The Effect of *Rusa unicolor* Antler Extracts from East Kalimantan in Bone Turnover Cell Models

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ABSTRACT

Objectives: Osteoporosis is a disease described by a skeletal degradation of bone tissue domination to increase risk of fracture. Traditionally, *Rusa unicolor* antler from East Kalimantan is used to treat many kinds of diseases, and one of them is disease related to bone turnover. The present research aims to analyze the effects of 70% ethanol and aqueous extracts of *Rusa unicolor* antler from East Kalimantan on nitric oxide inhibition, osteoblast differentiation, and mineralization related to bone turnover.

Materials and methods: The nitric oxide inhibition of the extracts in LPS-stimulated RAW 264.7 cells were evaluated by Griess reagent while osteoblast differentiations of extracts were evaluated by measuring alkaline phosphatase in p-nitrophenyl phosphate and their mineralization was determined using Alizarin Red Staining method.

Results: The 70% ethanol and aqueous extracts inhibited cells inflammation (40% and 80%) and stimulated osteoblast differentiation (65% and 52%), respectively. In mineralization test, the aqueous extract showed two times higher effect than that of 70% ethanol extract.

Conclusion: The extracts can be considered to successfully reduce expression of inflammatory markers on osteoblasts and maintain osteoblast functions.

Keywords: Antler, *Rusa unicolor*, alkaline phosphatase, mineralization, NO inhibition.

INTRODUCTION

Osteoporosis is a bone disorder described by a skeletal degradation of bone tissue domination to increase risk of fracture and being a silent disease in many complicated situations.¹ It is an important problem of elderly and expected to rise with increased age and life span. At present, 200 million people worldwide are estimated to suffer from osteoporosis.² The latest statistical data from the International Osteoporosis Foundation showed that 1 in 3 women over the age of 50 and 1 in 5 men will suffer osteoporosis fractures for the rest of their lives.³ This problem also occurs in Indonesia which has reached a level of caution because the number of osteoporosis sufferer is far greater than the latest data (>19.7%). The number of elderly people in Indonesia is expected to rise by 14% in the period of 1990-2025 while menopausal women in 2000 contributed to an increase of 15.5 million to 24 million in 2015.⁴

For thousands of years, natural plants and animal resources have played a vital role in the development of pharmaceutical drugs and food supplement for the treatment and prevention of diseases.⁵ One of such animals with high medicinal value is deer antler which belongs to the Cervidae family. It is an efficient traditional medicine for strengthening bones and tendons. Researchers believe that the imbalance between cartilage erosion and regeneration in people with osteoarthritis is caused by a lack of glycosaminoglycans. These substances have an essential in the cartilage structural integrity. Glycosaminoglycan is isolated from four parts of deer antler (*Cervus elaphus*), such as tip, upper, middle and base through cellulose acetate electrophoresis, enzymatic digestion and chromatography methods. Chondroitin sulfate which contains 88% uronic acid is the principal of glycosaminoglycan. Apart from chondroitin sulfate, deer antler also contains hyaluronic acid, keratan sulfate, and dermatan sulfate in little quantities.⁶ Then, some studies also show that deer antler can reduce or even eliminate symptoms associated with osteoarthritis.

For decades, the Chinese people have traditionally used the deer antler extract to strengthen bones, enhance virility, supplement vitality, feed blood, and enhance male and female sexual organs.⁷ A good number of products analogous to deer antler show

great potential effects on diseases associated with infection, immune dysfunction, and aging. However, the bioactive compounds accountable for this mechanism are unclear.⁸⁻¹³ According to some preclinical studies, deer antler products are able to reduce animals osteoporosis.¹⁴⁻¹⁶ Lee et al. (2011)¹⁷ showed facilitation of osteoblast proliferation and mineralization are some of the mechanisms underlying the effects of deer antler products. Furthermore, Choi et al. (2013)¹⁸ reported an inhibition of osteoclast differentiation by deer antlers. Protein, ash, lipids, collagen, and calcium are some of the chemicals contained in antler.¹⁹ Proteoglycan, testosterone, cholesterol, estradiol, glutamic acid, insulin-like growth factor 1 (IGF-1), iron, aspartic acid, and glycine are also contained in it.²⁰

In this study, we used 70% ethanol and aqueous extract of *Rusa unicolor* antler from East Kalimantan, Indonesia, to evaluate the effect of osteoblast differentiation, mineralization and expression of inflammatory markers by measuring alkaline phosphatase (ALP), Alizarin Red Staining (ARS) and nitric oxide (NO) inhibition values, respectively.

