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Attenuation of IL-1ß on the use of glucosamine as an adjuvant in meloxicam treatment in rat models with osteoarthritis

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Abstract:
Background: Osteoarthritis (OA) is the most prevalent joint disease and a common cause of joint pain, functional loss, and disability. The severity of this disease is always associated with increased levels of proinflammatory cytokines, which play an important role in cartilage damage, synovitis, and other damage to joint tissues. The discovery that many soluble mediators such as cytokines or prostaglandins can increase the production of matrix metalloproteinases by chondrocytes led to the first steps of an inflammatory state. Several studies show that cytokines, such as interleukin 1ß, have a major role in the development of inflammation that occurs in these joints. The use of glucosamine as an adjuvant to meloxicam therapy is expected to inhibit the development of inflammatory OA.
Methods: The OA model in rat was induced by single injection of intraarticular monosodium iodoacetate (MIA). The development of OA was observed for 21 days. Furthermore, the evaluation of glucosamine potency as an adjuvant of meloxicam therapy for reducing IL-1ß was done by combined treatment at a low dose of meloxicam 1 mg/kg BW with glucosamine at a dose of 125, 250, or 500 mg/kg BW orally for 28 days. Response to hyperalgesia and knee joint diameter was measured on days 0, 7, 14, 21, 28, 35, 42, and 49. IL-1ß levels were measured on day 21 and day 49 after MIA injection.
Results: MIA injection successfully induced OA as marked by a significant difference in the time of latency to heat stimulus ($p < 0.01$) and a significant increase in joint diameter ($p < 0.01$). On day 21, IL-1ß levels showed a significant decrease in MIA injection ($p = 0.05$). The administration of meloxicam and glucosamine did not induce significant decrease in knee joint diameter ($p > 0.10$), but was able to significantly increase the latency time to heat stimulus ($p < 0.01$). IL-1ß levels also showed a significant decrease after administering a combination of glucosamine and meloxicam ($p < 0.01$).
Conclusions: Taken together, the use of glucosamine as an adjuvant in meloxicam therapy may be caused by the synergistic mechanism of meloxicam for the attenuation of OA development through systemically reducing IL-1ß.

Keywords: glucosamine, IL-1ß, meloxicam, MIA, osteoarthritis
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Introduction

Osteoarthritis (OA) is the most common type of arthritis and is referred to as degenerative joint disease [1]. Osteoarthritis is ranked fifth as the highest cause of disability for all residents in high-income countries and the ninth highest cause in low- and middle-income countries [2]. The main characteristic of OA is the imbalance between the activity of anabolic and catabolic enzymes produced by chondrocytes [3]. Osteoarthritis is associated with damage to the cartilage accompanied by the formation of osteophytes at the edge of the joints with changes in the structure of bone thickness such subchondral, ligaments, synovial fluid, and other related tissues [4]. Osteoarthritis may cause stiffness, pain, and also impaired movement due to bone rubbing resulting from thinning of the cartilage in the joints [2]. Problems due to OA are poor quality of life, limitations in daily activities, and progressive disability [3].

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Osteoarthritis can be caused by various factors (multifactorial). Common predisposing factors for OA are age, sex, obesity, genetics, hypermobility, and also hormonal factors in rheumatic diseases. Other factors causing OA are mechanical factors, trauma, joint shape, and excessive joint use due to doing work or activities [5]. The severity and inflammation of OA can change according to the development of the disease, which is characterized by the presence of proinflammatory cytokines found in the early and advanced stages of the disease. Proinflammatory cytokines interleukin-1ß (IL-1ß) and tumor necrosis factor-α (TNF-α) are the main parts of proinflammatory cytokines involved in OA. IL-1ß and TNF-α change the homeostatic balance of chondrocytes by suppressing the activity of anabolic enzymes and stimulating the activity of catabolic enzymes in cartilages [6].

