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Glucosamine in the Egg Shells and Goat Fats as Therapy for Osteoarthritis Rat Model

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Abstract: NSAIDs (Non-Steroid Anti-Inflammatory) as systemic therapy of pain in osteoarthritis (OA) have side effects such as gastrointestinal and cardiovascular risks that cause limiting the long-term use. Therefore, it is necessary to look for alternative therapy that are safer to use. One of the alternative therapy by utilization of eggshells and goat fats. Eggshells contains glucosamine and goat fats contains omega-3 which both has role on wound healing of OA. The aim of this study was to determine the effect of the combination of glucosamine in the eggshell and omega-3 in the goat fats toward OA rats that induced by papain. This study using 30 male wistar rats that were divided into 5 groups: negative control (healthy), positive control (OA) and 3 treatments where dosage of glucosamine is same (135 mg / kg bw) while dosage of omega-3 is multilevel (9, 18 and 27 mg / kg bw). The data result are presented quantitatively. Analysis based on the pain response, knee swelling diameter and alterarion in behavior. The results showed that the combination of glucosamine with omega-3 from can reduce pain response and swelling diameter also return the behavior of OA rats close to normal again in P3 treatment.

Keywords : Osteoarthritis, omega-3, glucosamine, goat fat, eggshell.

Introduction

Osteoarthritis is degenerative joint disease affecting 20% of adult dogs and become the most prevalent joint disorder in dogs.[1,2] While in human, OA affects 151 million people in worldwide and reached 24 million people in Southeast Asia.[3] Incidence of canine OA is increased by trauma, obesity, aging and genetic abnormalities. Fifty percent of OA cases are reported in dogs aged between 8–13 years. Several joints that suffered OA have been reported in elbow, shoulder, hip and knee. Most commonly is knee joint. OA is a painful degenerative process involving progressive deterioration of all joint structures and remodeling of subchondral bone and followed by inflammatory.[2,4]

Until now, therapy of OA cause many side effects. Conventional drug that characterized by immediate release and repeated dosing of the therapeutic agent, however has many limitations, including undesirable side effects due to fluctuating drug level in plasma, poor drug efficacy, and poor patient compliance.[5] NSAIDs (Non-Steroid Anti-Inflammatory Drugs) that widely used for systemic treatment of OA pain, have side effects such as gastrointestinal (irritation or bleeding upon exposure to stomach) and cardiovascular risk thus limiting the use in the long term.[5,6] Therefore, it is necessary to look for alternative therapy that are safer to use. One of the alternative therapy by utilization of animal products waste such as eggshells and goat fats. Using wastes as raw materials could eliminate the wastes althought with high cost effectiveness.[7]

Chicken eggshells as economical and environment-friendly source are cheap, but also a waste that is easily obtainable.[7,8] Based on data from Bappenas, egg production in 2004 reached 1.1 million tons and in 2005 increased to 1.15 million tons with the major egg-producing areas are in the Java island.[8] Eggshell membranes are a new raw material that has abundant natural source of bioactive compounds such as glucosamine.[9] Glucosamine is one of amino glucose that has function to produce synovial fluid that working as lubricant of the cartilage thus the cartilage moves well.[10] *In vitro* studies shown the mechanism of glucosamine on repair processes involves stimulation of the glycosaminoglycans and collagen synthesis. Animal studies have shown the content of glycosaminoglycans within the site of partially ruptured muscles increased maximally five days after trauma and decreased thereafter. This suggests the timing of glucosamine supplementation may determine its therapeutic impact on wounds.[11]

Based on previous study, the combination of glucosamine sulfate and omega-3 fatty acids has more reduction effect in all WOMAC (Western Ontario and McMaster Universities Arthrosis index) scores (pain, stiffness and function 70,7%, 64,9%, 68,8%, respectively) than glucosamine sulfate alone (65,6%, 59,7%, 63,9%, respectively). From this results, it can be speculated that the combination product was statically and clinically superior compared with glucosamine sulfate alone. It is probably that the combination of glucosamine and omega-3 fatty acids act siynergistically, ie, omega-3 fatty acids inhibit the inflammation process in OA, whereas glucosamine sulfate further supports the rebuilding of lost cartilage substance. While glucosamine sulfate improves cartilage metabolism, the omega-3 fatty acids, EPA and DHA, reduce degradation of cartilage further by reducing the inflammation present in OA.[12] Even other in-vivo studies mentioned glucosamine also had antiinflammatory effect through inhibition of inflammation-mediating cell production.[13]

