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Original Article

DIHYDROTESTOSTERONE DOWNREGULATES BONE RESORPTION ACTIVITY OF OSTEOCLASTS IN DOSE DEPENDENT MANNER: AN *IN VITRO* MODEL USING RAW 264.7 CELLS

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ABSTRACT

Objective: Numerous studies have evidenced the bone regulatory potential of dihydrotestosterone in androgen-deficient osteoporosis. The present study was thus aimed to explore the translational mechanism of dihydrotestosterone to down-regulate the bone resorption activity of osteoclasts using RAW 264.7 cells as *in vitro* model.

Methods: Prior to analyze the efficacy of dihydrotestosterone (5α-DHT) to alleviate osteoclastic differentiation, their cell viability and cell proliferative ability was assessed using lactate dehydrogenase (LDH) and MTS assays. The osteoclastic differentiation capacity of dihydrotestosterone was evaluated by measuring TRAP activity and the expression of bone resorption-related proteins such as matrix metallopeptidase-9 (MMP-9), cathepsin-K, tartrate-resistant acid phosphatase (TRAP) and NFATc1. Moreover, the effects of dihydrotestosterone were also evaluated on superoxide (free radicals) generation and superoxide dismutase (SOD) activity in RANKL-induced osteoclasts.

Results: Dihydrotestosterone showed no toxicity towards RAW 264.7 cells and significantly enhanced their proliferation and growth rates in a dose-dependent fashion. It was also observed that dihydrotestosterone exhibits a remarkable inhibitory effect on differentiation, maturation and activation of osteoclasts. The marked inhibition of differentiation and activation of osteoclasts caused by 5α-DHT was due to down-regulation of the expression of MMP-9, cathepsin-K, TRAP, NFATc1, generation of superoxide and up-regulation of SOD activity in the RAW 264.7 cells.

Conclusion: Resulting data provided substantially *in vitro* evidence for the pronounced anti-osteoclastogenetic activity of dihydrotestosterone and its therapeutic value in treating osteoporosis and other bone-erosive disorders.

Keywords: Androgen-deficient osteoporosis, Dihydrotestosterone, RAW 264.7 cells, Bone resorption, Anti-osteoclastogenesis

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INTRODUCTION

Bone metabolism is a physiologic process that maintains homeostasis between the bone resorption (the activity of osteoclasts) and formation (the activity of osteoblasts). Any deregulation in the homeostasis of this process may lead to bone diseases [1-3] including osteoporosis [4]. Osteoclasts are activated for many reasons, one of which is the imbalance of hormones caused by the menopause which may lead to various biochemical alternations in biochemical parameters of bone [5]. The absence of estrogen, induced by the menopause, increases the formation and the activity of osteoclasts which play key roles in bone loss and osteoclasts ultimately increase the risk of menopausal osteoporosis [6]. Therefore, inhibition of osteoclast formation, maturation and activation is an important therapeutic strategy.

Osteoclasts are multinucleated cells generated from monocyte/macrophage precursor cells, and osteoclast formation requires receptor activator of nuclear factor-κB (NF-κB) ligand (RANKL). The receptor activator of NF-κB (RANK) is expressed on RAW 264.7 cell surfaces and conjugates with RANKL, which is essential for osteoclastogenesis [7]. RAW 264.7 cells have been well-recognized as osteoclast precursors. RAW 264.7 cells respond to RANKL stimulation *in vitro* to exhibit all characteristic features of fully-differentiated matured osteoclast [8–10]. Upon activation, RAW 264.7 cells stimulate activation of an important transcription factor, nuclear factor of activated T-cells cytoplasmic 1 (NFATc1), for osteoclastogenesis [11]. As a master transcription factor of osteoclastogenesis, NFATc1 regulates diverse osteoclastogenesis-related proteins such as TRAP, cathepsin-K and MMP-9 [12–14].