IL-1ß is synthesized by mononuclear cells (including synovial cells) in inflamed joints, such as the main mediators in the degradation of the cartilage matrix, and stimulates the synthesis and secretion of various degradative enzymes in the cartilage [7]. In the cartilage, IL-1ß stimulates the release of arachidonic acid and induces expression of cyclooxygenase-2 (COX-2) thereby increasing prostaglandin E2 (PGE2) production [8]. Osteoarthritis therapy is an attempt to reduce symptoms or modify the structure of the cartilage. Osteoarthritis therapy is used with the aim of relieving pain, reducing inflammation, reducing stiffness, increasing mobility, and increasing patient comfort. A measure of the success of OA therapy recommended by Outcome Measures in Rheumatology Clinical Trials (OMERACT) consists of four core categories, i.e., pain, physical function, general assessment by the patients, and general assessment by the doctors. Another key measure of success based on Disease-Modifying Osteoarthritis Drugs (DMOADs) is the narrowing of joint space, pain, and physical function [9].

Therapy using non-steroidal anti-inflammatory drugs (NSAIDs) is the first choice in OA [10]. NSAIDs acts by inhibiting COX and reducing prostaglandin production as well as reducing inflammation and pain [11]. Meloxicam is a group of NSAIDs that is included in the unselective category of COX-2 because it can partially inhibit COX-1 [12]. Meloxicam can work by inhibiting the biosynthesis of COX-2, inhibiting the levels of IL-1ß and TNF-α, which play a role in the inflammatory process [13], [14].

Glucosamine is the most common supplement used by OA patients [15]. The use of glucosamine in OA therapy is recommended with the aim of preventing, slowing down, or reversing structural and pathological changes in OA conditions [16]. Glucosamine naturally improves cartilage by increasing glycosaminoglycan synthesis (GAG) and preventing cartilage destruction. In OA, glucosamine inhibits the release of IL-1ß, where IL-1ß is the main proinflammatory cytokine, which in OA is produced in high amounts so that it triggers the expression of inflammatory factors, such as COX-2, iNOS, and IL-6 [17].

The combination of meloxicam and glucosamine is often used for OA therapy. A study by Fadhilah (2016) found that the percentage of meloxicam use combined with glucosamine in Universitas Airlangga Hospital was 18.30%. The percentage of combined use of meloxicam and glucosamine is the highest compared with the use of other drug combinations. From those results, further study needs to be done to determine changes in proinflammatory cytokine markers in osteoarthritis against the use of a combination of meloxicam and glucosamine.

The proinflammatory cytokine marker observed in this study was IL-1ß. IL-1ß has the most important role and is known to increase in high amounts in chondrocytes, synovial membranes, and synovial fluid in OA conditions [18]. By observing changes in IL-1ß, we can find the effectiveness of using glucosamine as an adjuvant to meloxicam therapy for decreasing inflammatory OA.

Materials and methods

Materials

Materials used in this study were meloxicam (Sun Pharmaceutical Industries Ltd, Mumbai, India) and glucosamine (PT Surya Windu Kartika, Banyuwangi, Indonesia) with pharmaceutical standards. Induction of OA in rats was done using MIA. Other materials used in this study included distilled water, 400 polyethylene glycol (PEG), physiological salts solution (0.9%), 2 N sulfuric acid, 10% ketamine, 1% xylazine, and IL-1ß ELISA kit.

Adaptation of experimental animals

Rats aged 3–4 months and weighing around 250–300 g were adapted to a cage for 7 days at the Animal Laboratory, Faculty of Pharmacy, Universitas Airlangga. Rat cages were made of acrylic material, and the cage chamber temperature was controlled at 22.5 ± 2 °C and at humidity of 55 ± 5% with a 12-h bright-dark lighting cycle [19], [20].

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Osteoarthritis induction

Healthy rats were induced with 4 mg of MIA dissolved in 50 µL of saline and then injected into the rat joint cavity using a 27-G needle. Intraarticular injection was performed under anesthesia using 10% ketamine (80 mg/kg) plus 1% xylazine of 5 mg/kg. After being injected, the animals were observed everyday to assess the condition of swelling and dysfunction of the joints [20], [21].