MATERIALS AND METHODS

Cell Culture and Reagents

Reagent chemicals such as Alkaline Phosphatase Colorimetric Assay Kit and Acid Phosphatase Leukocyte Kit were obtained from Sigma-Aldrich Co. (St Louis, MO, USA). All cell culture materials and solvents were bought from Thermo Fisher Scientific (Waltham, MA, USA) and analytical grade (J.T. Baker, USA). Macrophages (RAW 264.7) and mouse osteoblast-like cells (7F2) were purchased from Food Industry Research and Development Institute, Taiwan, and refined in Dulbecco's Modified Eagle's Medium (DMEM). They were further strengthened with 10% v/v Fetal Bovine Serum (FBS), 100 µg/mL streptomycin, and 100 units/mL penicillin. Cells were refined in a dabbled incubator with 5% CO₂ at 37°C.

Antler materials

Deer antler of *Rusa unicolor* was collected in middle of March 2017 in UPTD (Technical Implementation Service Unit) of East Kalimantan, Indonesia and voucher specimens were deposited at the UPTD of East Kalimantan, Indonesia.

Extraction of *Rusa unicolor* Antlers

Rusa unicolor antler was received from UPTD of East Kalimantan, Indonesia. It was powdered (991 g) and extracted with 70% ethanol-water (2.0 L x 3) using maceration method. The 70% ethanol solution was concentrated using rotary evaporator to get 70% ethanol extract (Et-TL, 35.0 g). In addition, the deer antler (430 g) was extracted with 100% water (1.0 L x 3) by applying continuous percolation method. The water solution was dried by freeze dried to get aqueous extract (A-TL, 6.1 g).

Cell Viability Assay

The RAW 264.7 cells were plated for cell growth studies at a density of 5×10^4 cells/well in 96-well plates. DMEM medium consisting of 100 units/mL penicillin, 10% FBS and 100 µg/mL streptomycin was used to restore the cell. After 24 hours, Et-TL and A-TL extracts of *Rusa unicolor* antler were incubated at various concentrations (10, 50, and 100 µg/mL) at 37°C for another 24 hours. The cell supernatants were subsequently extracted, after 200 µL 3-(4,5-dimethylthiazol-2-yl)- and 100 µL 2,5-Diphenyltetrazolium Bromide (MTT) reagent (100 µg/mL) were incubated for 4 hours. Similarly, to dissolve the formazan crystals, 100 µL dimethyl sulfoxide (DMSO) was added. The absorbance was ruminated at 570 nm using an enzyme-linked immunosorbent assay (ELISA) reader. All experiments were carried out in triplicate, with the relative cell viability (%) declared as a portion relative to the unprocessed control cells. The same method was carried out on 7F2 osteoblast-like cells (10^4 cells/well) in DMEM containing 10% FBS, 100 units/mL penicillin, and 100 µg/mL streptomycin.²¹

Nitrite Measurement

The 24-well plates were used to seed the RAW 264.7 cells (5×10^5 cells/well) with DMEM containing 100 unit/mL penicillin, 10% FBS, and 100 µg/mL streptomycin at 37°C with a 24 hours incubation duration. The cells were aroused with 500 ng/mL lipopolysaccharide (LPS) and examined with Et-TL and A-TL extracts at 10–100

µg/mL for 1 day. The emitted NO was estimated by weighing the nitrite concentration. The 100 µL of samples (Et-TL and A-TL extracts) were added with 100 µL of Griess reagent in 96-well plates for 15 minutes at room temperature. ELISA reader was used to deliberate the absorbance at 550 nm. Furthermore, standard calibration curves were processed using sodium nitrite to evaluate nitrite expression from cells with sensitivity and linear range values of 2.5 µM and 20-100 µM, respectively.²¹

Differentiation of Cellular Alkaline Phosphatase Activity (ALP)