Numerous studies have evidenced the substantial roles of androgens (dihydrotestosterone) in the regulation of skeletal homeostasis in humans [15, 16] and rodents [17–20]. According to a recently published review [21], testosterone imparts promising roles in improving the bone health by enhancing the proliferation and differentiation of osteoblasts and down-regulating the maturation of osteoclasts. Despite of the numerous studies demonstrating the roles of dihydrotestosterone in regulating bone health [22, 23], the molecular and translational mechanism by which dihydrotestosterone regulates bone milieu and maintain homeostasis between bone formation and bone resorption is yet to be explored.

Thus, the present study was aimed to evident the potential effects of dihydrotestosterone on the proliferation, differentiation and maturation of osteoclasts *in vitro*. The cell proliferation activity of dihydrotestosterone was assessed using MTS assay. The cells differentiation ability was evaluated in terms of TRAP activity and the expression of specific osteoclast-related proteins including MMP-9, cathepsin-K, TRAP and NFATc1 *in vitro*. Moreover, the effects of dihydrotestosterone on the RANKL-induced generation of superoxide (free radicals) and SOD activity were also studied.

MATERIALS AND METHODS

Materials

RAW 264.7 cells were purchased from the American type culture collection (ATCC) cell bank (Manassas, VA, USA) and were used as *in vitro* model. Cell culture reagents Dulbecco modified eagles medium (DMEM), penicillin, streptomycin and foetal bovine serum (FBS) were sourced from Gibco laboratories (Grand Island, NY, USA). RANKL was purchased from Oriental Yeast Co., Ltd. (Tokyo, Japan). MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H tetrazolium) assay kit was purchased from Sigma-Aldrich, USA. Lactate dehydrogenase (LDH) cytotoxicity

detection kit was purchased from Roche Diagnostics, Switzerland. 5α-dihydrotestosterone (5α-DHT) was purchased from Abcam, USA. ELISA kits for the expression of MMP-9, TRAP, cathepsin-K and NFATc1 were purchased from Elabscience Biotechnology Co., Ltd. (Wuhan, China). All other chemicals and reagents were sourced from pharmacology and cell culture laboratories of Faculty of Pharmacy, Universiti Teknologi MARA (UiTM), Malaysia.

Treatment

Prior to drug treatment, a stock solution (1 ng/ml) of 5α-DHT was prepared using either DMEM or differentiation media. Different concentrations (0.1, 0.01 and 0.001 ng/ml) of 5α-DHT were then prepared from the stock solution and were sterilized using 0.2 µm syringe filter (Sartorius, Germany). RAW 264.7 cells were then treated with different concentrations of 5α-DHT and culture media was replaced every other day throughout the experimental period.

Cell culture and sub-culture

RAW 264.7 cells were used as the osteoclast precursor cells. Cell culturing and sub-culturing were performed by growing active RAW 264.7 cells in a growth media consisting of DMEM supplemented with 10% heat-inactivated FCS and 1% penicillin/streptomycin (Antibiotic/Antimycotic). Cells were then incubated in a humidified chamber (95% air and 5% CO_2) at 37 °C until they reached 80% confluence. The adhered cells were then released from the flask using cell scrapper and the removed cells were counted using a hemocytometer. For analyzing osteoclastic differentiation, cells were sparsely seeded at a density of 1×10^4cells/well in 24-well plate and were cultured under the same incubation circumstances. Cells were cultured for 24 h to obtain monolayers containing DMEM with 10% FCS to promote cell survival, division and metabolism. The cells were subsequently treated with or without 5α-DHT at different concentrations (0.1, 0.01 and 0.001 ng/ml). These cultures were fed every other day by replacing the differentiation media with an identical volume of fresh media.

Cytotoxicity assay

LDH assay is a widely accepted measure of cellular toxicity. Changes in cellular health status are determined by use of indirect measures related to the formation of a colored tetrazolium dye product that can be measured spectrophotometrically at 490 nm. An increase in the absorbance is indicative of an elevated release of cellular LDH, consequent to loss of cell membrane viability: a positive marker of toxicity (LDH assay).