Experimental animals grouping

This study was conducted using 42 rats that were divided into seven groups; each group consisted of six rats. The division of groups was based on the treatment given: naive group (injected with 50 µL of saline and treatment with PEG 400 and distilled water), OA group (induced with 4 mg MIA dissolved in 50 µL of saline and treatment with PEG 400 and distilled water), meloxicam group (receiving meloxicam drug in a dose of 1 mg/kg BW), glucosamine group (receiving glucosamine drug in a dose of 500 mg/kg BW), meloxicam-glucosamine 125 group (receiving a combination of 1 mg/kg BW of meloxicam dose and 125 mg/kg BW of glucosamine), meloxicam-glucosamine 250 group (receiving a combination of 1 mg/kg BW of meloxicam with 250 mg/kg BW of glucosamine), and meloxicam-glucosamine 500 group (receiving a combination of mg/kg BW of meloxicam with 500 mg/kg BW of glucosamine). The naive group was injected intraarticularly with saline, while the OA group and the treatment groups were induced OA by means of intraarticular MIA injection in the ipsilateral knee. Some parameters used to assess the development of OA in rats were, physically, the presence of an increase in diameter of the rat ipsilateral knee where the injection was done, and, functionally, through a decrease in the latency time (hyperalgesia) to heat stimulus using the hot plate method.

Preparation of MIA solution

Osteoarthritis induction solutions for the rats were prepared by dissolving 4 mg of MIA in 50 µL of saline (0.9%) [20]. The induction solution must be sterile so filtration needs to be carried out using a pore size of 0.22 µm [22].

Preparation of meloxicam solution

Meloxicam solution was made by dissolving meloxicam into (PEG) 400: water with a ratio of 50:50 [20]. Meloxicam solubility data in PEG 400 was 7 mg/mL [23], while meloxicam solubility data in the water was 0.012 mg/mL [24].

Preparation of glucosamine solution

Glucosamine solution was prepared by dissolving glucosamine in the water with data on the glucosamine solubility in the water of 100 mg/mL [25].

Meloxicam and glucosamine administration

Meloxicam and glucosamine solutions were given to each group of rats per oral (PO) with needles according to the dose in each treatment group at the same hour, between 9 and 10 am everyday for 4 weeks (28 days).

Hyperalgesia testing

Hyperalgesia testing was carried out in the control and treatment groups on days 0, 7, 14, 21, 28, 35, 42, and 49 using hot plates. Rats were placed one at a time on a hot plate with the temperature set to 55.0 ± 0.5 °C, and the temperature was maintained at a constant intensity. Exposure to heat was given until a nociceptive reaction in the rats occurs. The typical rat response observed was shaking and/or withdrawal hind limb. The rats were immediately removed from the hot plate after the response occurred. Latency response was measured manually with a stopwatch.

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Measurement of knee joint swelling

Swelling measurements were performed on the ipsilateral rat's knee after MIA intraarticular injection. Measurements were made on days 0, 7, 14, 21, 28, 35, 42, and 49 using a calibrated screw micrometer.

ELISA analysis

Blood, as much as 0.5–1 mL, was taken from the rats' tail, then put into a venoject tube. Centrifugation at a speed of 3000 rpm was carried out for 5 min. The centrifuge was inserted into an aliquot tube and stored at −20 °C. A microplate was coated with murine monoclonal antibody specific to IL-1ß in the well. Thereafter, 100 µL of HD1C assay diluent and 150 µL of standard (IL-1ß standard) or samples were put into each well and covered with an adhesive covering. Incubation was carried out for 14–20 h at room temperature and washed four times. Then, 200 µL of conjugate solution containing polyclonal antibodies labeled with enzymes specific to IL-1ß was added to each well. Incubation was carried out for 3 h at room temperature and washed again four times. As much as 50 µL of substrate solution was added to each well, then incubated for 45 min at room temperature. A total of 50 µL of the amplifier solution was added and covered with a new adhesive cover. Incubation was carried out for 45 min at room temperature. An amplifier solution was added until the stain started to appear. A total of 50 µL of stop solution was added to each well, then read with a microplate reader at a wavelength of 490 nm (in 30 min). The reading was corrected at a wavelength of 650 nm or 690 nm.