In the last decade, omega-3 plays an important role in the prevention and treatment of coronary diseases, hypertension, arthritis and impaired immune system.[14,15] In bone disease, omega-3 has the ability to modulate osteoclastogenesis, osteoblastogenesis and eicosanoid production in bone calcium metabolism.[14] Marine fish as the traditional source of PUFA (omega-3), however has many lack such as limited fishing seasons, geographical locations, declining fish populations, contain cholesterol and have unpleasant odour. Therefor it's a need to look for alternative sources of PUFA.[16] The number of slaughter goat enhancement (slaughter recorded in 2008 amounted to 309 930 goats and increased in 2012 to 540 110 goats) followed by an increase waste.[17] One of the wastes is goat fats. Fatty acids can be obtained from goat fats because high of omega-3 than other ruminantia fat.[7,18] Goat lipids has more PUFA (i.e., C18:2, C18:3 and C20:4) than noted in lamb and beef. For the individual PUFA, the species rankings for C18:3 (omega-3 fatty acids) are goats>lamb> beef>pork.[18] From this results, researchers want to combine glucosamine hydrochloride of the eggshells with new source of omega-3, goat fats.

Methods

Study Design: Thirty male Wistar rats weighting 150 - 250 g were evaluated and approved by Brawijaya University Biosains Department local ethic committee with ethical clearance certificate no. 520-KEP-UB. Rats were kept under standard laboratory conditions (temperature 24°C, 12h light-dark cycle). Feed and water were provided *ad-libitum*. Rats were divided into five groups: 6 rats as negative control group (healthy rats), 6 rats as positive control group (OA rats) and 18 rats were given therapy of combination of glucosamine (dosage remain the same: 135 mg/kg weight of body) and omega-3 (3 multilevel dosage: 9, 18 and 27 mg/kg weight of body).

Induction of OA: Rats were anesthetized with ketamine and xylazine before every injection. Osteoarthritis was induced in the right knees joints by injecting 0.2 ml of 4% papain solution intraarticularly. Injection was repeated on the fourth and seventh days.[19]

Pain assessment: Pain threshold was assessed in control and OA groups on days 9th by the hot water tail flick assay. The dependent variable was the latency (in seconds) for the rats to flick its tail from the hot water bath. The water was maintained at 55^{o}C in a constant temperature water bath and monitored by an electronic thermometer. Rats were wrapped in a breathable towel and gently held. The distal third of the rat's tail was dipped in water bath and time required for the rat to flick its tail from heat stimulus was counted with the help of stop watch. The tail flicking score was calculated as the mean of the last two of three readings separated by 30 seconds interval. Clinical score allocated for pain was one if tail retained in hot water for 0.1 to 1 seconds. Two clinical score for pain at retention time of 1.1 to 2 seconds, three for 2.1 to 3 seconds and four for retention time of 3.1 to 4 seconds respectively.[20]

Measurement of swelling: Knee diameter was measured using calibrated digital caliper in mm (millimeter) to assess the developmental stages of OA with time interval in days. Knee diameters were scored as 0.1-2mm as 01, 2.1-4.0 mm was allotted score 02, 4.1-6.0 mm was allotted score 03 and 6.1-8.0mm was allotted score 04.[20]

Glucosamine extraction from eggshells: Eggshells are washed and discarded from its residuals. Eggshell soaked with hot water 80ºC for 15-30 minutes and then cleaned, dried, ground and sieved to equalize the size of the powder.[10,21] Flour turned into chitin through a chemical process to remove organic components. The stages are demineralization (mineral removal) by submersion on 3.5% NaOH solution for 2 hours, temperature at 65ºC and constant stirring (ratio of flour and NaOH is 1:10) and deproteinization (protein removal) by submersion on 1 N HCl for 30 minutes at room temperature (ratio flour and HCl is 1: 5). After each stage, precipitates are dried under the sun for 1 days. Chitin obtained using chemically hydrolyzed by soaking it in a solution of HCl 37% (ratio 9: 1 w/v) for 4 hours at temperature of 90°C. Glucosamine hydrochloride obtained from slurry hydrolyzed with a centrifuge (speed of 10,000 rpm) for 15 minutes. The precipitate that obtained are washed with 100% ethanol (p.a) and centrifuged again with the speed of 10,000 rpm for 15 minutes. The precipitate is dried under the sun for 1 day.[22]