The 7F2 osteoblast-like cells were cultured in 24-well plates at a density of 10^4 in DMEM containing 5 mM β-glycerol phosphate (β-GP), 10% FBS and 50 µg/mL ascorbic acid with or without 10–100 µg/mL Et-TL and A-TL extracts with a 4 days incubation period at 37°C in a 5% CO₂ atmosphere. Phosphate buffer saline (PBS) was used to wash the extracted supernatants. After that, a percentage of v/v triton solution was inserted and incubated at 37°C for 10 minutes. After the incubation, the cell lysates were examined for ALP by adding 200 µL of p-nitrophenyl phosphate (PNPP) and di-ethanolamine buffer into each well for a period of 30 minutes and at room temperature. The 50 µL/well of stop solution was inserted to end the reaction while ELISA reader at 405 nm was used to evaluate the absorbance.²¹

Mineralization of Extracellular Matrix

One mL of DMEM comprising of 10% FBS, 5 mM β-GP and 50 µg/mL ascorbic acid (2GF medium) was used to seed a portion of the osteoblast-like cells at a density of 10^4 cells/well in an incubator for 7 days. The Et-TL and A-TL extracts were also added to the growth medium at the concentration of 10 – 100 µg/mL. PBS and 75% v/v ethanol were used to wash the samples and then dried at room temperature. The cells were stained with 1% ARS (200 µL) for one hour. A microscope (Nikon TI-E) and SPOT RT3 camera were used to note the images of the cell morphology. Each well received 400 µL of 10% w/v cetylpyridinium chloride and shaken for 10 minutes in order to dissolve the calcium. The absorbance was finally evaluated using an ELISA reader at 560 nm.²¹

Statistical Analysis

The experiments were carried out for three more consecutive times using similar results. It then presented as means $\pm$ standard deviations. Paired t-test was used to illustrate data analysis. The differences proved to be statistically significant at $P < 0.05$.

RESULTS

The Effect of *Rusa unicolor* Antler Extract on Cell Viability and NO Production

In this research, we analyzed the effects of Et-TL and A-TL extracts toward anti-inflammatory related to bone turnover. Several researches have reported the pharmacological effects of deer bones and antlers from several countries, but there have been no reports on deer antlers from Indonesia, which is *Rusa unicolor*.^{7,22-24}

In vitro cytotoxic samples were examined using MTT test in RAW 264.7 cells. Samples (Et-TL and A-TL extracts) with different concentrations were used to incubate cells for 24 hours and were examined using MTT method. After 24 hours, the data were represented as the mean cell viability. The A-TL extract did not show cytotoxicity compare to Et-TL extracts (Fig. 1). The cell viability significantly decreased in Et-TL extracts (50 and 100 $\mu\text{g/mL}$). The result showed that the aqueous extract was not toxic toward the RAW 264.7 cells at the concentration range of 10–100 $\mu\text{g/mL}$. It could also decrease cytotoxicity and increase absorption of cellular uptake of compounds in site. The anti-inflammatory activity of samples in LPS-stimulated RAW 264.7 cells was conducted by observing the NO inhibition. The Griess reagent was used to evaluate the nitrite value that was directly related to the amount of NO production using RAW 264.7 cells. These cells were incubated with samples, after which the NO produced in the LPS-stimulated RAW 264.7 cells deteriorated significantly (Fig. 2). The Et-TL extract stopped NO production until it was at 40% in 10 $\mu\text{g/mL}$. Similarly, A-TL extract inhibited until 80% in the same concentration. LPS was not used on the cells of the control group. The total nitrite was manufactured by LPS group (500 ng/mL). This group had a value of 100%.

The Effect on ALP Stimulation of 7F2 Osteoblasts of 70% Ethanol and Aqueous Extracts of Deer Antler

The effect of samples (Et-TL and A-TL extracts) in 7F2 osteoblastic cell line proliferation was carried out using MTT test. The viability cells in Et-TL extract

decreased significantly (50 and 100 µg/mL), but the value was more than 100%. It showed that high concentration of Et-TL extract was not toxic even though the cell proliferation value decreased. Whereas, cells viability of A-TL extract increased significantly (Fig. 3). As a result, all deer antler extracts decreased cytotoxicity and raised cellular uptake. Then, the ALP and mineralization experiments continued.

The ALP stimulation of 7F2 osteoblast cells using Et-TL and A-TL extracts of deer antler was incubated for 4 days. The effects of test samples on the ALP assay increased in the 7F2 osteoblasts opposed to the 2GF group. After 4 days, the A-TL extract stimulated their ALP activity to 65% while the Et-TL was stimulated to 52% (Fig. 4).