Data analysis

To test and establish the diagnosis of IL-1ß levels, a paired t-test statistical test was used. IL-1ß proinflammatory cytokine levels were analyzed using one-way ANOVA and two-way ANOVA statistical tests with 95% confidence intervals. The Kolmogorov Smirnov test was used in each group to determine the normality of the data distribution. Then, to find out the different groups, the test continued with the post hoc test with LSD (least significant differences).

Results

Development of osteoarthritis model in rats induced using intraarticular monosodium iodoacetate (MIA)

The physical parameters used to determine the success of induction and formation of OA models were the measurement of the ipsilateral knee diameter of the rats injected with MIA using a screw micrometer. Data from the rat knee diameter measurements are presented in the form of X ± SD and can be seen in Table 1.

Table 1: Diameter of rat ipsilateral knee after intraarticular MIA injection.

Groups	Rat ipsilateral knee diameter (mm) after intraarticular MIA injection			
	Day 0	Day 7	Day 14	Day 21
Naïve (n = 5)	7.45 ± 0.75	7.81 ± 0.61	8.26 ± 0.46	8.20 ± 0.51
OA (n = 5)	7.41 ± 0.22	9.15 ± 0.48	9.59 ± 0.21	10.30 ± 0.08

Statistical tests showed a significant difference between the negative control and positive control groups ($p < 0.01$) on parameters of the rat right knee diameter from day 7 to day 21 after MIA intraarticular injection.

The functional parameter to determine the success of induction and formation of OA models is the time of resistance (hyperalgesia) to heat stimulus by the hot plate method. Data on the measurement of time resistance to heat stimulus in rats with intraarticular injection of MIA is presented in the form of X ± SD and can be seen in Table 2. There is a significant difference between the negative control group and the positive control group

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($p < 0.01$) on the parameters of resistance time (hyperalgesia) to heat stimulus in rats from day 7 to day 21 after MIA injection.

Table 2: Time of latency (hyperalgesia) to heat stimulus in rat after intraarticular injection of MIA.

Groups	Rat time of resistance to heat stimulus (second) after intraarticular MIA injection?			
	Day 0	Day 7	Day 14	Day 21
Naïve (n = 5)	23.40 ± 1.59	23.33 ± 1.18	23.47 ± 2.91	23.20 ± 1.13
OA (n = 5)	22.73 ± 1.32	18.47 ± 0.51	18.07 ± 0.28	17.40 ± 0.49

On day 21 after MIA injection, blood sampling was performed for IL-1ß pro-inflammatory cytokine levels. Data are presented in the form of $X \pm SD$ and can be seen in Table 3.

Table 3: Levels of rat IL-1ß after intraarticular injection of MIA.

Groups	Rat pro-inflammatory cytokine IL-1ß (pg/mL) after intraarticular MIA injection
Naïve (n = 5)	571.67 ± 69.66
OA (n = 5)	777.04 ± 15.85

The statistical test results showed a significant difference between negative control and positive control groups ($p = 0.05$) in the parameters of pro-inflammatory IL-1ß cytokine.

Effect of glucosamine and meloxicam administration in rats with osteoarthritis

After experiencing OA, rats in each treatment group were given 1 mg/kg BW of meloxicam; 500 mg/kg BW of glucosamine; a combination of 1 mg/kg BW of meloxicam and 125 mg/kg BW of glucosamine, a combination of 1 mg/kg BW of meloxicam and 250 mg/kg BW of glucosamine, and a combination of 1 mg/kg BW of meloxicam and 500 mg/kg BW of glucosamine orally. Treatment was given to test the extent of the influence of meloxicam, glucosamine, as well as the combination of meloxicam and glucosamine administration on the development of OA. The treatment was started on day 21 after the rats were injected with MIA intraarticularly. Measurement of rat ipsilateral knee diameter and time of latency (hyperalgesia) to heat stimulus was done every week for 4 weeks, which were on days 28, 35, 42, and 49. At the end of the fourth week (day 49) after treatment, rat blood was taken through the tail to determine the level of pro-inflammatory cytokines IL-1ß. During the therapeutic process, the measurement of ipsilateral knee diameter of rats injected with MIA was carried out using a screw micrometer. Data from the rat knee diameter measurements are presented in the form of $X \pm SD$ and can be seen in Table 4. The data shows the absence of significant differences between OA group and treatment group ($p > 0.10$) in the parameter of rat ipsilateral knee diameter from day 28 to 49 during treatment.