Isolation of omega-3 from goat fats: Waste animal (goat) fats weighed and comparable the size into 1x1x1 cm, then added distilled water as much as 10% of the weight of the fat. Fat was cooked in a vessel to remove the moisture. The temperature was gradually increased to 80˚C to extract the oil from the fat.[23,24] Stired gently for 20 minutes for decreased the temperature. Stewed fat coarse are filtered to separate oil and solids. Crude oil is purified by adding a solution of NaCl 2.5% as much as 50% of the weight of the oil and heated at a temperature of 50°C. Layers of oil and water are separated by a separating funnel. Bentonite is added to the oil layer as much as 1% of the weight of the oil while keep stirring. Settled then filtered to obtain a clean oil. Clean oil is stored in a sealed container and protected from direct contamination of sunlight.[16] Clean oil gained as much as 20% of the amount of fat extracted.[24]

Concentrate omega-3 with high EPA made using low temperature solvent crystallization method (-40°C). NaOH solution was prepared by dissolving 48 g NaOH and 0.5 g Na 2 EDTA in 160 ml of water, then added with 160 ml of ethanol. A total of 100 g of oil mixed with 200 ml of NaOH in distilled water and ethanol, stirred for 30 minutes at 60°C. After saponification, add 40 ml of distilled water. Fatty acids extracted by adding 400 ml of hexane and stirred for 1 hour. The top layer containing soaped material are not discarded. HCl is added to the bottom layer and stirred until a pH of 1. The two layers are formed when the pH reached 1. The lower layer is discarded and the upper layer (hexane layer) was taken and evaporated using a vacuum rotary evaporator until all the solvent 200mBar pressure discharged at a temperature of 40°C, Oil free fatty acids from the extraction of fatty acids dissolved in acetone with oil ratios: acetone 1: 6 (w/v). The mixture is then incubated using dry ice at a temperature of 40°C for 24 hours in the ice box. After 24 hours, the crystals were separated from the solvent by filtering at low temperatures. The oil that has not crystallized are separated from the solvent that has been crystallized. The oil layer and the solvent is evaporated vacuum.[24] Then obtained the concentrate of omega-3 goat fats.

Therapy: Suspension with total volume of 2 ml made by mixing 135 mg of glucosamine and omega-3 goat fats (9/18/ 27 mg) using solvent (CMC-Na). First, 2 ml of distilled water mixed with glucosamine powder, then added to mixture of omega-3 with CMC-Na (1% of volume) slowly. Suspension are given orally once daily for 3 weeks.

Data analysis: Results of therapy were evaluated by hot water tail flick assay, measuring the diameter of knee swelling and behavioral observations.

Results

Pain Response

OA development is measured by the speed of tail response to heat. The slower the response, the greater pain experienced due to swelling of the joints. The results of measurements on the 9th day post-injection of papain on day-1 is described as follows (table 1):

Table 1. The results of pain response measurement to the negative control and 5 random rats positive control

Rat	Pain Response (seconds)	
	Negative Control	Positive Control
1	0	4
2	0	4
3	0	3
4	0	4
5	0	3
Average	0	4
Clinical Score	0	4

From above results showed after 2 days injected by papain, 5 rats (selected randomly) have pain response average of 4 seconds (conclusion of the clinical score is 4). Compared with previous studies on the experimental of clinical assessment rats induced OA by papain in sodium acetate buffer solution without repetition, Highest statistical mean retention time (3.43±.44) was observed on day 21st post papain injection, followed by day 14th (2.80±.59), 28th (2.46±.14), 7th (1.95±.47) and 1st (1.89±.47). Clinical score analyzed by Duncan's multiple range test (DMRT).[20] Comparison shows papain without buffer solution that repeated 2 times were faster to produce OA (within 7 days only).