The Effect on Mineralization of 7F2 Osteoblasts of 70% Ethanol and Aqueous Extracts of Deer Antler

The 7F2 osteoblast cells were cultured in 2GF medium to prompt osteoblast differentiation and mineralization. After 7 days of incubation, the effects of Et-TL and A-TL extracts of deer antler on osteoblast mineralization were examined using ARS, which determined the calcium content in the bone matrix. The Et-TL extract displayed the stimulation on cell differentiation (Fig. 4) and after 7 days, this extract stimulated the mineralization. The A-TL extract displayed the best stimulation mineralization than 70% ethanol extract of deer antler (Fig. 5).

The stains on ARS represented mineral deposits and quantified by cetylpyridinium chloride extraction method. The treated 7F2 osteoblast cells increased the ARS accumulation compared to the 2GF group. Photographs were used to represent mineralization process of osteoblast cells under bright field. Several mineralized nodules formation (red) was high in 7F2 osteoblast cells treated with Et-TL and A-TL extracts on the 7th day under microscope observation (Fig. 6). Identical patterns were also observed in ALP activity.

DISCUSSION

Bone remodeling is a process created between bone resorption (osteoclast activity) and formation (osteoblast activity). Osteoclasts are multinucleated cells produced from macrophage precursor cells and osteoclast formation that requires RANKL as receptor

activator. The RAW 264.7 macrophages have been shown to play an important role in osteoclast formation and function.²⁵ Osteoclast function can be impaired due to degeneration of articular cartilage and synovial inflammation involving chemokines, interleukin (IL)-1 β , IL-6, IL-11, IL-17, tumor necrosis factor- α (TNF- α), and a number of inflammatory cytokines. They produce bone loss by reducing osteoprotegerin (OPG) production and stimulating receptor activator of nuclear factor kappa-B ligand (RANKL) expression in stromal cells and osteoblasts. The NO inhibition of RAW 264.7 macrophages showed a decrease in cytokine inflammation thus preventing bone loss by increasing the amount of OPG and RANKL.²¹

The NO inhibition through inhibition of the JNK, p38, and NF- κ B signaling pathways is associated with inflammatory response inhibition and osteoclastogenesis suppression. Yeh et al.²¹ showed that Cur liposomes, as sample, inhibited NO production in RAW 264.7 macrophages and prevented osteoclast differentiation by decreasing cathepsin K regulation, tartrate-resistant acid phosphatase (TRAP) expression and increasing OPG/RANKL ratio. The high ratio of OPG/RANKL and NO inhibition caused a decrease in osteoclast activation and an increase in the number of osteoblasts, so the osteoporosis effect can be reduced.

In this study, we evaluated NO production of 70% ethanol and aqueous extracts in LPS-stimulated RAW 264.7 macrophages. LPS markedly stimulated NO production in these macrophages compared to its effects in control group. The 70% ethanol extract of deer antler showed higher reaction rate in lowering LPS-stimulated RAW 264.7 than that of aqueous extract (Fig.2). Both of them did not have any cytotoxic effect on RAW 264.7 macrophages (Fig.1). According to Choi et al. (2016),²⁶ the ability of deer bone methanol fraction in reducing NO production was a result of decreased regulation and expression mRNA from pro-inflammatory mediators, such as cyclooxygenase (COX-2), IL-12 β , and IL-1 β . The methanol fraction containing sugar played an essential role in lowering inflammatory responses by controlling pro-inflammatory cytokines and mediators. Exorbitant production of pro-inflammatory cytokines has been discovered in variety of diseases, such as cancer, arthritis, rheumatoid arthritis, osteoarthritis and osteoporosis.^{27,28}

The osteoblast phenotype for bone mineralization is obtained in two stages. In the first stage, the mature matrix and specific protein associated with the bone cell phenotype (ALP) are detected. After that, the matrix becomes mineralized by calcium deposition in the second stage. Then, a sponge bone layer is formed around the original cartilage

and the space between the sponge bones is filled with a bone matrix and becomes a compact bone.