Table 4: Diameter of rat ipsilateral knee during treatment.

Groups	Diameter of rat right knee during therapy				
	Day 21	Day 28	Day 35	Day 42	Day 49
OA	10.30 ± 0.08	10.41 ± 0.30	10.42 ± 0.22	10.45 ± 0.16	10.47 ± 0.07
Meloxicam 1 mg	10.25 ± 0.25	10.44 ± 0.13	10.46 ± 0.09	10.49 ± 0.11	10.51 ± 0.17
Glucosamine 500 mg	10.26 ± 0.05	10.40 ± 0.21	10.44 ± 0.15	10.51 ± 0.19	10.55 ± 0.17
Meloxicam-glucosamine 125 mg	10.24 ± 0.59	10.29 ± 0.50	10.31 ± 0.16	10.44 ± 0.12	10.47 ± 0.14
Meloxicam-glucosamine 250 mg	10.36 ± 0.13	10.39 ± 0.14	10.45 ± 0.19	10.52 ± 0.12	10.53 ± 0.17
Meloxicam-glucosamine 500 mg	10.21 ± 0.33	10.47 ± 0.11	10.50 ± 0.10	10.56 ± 0.09	10.58 ± 0.07

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During treatment, measurement of the time of latency (hyperalgesia) to the heat stimulus was performed by hot plate approach. Data on the measurement of the time of latency to heat stimuli in rats during therapy is presented in the form of $X \pm$ SD and can be seen in Table 5.

Table 5: Time of latency (hyperalgesia) of rat to heat stimulus during treatment.

Groups	Rat time of latency to heat stimulus (second) during therapy				
	Day 21	**Day 28**	**Day 35**	**Day 42**	**Day 49**
OA	17.40 ± 0.49	16.53 ± 0.18	16.33 ± 0.24	16.27 ± 0.28	15.67 ± 0.67
Meloxicam 1 mg	17.56 ± 1.93	18.83 ± 0.62	19.56 ± 0.54	19.67 ± 0.94	21.11 ± 1.22
Glucosamine 500 mg	18.00 ± 0.73	19.11 ± 2.21	19.33 ± 1.62	19.39 ±1.04	20.17 ± 2.85
Meloxicam-glucosamine 125 mg	16.72 ± 1.47	18.39 ± 0.49	19.05 ± 0.25	19.61 ± 0.90	20.33 ± 1.98
Meloxicam-glucosamine 250 mg	16.93 ± 2.47	19.53 ± 2.30	20.33 ± 2.21	21.33 ± 1.61	22.27 ± 4.39
Meloxicam-glucosamine 500 mg	17.28 ± 0.33	19.17 ± 0.78	20.95 ± 1.81	20.89 ± 1.30	22.33 ± 0.79

Statistical tests showed a significant difference between the OA group and the treatment group ($p < 0.01$) in the parameter of time of resistance to heat stimulus in rats from day 28 to day 49 during therapy administration.

On day 49 after administration of meloxicam, glucosamine, or a combination of both, rat blood was taken to see changes in levels of pro-inflammatory cytokines IL-1ß. Data on the measurement of pro-inflammatory cytokine IL-1ß levels are presented in the form of $X \pm$ SD and can be seen in Table 6.

Table 6: Levels of pro-inflammatory cytokine IL-1ß before and after glucosamine and meloxicam administration.