Meanwhile 5 rats on negative control have 0 seconds (result under 1 seconds assumed as 0 second) and clinical score as 0. It can be hypothesized, the greater pain that the rats felt, the slower reacts toward hot water. After rats were confirmed of OA, positive control divided in to 4 groups which 3 groups given therapies that have different dosage of omega-3 and glucosamine dose remain the same (135 mg/kg of body weight). Therapies were given on day 3 after the last OA induction. The development of therapy was measured once a week on day 8, 15 and 22 days post first therapy on day-1. The measurement results are tabulated below (table 2):

Table 2. The results of pain response measurement on 2 random rats in the negative control, positive control and treatment groups in 3 weeks

Week	Pain Response (seconds)				
	Negative Control	Positive Control	Treatment 1[a]	Treatment 2[b]	Treatment 3[c]
1	0	4	3	4	3
	0	4	4	4	4
2	0	4	2	2	2
	0	4	3	2	3
3	0	3	1	2	1
	0	4	2	2	1

a = Omega-3 dose 9 mg/kg of body weight; b = Omega-3 dose 18 mg/kg of body weight; c = Omega-3 dose 27 mg/kg of body weight

Based on the above table, after 3 weeks of therapy rats on treatment 3 group have more quickly respond to heat. Average time of pain response is 1 seconds with clinical score of 1. Compared with treatment 1 and 2 group that has pain response average of 2 seconds (1,5 seconds assumed as 2 seconds), the pain response of rats in treatment 3 is faster which signify the reduction of OA pain effect. If pain due to OA are reduced, it make tail reflex become more faster to the hot water stimulate. From this results, can be concluded that treatment 3 (dose omega-3 27 mg/kg of body weight) have faster cure to clinical of OA compared to other treatment.

These results strengthen previous studies on the effect of the combination of glucosamine and omega-3 in humans for 26 weeks. Where OA symptoms (stiffness and pain in the hip and knee) in group A that were given the combination therapy is greater decline (from 48.5 to 55.6%) than in group B (41.7 to 55.3%) that were given glucosamine therapy alone.[12] The higher the dose is, the faster the decreasing symptoms of OA.

Diameter of Swelling

Measurement the diameter of knee performed on the same day with the measurement of pain response. The procedure of measurement using caliper is the low jaw of the caliper touches the widest part of the joint then calculated the number results. The result obtained by reduced the left knee diameter (OA) with the right knee diameter (normal). Measurements on the 9th day is described in the following table 3:

Table 3. The results of swelling diameter measurement to the negative control and 5 random rats positive control

Rat	Swelling Diameter (mm)	
	Negative Control	Positive Control
1	0	8,2
2	0	6,2
3	0	7,05
4	0	7,3
5	0	7,4
Average	0	7,23
Clinical Score	0	4

The results of swelling diameter measurement in positive control group is between 6.2 to 8.2 mm with an average of 7.23 mm and clinical score as 4. These results showed two days post last injection, all rats have becomes OA positive. The results are not much different than previous research which the highest clinical scores swelling of OA group on the first day is 6:31 ± 0.54 mm. While the swelling in the days among others the 7th day (2.56 ± 0.28), 14 (2:30 ± 0.09), 21 (1.45 ± 1.01) and 28 (1.14 ± 0, 39), respectively, is not significant.[20] Rats that injected by papain without buffer, has the same swelling result as rats that injected papain with buffer within the same time frame (one week).

Meanwhile rats on negative control don't have differences knee diameter (0 mm) as same as previous study. After that, rats divided into 4 groups like the explanation before. Measurement of the swelling diameter were repeated when rats is given therapies with interval 1 week (the same time with measurement of pain response). Measurement procedure is the same as described above. The following table 4 is the measurement results:

Table 4. The results of swelling diameter measurement on 2 random rats in the negative control, positive control and treatment groups in 3 weeks

Week	Swelling Diameter (mm)				
	Negative Control	Positive Control	Treatment 1[a]	Treatment 2[b]	Treatment 3[c]
1	0	8,35	6,3	6,15	5,35
	0	8,2	7,25	5,4	5,1
2	0	7,25	4,7	2,75	4,3
	0	7,475	3,15	4,1	3,45
3	0	6,35	0,7	1	0,1
	0	7,1	1	1,2	1,05

a = Omega-3 dose 9 mg/kg of body weight; b = Omega-3 dose 18 mg/kg of body weight; c = Omega-3 dose 27 mg/kg of body weight