Antler increased the proliferation of osteoblasts and bone matrix proteins, such as collagen type I and bone sialoprotein (BSP). These increases triggered osteoblast differentiation, such as mineralized nodule formation. As expected, the 70% ethanol and aqueous extracts of deer antler increased ALP activity constantly (Fig.4). The cells cultured in the medium containing aqueous extract had higher ALP activity than the 70% ethanol extract which is similar to the results obtained by Lee et al. (2011).¹⁷ Deer antler increased osteoblasts proliferation significantly up to 119% of the basal value. Significant increases in mRNA expression and ALP activity were found at 50-100 µg/mL. It also increased the expression levels of type I collagen mRNA and mineralization to be more than 183%.¹⁷

Interestingly, ALP has been proven as an important enzyme in the mineralization process. Human inherited bone abnormalities and hypophosphaphia, can occur due to the absence of expression of ALP bone form^{29,30} and was characterized by the absence of mineral deposition in bone (rickets). ALP is important for mineralization but its role has not been fully clarified. We continued to evaluate the effect of 70% ethanol and aqueous extracts of deer antler in mineralization activity (Fig.5).

Bone formation implicated the proliferation, differentiation, and mineralization of osteoblasts. The 7F2 osteoblast cells started differentiating at the 4th day. The ALP activity is displayed as a marker of the initial period of cell differentiation. Then, the mineralization of 7F2 osteoblast cells produced by ARS was monitored. Figure 6 showed that the extracts stimulated the mineralization of 7F2 osteoblast cells. The stimulation of aqueous extract at 100 µg/mL was two times higher than that of 70% ethanol extract. This difference may be as a result of reduction of deer antler extracts toxicity or chemical constituents inside both extracts.

CONCLUSION

Both of 70% ethanol and aqueous extracts of *Rusa unicolor* antler from Kalimantan had a role in bone remodeling. The aqueous extract stimulated higher bone differentiation and mineralization than the 70% ethanol extract with ALP value of 65% and twice ARS value. However, the 70% ethanol extract showed higher NO inhibition

activity than aqueous extract with the inhibition value of 40%. Therefore, the extracts can be considered to successfully reduce expression of inflammatory markers on osteoblasts and maintain osteoblast functions.

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Conflict of Interest: No conflict of interest was declared by the authors.

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Figure 1. The effects of 70% ethanol extract (Et-TL) and aqueous extract (A-TL) of deer antler from East Kalimantan at 10, 50, and 100 µg/mL on RAW 264.7 cell viability; C was the medium without sample. Data are expressed as the mean of percent cell viability compared to control after exposure for 24 hours $\pm$ standard deviation.

Figure 2. The effects of 70% ethanol extract (Et-TL) and aqueous extract (A-TL) of deer antler from East Kalimantan at 10 µg/mL on NO production in LPS (500 ng/mL)-induced RAW 264.7 macrophages for 24 hours. C-LPS: control of LPS, C: cells of the control group were not induced with LPS. The total nitrite produced by the cells of the LPS group is expressed as 100%. Results are expressed as percentage with mean $\pm$ standard deviation. *P<0.05 with respect to control.

Figure 3. The effects of 70% ethanol extract (Et-TL) and aqueous extract (A-TL) of deer antler from East Kalimantan at 10, 50, and 100 µg/mL on 7F2 cell viability; C was medium without sample. Data are expressed as the mean of percent cell viability compared to control after exposure for 24 hours ± standard deviation.

Figure 4. The effects of 70% ethanol extract (Et-TL) and aqueous extract (A-TL) of deer antler from East Kalimantan at 10, 50, and 100 µg/mL on osteoblast differentiation (ALP) during 4 days incubations. The 7F2 osteoblast cells were cultured in 2GF (50 µg/mL ascorbic acid and 5 mM β-GP) medium to induce osteoblast differentiation. Results are expressed as percentage with mean ± standard deviation. *P<0.05 with respect to 2GF.

Figure 5. The effects of 70% ethanol extract (Et-TL) and aqueous extract (A-TL) of deer antler from East Kalimantan at 10, 50 and 100 $\mu\text{g/mL}$ on quantification mineralization assay during 7 days incubations. ARS stain was quantified by cetylpyridinium chloride extraction method. Results are expressed as percentage with mean $\pm$ standard deviation. * $P < 0.05$ with respect to 2GF.

Figure 6. Histochemical staining of mineral deposition of 70% ethanol extract (Et-TL) and aqueous extract (A-TL) of deer antler from East Kalimantan at 10, 50, and 100 $\mu\text{g/mL}$. They were visualized using ARS staining ($\times 100$ magnification). Red staining represents mineral deposition.