Groups	Rat IL-1β level (pg/mL) before treatment	Rat IL-1β level (pg/mL) after treatment	Rat IL-1β (pg/mL) level reduction after treatment
OA	777.04 ± 157.85	799.17 ± 46.05	−22.13
Meloxicam 1 mg	788.37 ± 144.68	500.49 ± 162.23	287.88
Glucosamine 500 mg	677.00 ± 88.56	473.83 ± 72.43	203.17
Meloxicam-glucosamine 125 mg	800.02 ± 73.66	546.09 ± 64.81	253.93
Meloxicam-glucosamine 250 mg	600.09 ± 117.28	453.56 ± 49.56	146.53
Meloxicam-glucosamine 500 mg	750.43 ± 119.75	462.52 ± 80.87	287.91

Discussion

This study aims to determine the role of glucosamine as an adjuvant to meloxicam therapy in changes of IL-1ß pro-inflammatory cytokine levels using osteoarthritic rat model. To induce pathophysiological OA in rats as that in humans, we injected MIA intraarticularly in the ipsilateral knee of the rats. MIA is a metabolic inhibitor that breaks down cellular aerobic glycolysis pathways so that it induces cell death. The mechanism of action of MIA is to inhibit glyceraldehyde-3-phosphate dehydrogenase, which causes a disturbance of chondrocyte metabolism so that it affects the production of reactive oxygen species (ROS) and catabolism of the cartilage matrix [26]. MIA can induce an increase in pro-inflammatory cytokines such as IL-1ß and TNF-α, which can induce COX-2 and matrix metalloproteinases (MMPs), which have an active role in the inflammatory process [4], [22], [26].

The development of intraarticular MIA-induced OA in rats was observed for 21 days. The rate of development of OA was assessed through three parameters, physical parameters of the rats' knee diameter, functional parameter of the latency time to heat stimulus, and biochemical parameters of IL-1ß pro-inflammatory cytokine

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level. Prior to intraarticular MIA injection, rat body weight measurement was performed. The analysis of the two-way ANOVA test showed no significant difference in body weight of the rats between the OA group receiving MIA injection compared to the naive group receiving normal saline injection ($p = 0.09$). The absence of significant differences related to the rat's weight showed that those rats grew normally.

After intraarticular MIA injection, the measurement of rat ipsilateral knee diameter was performed with the aim of knowing the presence of tissue swelling as an index of inflammation resulting from the injection. The two-way ANOVA test showed significant increase in the right knee diameter in the OA group, which received MIA injection compared to the negative control group that was injected with SN ($p < 0.01$). An increase in right knee diameter of the rats injected intraarticularly with MIA indicates swelling or edema. The resulting knee swelling implies synovial inflammation, which has a clinical correlation with the development of OA [27].

The inflammatory state in osteoarthritic rats after intraarticular MIA induction can also be determined by observing the rat behavior. The observations were performed by measuring the resistance time of mice to heat stimulus using hot plates. A two-way ANOVA test showed a significant decrease in the time of resistance of the rats to heat stimulus in the OA group receiving MIA injection compared to the naive group, which received SN injection ($p < 0.01$). Fisher's LSD test showed that a decrease in the time of resistance of the rats to heat stimulus started to appear on day 7 after MIA injection ($p < 0.01$). The decrease showed hyperalgesia, which was a sign of pain development in the rats induced by OA.

On day 21 after intraarticular MIA injection, rat blood was taken and tested with ELISA to determine the level of pro-inflammatory cytokines IL-1ß before the therapy was provided. The t test revealed a significant difference in the level of pro-inflammatory cytokines IL-1ß in the positive control group receiving MIA injection compared to the negative control group receiving SN injection ($p = 0.05$). This difference was due to the MIA action mechanism that induced chondrocytes and synovial cells to respond to intracellular signals, thereby increasing the production of pro-inflammatory mediators, such as cytokines and proteinases [28].