Briefly, RAW 264.7 cells were seeded at a density of 1×10^4 cells/well in 24-well plate and cultured for 24 h. After the cell adherence, culture media was changed and different doses of 5α-DHT (0.1, 0.01 and 0.001 ng/ml) were added. Cells were cultured for additional 24 h and culture media was collected to test cytotoxicity.

LDH cytotoxicity assay was performed according to the manufacturer's protocol. This colourimetric assay quantifies the LDH released from the cytosol of damaged cells into the supernatant and thus serves as an index of cell death. Results were presented as optical density values (O. D) and percentage of cell viability of test groups relative to the control cells (100% of cell viability) and of cells treated with 1% Triton X-100 (0% cell viability) using the following equation.

$$\text{Cell viability (\%)} = \frac{\text{5α DHT treated cells} - \text{control cells}}{\text{Triton treated cells} - \text{control cells}} \times 100$$

Cell proliferation assay

Cell proliferation ability of 5α-DHT was performed by using colorimetric [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxy-methoxy-phenyl)-2-(4-sulfophenyl)-2H-tetrazolium] (MTS) assay. This colourimetric assay is based on the metabolic reduction of the MTS tetrazolium by the living cells to a formazan product. The absorbance of the formazan product was quantified at the wavelength of 490 nm. Briefly, RAW 264.7 cells were seeded at a density of 1×10^4 cells/well in 24-well culture plates with six duplicate wells per treatment for cell proliferation assay and maintained in growth media for 24 h at 5% CO_2 at 37 °C. After 24h incubation, media was removed and the cells were treated with fresh growth media containing different concentrations (0.1, 0.01 and 0.001 ng/ml) of 5α-DHT and were cultured for additional 24 h. Normal growth medium (without 5α-DHT) was used as untreated (normal control). After 24 h of incubation, 20 µl of diluted MTS solution was added to each well and cells were further incubated at 37 °C in the dark after being covered with aluminium foil for additional 2 h. The absorbance of each well was recorded using microplate reader.

Cell differentiation and tested groups

RAW 264.7 cells differentiate into mature osteoclasts after stimulating with RANKL. For that, RAW 264.7 cells were cultured in DMEM with 10% FBS and 1% penicillin/streptomycin and seeded in 24 wells culture plates at a density of 1×10^4 cells/well and placed in the CO_2incubator overnight to allow the cells to adhere to the well surfaces. After 24 h, the culture media was replaced with osteoclastic differentiation media and five groups were made: (1) first group was untreated (normal control) containing RAW 264.7 cells cultured with normal growth media, (2) Second group was RANKL-treated cells (RANKL induced control) in which RAW 264.7 cells were incubated with differentiation media containing 50 ng/ml RANKL, (3) third group was RANKL-induced RAW 264.7 cells that were treated with 0.1 ng/ml of 5α-DHT, (4) fourth group was RANKL-induced RAW 264.7 cells that were treated with 0.01 ng/ml of 5α-DHT, (5) fifth group was RANKL-induced RAW 264.7 cells that were treated with 0.001 ng/ml of 5α-DHT.

TRAP activity

To analyze the extent of osteoclastic differentiation, RAW 264.7 cells were cultured in 24-well cell culture plates at a density of 1×10^4 cells/well and induced with differentiation medium after being treated with 5α-DHT at different concentrations (0.1, 0.01 and 0.001 ng/ml) or left untreated (normal control) for 5 d. The medium was changed every other day. Afterward, the medium was removed and the cell monolayer was gently washed twice using ice-cold PBS. The cells were fixed in 3.5% formaldehyde for 10 min and ethanol/acetone (1:1) for 1 min. Subsequently, the dried cells were incubated in a 50-mM citrate buffer (pH 4.5) containing 10 mmol sodium tartrate and 6 mmol of *p*-nitrophenyl phosphate (PNPP). After 1 h of incubation, the reaction mixtures were transferred to new well plates containing an equal volume of 0.1 N NaOH. Absorbance was measured at 405 nm using an enzyme-linked immunoassay reader and TRAP activity was expressed as optical density compared to the controls.