The hypothesis that can be drawn are treatment 3 decrease swelling diameter larger than other treatment. Where the differences diameter is 0.1 mm (closer to normal) and swelling diameter average is 0,575 mm. The treatment 1 and 2 also has a small difference in knee diameter but the average is still higher. Which average swelling diameter on treatment 1 and 2 is 0.85 mm and 1.175 mm. The conclusion is omega-3 with dose of 27 mg/kg of body weight decrease swelling diameter more faster than omega-3 with dose of 9 and 18 mg/kg of body weight.

Reduced inflammation results in reduced swelling and a reduction of pain. 52.5% of the patients treated with the combination product and 37.9% of the patients treated with glucosamine sulfate alone reported a WOMAC pain score reduction of 80%.[12]

Behaviour Observation

In addition to the above observations, behavior (gait, the walking speed, ability to climb) of all rat groups is also observed. Rats on positive control experienced lameness (some suffer shuffled feet), difficulty to climbing (only capable to standing up for take the food) and the enlargement of the left foot clearly visible. Conditions remain the same after three weeks post last OA induction. In the treatment group, the conditions on the first week of therapy are not much different from the positive control group. In the second week, rats are more active with lameness reducing but still trouble to climbing. In the third week of treatment, rats become more active (capable to climb) compared to the previous week and nearly same condition with the negative control in terms of gait, how to grab the food and the walking speed. The walking speed on treatment groups 1, 2 and 3 are 4, 3 and 2 seconds (respectively) within a distance of 100 cm. Meanwhile in the OA group, the walking speed is 6 seconds with the same distance. Supported by previous study, OA rats has slower walking speed than other groups.[2] The hypotheses that can be taken is macroscopically OA rats that given therapy for 3 weeks has been close to normal.

Third sign observed on previous study was lameness which was based on position of posture and utility of the joint during walk. Again converted to scores for quantification. Highest mean clinical score for lameness was recorded on days 1st, 14th and lowest on 28th (1.40) post papain injection. Clinical signs were countered by the natural immune system of rats but severity was observed around days 21st and 28th.[20]

Discussions

Arthritis is a painful and associated with several inflammations in joints. The exact mechanism of arthritis toward the inflammation and pain is still unknown. Degeneration of bone cartilage and generation of free radicals that leads to severe inflammation and unbearable pain.[24] Some literatures advocated and state that arthritis is contributed due to denaturation of proteins in-vivo. In-vitro screening of protein denaturation and membrane stabilization are considered as a significant index for Anti-arthritic, anti-inflammatory activity.[23] On other study, additional observation is using paw volume and joint edema increased. The acute inflammatory can be known if paw volume and joint edema increased is 160% and 120%, respectively.[26]

Previous study revealed individuals with knee OA that experience pain, stiffness and decreased range of joints motion has limited ability to stand comfortably or climb. Impaired strength and mobility limitation in OA causes alteration in gait system which in turn increase mechanical energy expenditure or moments at the ankle and hip. Weaken of body ability awareness (proprioceptive) toward the knee joint position and movement affects the coordination knee inter-joint, impaired the balance and increased the risk of fall. Impaired proprioceptive sense effected impairment in walking rhythm, shortened distance of step, and decreased walking speed.[2] This study results support the current study that OA rats have lameness, imbalance in gait and slow walking speed. While rats given therapy has faster walking speed along with the higher dose. Moreover, necropsy result found muscle enlargement in OA foot rats that indicated increasing of energy expenditure. The same situation with treatment but not not as thick as the control group.

Conclusions

The results showed that the combination of glucosamine with omega-3 from goat fats can reduce pain response and swelling diameter also return the behavior of OA rats close to normal again with the best treatment is the treatment 3 (omega-3 doses of 27 mg/kg of body weight). While the positive group (OA rats) decreased less significantly compared to the treatment groups. It can be concluded 135 mg / kg of body weight of glucosamine that combine with 27 mg / kg of body weight of omega-3 can reduce pain response and swelling diameter faster within 21 days (3 weeks).

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