al., 2016). So, we conclude that oral administration of deer antler extract effectively reduces levels of the pro-inflammatory cytokine IL-I β induced by MIA injection in animal models. Hence, the deer antler extract is a potential agent for OA therapy.

5. Conclusion

This study showed that 70% ethanol extract from deer antlers reduced levels of the pro-inflammatory cytokine IL-1β, reduced joint swelling, and increased latency time by MIA injection in animal models. These findings suggest that deer antler extracts 250 and 500 mg/kg BW may be potential treatments for OA as well as glucosamine sulfate.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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