Expression of protein markers

In this experiment, the potential biological influence of 5α-DHT on the expression and regulation of specific RANKL-induced osteoclast-functional protein biomarkers such as MMP-9, TRAP, cathepsin-K and NFATc1 was investigated. These proteins are major phenotypic markers for osteoclast differentiation during bone resorption.

The expression of the above bone-resorption protein markers was measured using sandwich ELISA method. In this technique, the culture plates were pre-coated with antibodies specific to the protein marker. These pre-coated antibodies specifically bind to their protein markers in the sample. The biotinylated detection antibody specific for each protein markers were added to sandwich the bound protein markers and were detected as changes in color. The optical density (OD) values were recorded at 450 nm and the intensity of the color is directly proportional to expression concentration of each protein marker in the sample. The sensitivity of detection for MMP-9, TRAP, cathepsin-K and NFATc1 were 18.75, 78.5, 10, and 1.0 pg/ml, respectively.

Superoxide production

Superoxide concentration was measured using a green chemiluminescence CD kit according to the manufacturer's instructions. The green chemiluminescent CD is a highly sensitive chemiluminescence probe that reacts with the superoxide anion and a luminescence dye specific for the detection of superoxide. Briefly, RAW 264.7 cells were cultured in 24-well cell culture plates at a

density of 1 × 10^4 cells/well and were left untreated or treated with different concentrations of 5α-DHT (0.1, 0.01 and 0.001 ng/ml). The contents of the kit were dissolved in hot methanol: water (1:1 v/v) containing 0.1% (w/v) trifluoroacetic acid. To measure superoxide generated into the media, 200 µl of the media was mixed with 150 µl of the reagent. The luminescence intensity of each sample was measured using a luminescence plate reader. The value for each treated group was converted to a percentage of the control luminescence.

SOD activity

SOD activity expressed in each cell culture media was measured using an SOD assay kit-WST according to the manufacturer's instructions (Dojindo Molecular Technologies, Inc., Kumamoto, Japan). Briefly, RAW cells were seeded in a 24-well plate at a density of 1 × 10^4 cells/well and were left untreated or treated with different concentrations of 5α-DHT (0.1, 0.01 and 0.001 ng/ml). The SOD activity was measured by mixing the reagents from 220 µl of the WST kit with 20 µl of the culture medium. After 20 min incubation at 37 ˚C, absorbance was measured at 450 nm using a microplate reader (Bio-Rad Model 680; Bio-Rad, Hercules, CA, USA). The value for each treated group was converted to a percentage of the value obtained for the control group.

Statistical analysis

Data were analyzed using one-way analysis of variance (one-way ANOVA) followed by Student's t-test using SPSS version 21.0. All the experiments were performed independently three times with quadruplicate sampling (n=12). Data were presented as mean±standard deviation (SD). A significant difference was recognized by$^{\#\#}$P<0.01 represent a significant difference between normal control and RANKL induced control group and ** P<0.01 and *P<0.05 represents significant differences between treated (5α-DHT) and RANKL induced control groups.

RESULTS

Effect of 5α-DHT on cell viability and cytotoxicity of RAW 264.7 cells

The cytotoxicity of 5α-DHT on RAW 264.7 cells was investigated by measuring cell viability using LDH assay. RAW 264.7 cells were incubated with different concentrations of 5α-DHT and their cell viability was measured on days 1 and 5 compared to the control groups (normal control and RANKL induced control) (table 1). Results demonstrated no cytotoxicity of 5α-DHT at different concentrations (0.1, 0.01 and 0.001 ng/ml) in RAW 264.7 cells with or without RANKL-induction on days 1 and 5 (table 1). The viability of cells at all the tested concentrations was>100%.