After the rats became a model of OA, meloxicam and glucosamine therapy was given from day 21 to day 49 (for 28 days). Meloxicam, which is a class of NSAIDs, can also act by blocking IL-1ß and TNF-α, which play a role in inflammatory process [14]. Glucosamine is a glycosaminoglycan (GAG) constituent in the cartilage matrix and synovial fluid that exerts certain pharmacological effects on cartilages and chondrocytes Roman et al. Glucosamine has another mechanism, which inhibits the activation of IL-1ß in chondrocytes under osteoarthritic condition [29].

In this study, the treatment provided to each treatment group was 1 mg/kg BW of meloxicam; 500 mg/kg BW of glucosamine; 1 mg/kg BW of meloxicam and 125 mg/kg BW of glucosamine; 1 mg/kg BW of meloxicam and 250 mg/kg BW of glucosamine; and 1 mg/kg BW of meloxicam and 500 mg/kg BW of glucosamine. During treatment, we measured the rats' body weight (data not shown), ipsilateral knee diameter using a micrometer screw, and time of resistance to heat stimulus using hot plates every week in order to determine the effect of therapeutic administration on each treatment group.

The measurement of rat body weight during treatment showed no significant difference in the positive control group, which did not receive therapeutic agent compared to the treatment group with therapy or between treatment groups, which received therapy ($p = 0.17$) based on the two-way ANOVA test. This shows that the rats experienced normal growth from day to day, and the administration of meloxicam and glucosamine did not have an impact on the significant increase in body weight. The results of the rat knee diameter measurements were further analyzed by two-way ANOVA and showed no significant difference in the right knee diameter of the rats that received MIA injection between the OA group and treatment group and between treatment groups ($p > 0.10$). The absence of differences in the rats' knee diameter can be caused by the growth of those rats that occurred during the administration of the therapy, which was marked by an increase in body weight so that the knee size became larger.

Two-way ANOVA test at the rat time of resistance to heat stimulus showed a significant difference between the OA group without therapy and the treatment groups with therapy ($p < 0.01$). The Fisher's LSD test revealed that significant differences in the rats' time of resistance to heat stimulus began to appear on day 28, while each treatment group that received therapy did not show significant differences in the time of resistance to heat stimulus. Some irregularities in the results of statistical tests on the resistance time of those rats could be caused by the weakness of the test method using hot plates. The hot plate method is done by placing the experimental animals on a hot board with a constant temperature and the animal activity on heat stimulation in the form of licking the legs (paw licking) or jumping/lifting body movements are observed. These movements, called chaotic defensive movements, are very complex and make observing and identifying the response of the experimental animals more difficult, and it is in itself a less consistent test [30].

The results of the two-way ANOVA test showed a significant difference between the levels of pro-inflammatory cytokines IL-1ß before therapy and the levels of pro-inflammatory cytokines IL-1ß after therapy ($p < 0.01$). The Fisher's LSD test showed that pro-inflammatory cytokine levels decreased or changed significantly before and after therapy in the positive control group and treatment group (P1–P5). The decrease is

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caused by the synergistic mechanism of action of meloxicam and glucosamine. At a more fundamental level, meloxicam, which belongs to the NSAID class can interact with transcriptional factors and affect the production of cytokines, so as to inhibit levels of IL-1ß and TNF-α, which play a role in the inflammatory process [14]. Meanwhile, the mechanism of action of glucosamine is to inhibit the release of pro-inflammatory cytokines IL-1ß, which in OA IL-1ß is produced in high quantities so that it can trigger the expression of inflammatory factors such as COX-2, iNOS, and IL-6 [17].

Conclusions

Taken together, the use of glucosamine as an adjuvant of meloxicam therapy may be caused by the synergistic mechanism of meloxicam for attenuation of OA development through systemically reducing IL-1ß.

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Ethical approval: All experiments were performed at the Animal Research Laboratory of Faculty of Pharmacy, Universitas Airlangga, Surabaya, Indonesia in accordance with the guide for care and use of laboratory animal issued by National Institute of Health revised in 1985. The protocol of this reseach was approved by the Ethical Committee of Faculty of Veterinary, Universitas Airlangga.

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