Table 1: Effect of 5α-DHT on RAW 264.7 cells viability. RAW cells were treated with different concentrations of 5α-DHT (0.1, 0.01, and 0.001 ng/ml) for different time durations (days 1 and 5) with or without stimulating by RANKL. Data are representative of results from three independent experiments in quadruplicate sampling (n=12). Data are expressed as percentages of the value of the control cells (mean±Standard deviation)

Test groups	Normal control		DHT–0.1 (0.1 ng/ml)			DHT–0.01 (0.01 ng/ml)			DHT–0.001 (0.001 ng/ml)		
Time duration	Day-1	Day-5	Day-1	Day-5 (–RANKL)	Day-5 (+RANKL)	Day-1	Day-5 (–RANKL)	Day-5 (+RANKL)	Day-1	Day-5 (–RANKL)	Day-5 (+RANKL)
O. D values (490 nm)	0.23±0.05	0.28±0.03	0.25±0.02	0.34±0.05	0.32±0.03	0.24±0.02	0.30±0.06	0.31±0.03	0.24±0.05	0.31±0.07	0.30±0.06
% cell viability	100±6	100±7	105±9	112±5	106±6	102±8	104±4	105±8	101±7	105±6	104±4

Effect of 5α-DHT on proliferation of RAW 264.7 cells

To evaluate the effect of different doses of 5α-DHT on the growth rate of RAW 264.7 cells, cell proliferation assay was performed. Briefly, RAW 264.7 cells were subjected different concentrations of 5α-DHT (0.1, 0.01 and 0.001 ng/ml) and their cell growth rate was measured using MTS assay and results were compared to the control group (table 2). A significant increase in cell growth was observed in RAW 264.7 cells treated with different concentrations of 5α-DHT particularly 0.1 and 0.01 ng/ml, on day-1 (table 2) compared to the untreated (normal control) group. The cell growth was further increased when the duration of incubation was increased (day-5). The increase in cell growth was dose-dependent and the highest cell growth was observed in RAW 264.7 cells treated with 0.1 ng/ml concentration. These results indicated that 5α-DHT is safe and significantly (*P<0.05) enhance cells proliferation in RAW 264.7 cells (table 2).

Table 2: Effect of 5α-DHT on RAW 264.7 cells proliferation. RAW cells were treated with different concentrations of 5α-DHT (0.1, 0.01, and 0.001 ng/ml) for different time durations (days 1 and 5) with or without stimulating by RANKL. Data are representative of results from three independent experiments in quadruplicate sampling (n=12). Data are expressed as percentages of the value of the control cells (mean±standard deviation)

Test groups	Normal control		DHT–0.1 (0.1 ng/ml)			DHT–0.01 (0.01 ng/ml)			DHT–0.001 (0.001 ng/ml)		
Time duration	Day-1	Day-5	Day-1	Day-5 (–RANKL)	Day-5 (+RANKL)	Day-1	Day-5 (–RANKL)	Day-5 (+RANKL)	Day-1	Day-5 (–RANKL)	Day-5 (+RANKL)
O. D values (490 nm)	0.27±0.08	0.34±0.06	0.36±0.11	0.48±0.15	0.46±0.13	0.34±0.07	0.44±0.09	0.43±0.12	0.31±0.09	0.41±0.07	0.39±0.09
% cell proliferation	100±8	100±6	110±5	121±9	119±6	108±3	117±6	115±7	105±6	112±8	110±5

Effect of 5α-DHT on RANKL-induced differentiation of RAW 264.7 cells

RAW 264.7 cells were exposed to RANKL (50 ng/ml) to fully differentiate them into the mature multinucleated TRAP-positive cells to further examine the effect of 5α-DHT on osteoclastogenesis.

The RANKL-induced differentiation potential of RAW 264.7 cells was evaluated by measuring TRAP activity of different cell cultures treated with different concentrations of 5α-DHT compared to the control groups (normal and RANKL induced controls) (fig. 1). Results showed that TRAP activity was negligibly low in all the tested groups on day-1;

however, it was enormously high at day-5. At day-5, a significantly (##P<0.05) higher TRAP activity was observed in the RANKL-induced control group compared to the normal control group (fig. 1). However, in RAW 264.7 cells treated with 5α-DHT, a dose-dependent significant decrease in TRAP activity was observed compared to the RANKL induced control group. Comparative analysis revealed that the decrease of TRAP activity was more obvious in RAW 264.7 cells treated with 0.1 ng/ml (**P<0.05) (fig. 1).

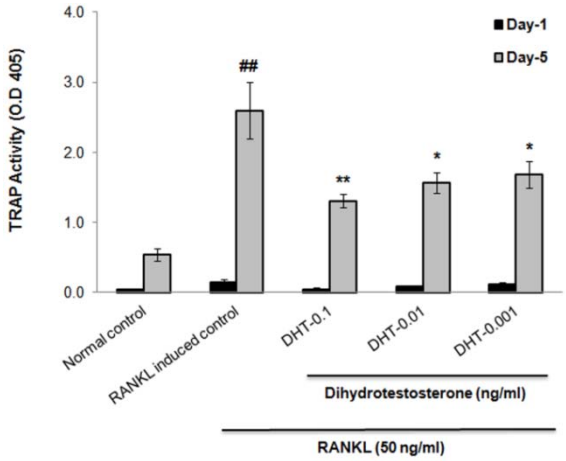

Fig. 1: Effect of 5α-DHT on tartrate-resistant acid phosphatase (TRAP) activity in RANKL-induced RAW 264.7 cells. TRAP activity was measured using ELISA reader (optical density, 405 nm). Data are represented as the means±standard deviation of three independent experiments in quadruplicate sampling (n=12). ##P<0.01 compared with normal; **P<0.01 and *P<0.05 compared with RANKL induced control group

Effect of 5α-DHT on protein biomarkers

To evaluate the effect 5α-DHT on the bone resorption capacity of osteoclasts, we measured the expression of various phenotypic bone resorption-related proteins including MMP-9, cathepsin K, TRAP and NFATc1 in RAW 264.7 cell cultures treated with 5α-DHT.

Expression of MMP-9

To examine the effect of 5α-DHT on the expression of MMP-9, a sandwich ELISA test was performed and the results were compared with an untreated (normal control) group (fig. 2A). Resulting data demonstrated that the expression of MMP-9 was negligibly low at day-1; however, an enormous increase in its expression was observed on day-5 in all the experimental groups (fig. 2A). Comparative analysis revealed that the expression of MMP-9 was significantly (##P<0.01) high in RANKL induced control group compared to the normal control on day-5. A significant dose-dependent decrease in the expression of MMP-9 was observed in 5α-DHT treated RAW 264.7 cells at different concentrations compared to RANKL induced control group. The decrease in the expression of MMP-9 was more pronounced in RAW 264.7 cells treated with 0.1 ng/ml (**P<0.01) (fig. 2A).

Expression of cathepsin-K

To evaluate the effect of 5α-DHT on the expression patterns of cathepsin-K in RANKL-induced RAW 264.7 cells was estimated using sandwich ELISA technique and the results were compared with normal control group (fig. 2B). Results showed that the expression of cathepsin-K was enormously high at day-5 compared to day-1 in all the experimental groups. A significantly highest expression of cathepsin-K was observed in RANKL induced control group on day-5 compared to the other tested groups (##P<0.01). However, the treatment of RANKL induced RAW 264.7 cells with 5α-DHT showed a dose-dependent decrease in the levels of cathepsin-K. The decrease was more obvious at 0.1 ng/ml (**P<0.01) compared to other treated and untreated groups (fig. 2B).

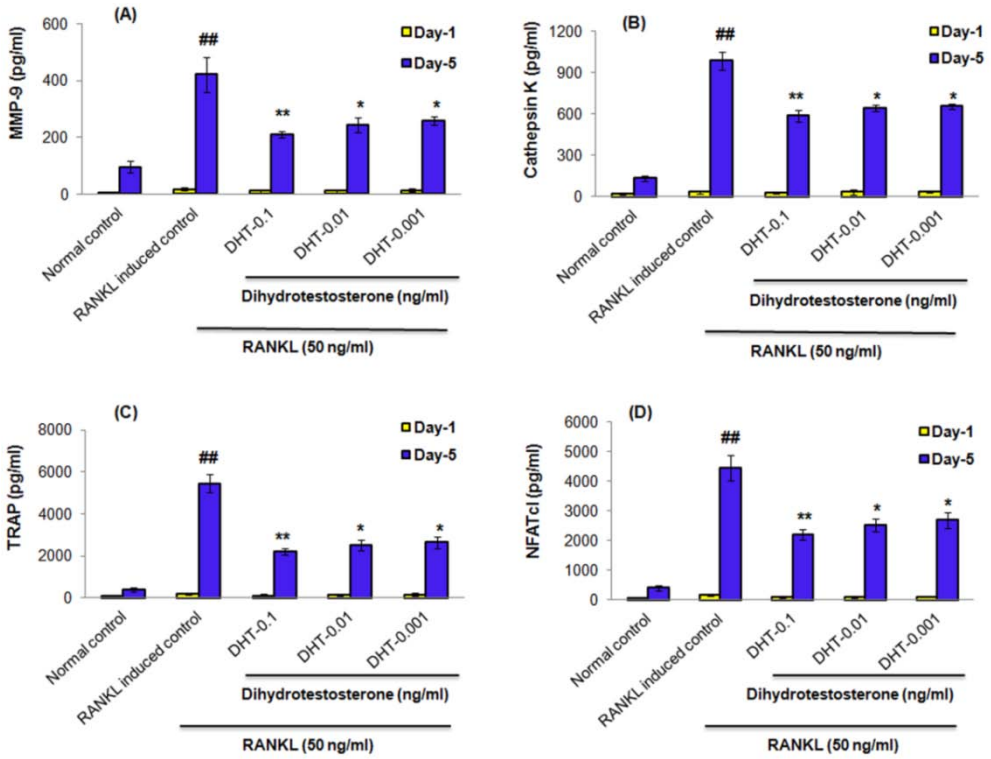

Fig. 2: Effect of 5α-dihydrotestosterone (5α-DHT) on the expression of matrix mettaloproteinase-9 (MMP-9) (A), Cathepsin-K (B), tartrate-resistant acid phosphatase (TRAP) activity (C), and NFATc1 (D) in RANKL-stimulated RAW 264.7 cells using sandwich ELISA. Data are represented as the means±standard deviation of three independent experiments in quadruplicate sampling (n=12). ##P<0.01 represent significant difference between RANKL induced control and normal control groups; **P<0.01 and *P<0.01 represent significant difference between 5α-DHT-treated and RANKL-induced groups

Expression of TRAP

In this experiment, the effect of 5α-DHT on the sequential expression of TRAP was evaluated in RANKL induced RAW 264.7 cells and the results were compared with RANKL induced control and normal control groups (fig. 2C). Results demonstrated that the expression of TRAP was lowest on day-1 in all the experimental groups; however, an enormous increase in the expression of TRAP was observed on day-5. Results showed that the expression intensity of TRAP in RANKL induced control group was significantly high ($^{\#\#}P<0.01$) compared to the normal control group at day-5. The resulting pattern demonstrated a systematic physiological correlation between the expression of TRAP and osteoclastogenesis. Notably, results showed a significant dose-dependent decrease in the expressional intensity of TRAP in 5α-DHT treated RAW 264.7 cells compared to the RANKL induced control group. Further analysis revealed that highest decrease in the TRAP expression was observed at 0.1 ng/ml ($^{**}P<0.01$) compared to the other tested groups (fig. 2C).

Expression of NFATc1

To examine the effect of 5α-DHT on osteoclastogenesis and bone resorption, we have also measured the expression of one NFATc1. NFATc1 is known to be a master transcription factor in osteoclastogenesis [24–26]. The effect of different concentrations of 5α-DHT on the expression of NFATc1 was evaluated using sandwich ELISA (fig. 2D). The resulting data showed that the expression of NFATc1 was significantly ($^{\#\#}P<0.01$) high in RANKL induced control group compared to the normal control group at day-5. At day-1, negligibly lower expressions of NFATc1 were observed in all the experimental groups. Results also showed that a significant dose-dependent decrease in the expression intensity of NFATc1 was observed in 5α-DHT treated RAW 264.7 cell cultures compared to the RANKL induced control group and the highest decrease was observed in RAW 264.7 cells treated with 0.1 ng/ml ($^{**}P<0.01$) (fig. 2D).

Effect of 5α-DHT on superoxide production and SOD activity

This experiment was performed to evaluate the effect of 5α-DHT on the reactive oxygen species (ROS) and superoxide production in RANKL-induced RAW 264.7 cells and the results were compared with normal and RANKL induced control groups (fig. 3A). The resulting data depicted that the production of superoxide was significantly high in RANKL induced group (taken as 100%) on day-5. However, a dose-dependent decrease in superoxide production was observed in 5α-DHT treated RAW 264.7 cells compared to the RANKL induced control group. Comparative analysis revealed that the inhibition of superoxide production was more obvious at 0.1 ng/ml of 5α-DHT compared to other treatment groups.

On the other hand, we have also examined the effect of 5α-DHT on SOD activity in RANKL-induced RAW 264.7 cells and the results were compared with normal control group (fig. 3B). Resulting data showed that higher SOD levels were observed in 5α-DHT treated RAW 264.7 cells compared to the RANKL induced control and normal control groups. The comparative analysis revealed that the highest SOD activity was observed in RAW 264.7 cells treated with 0.1 ng/ml ($^{**}P<0.01$) compared to other treatment groups. Further analysis revealed that SOD levels were increased in a dose-dependent manner in 5α-DHT treated groups (fig. 3B).

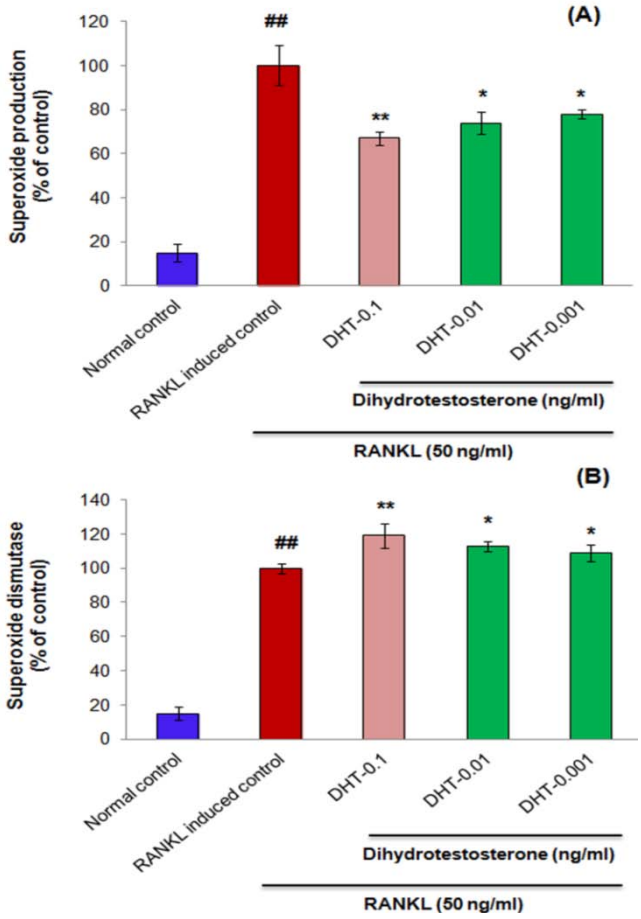

Fig. 3: Effect of 5α-dihydrotestosterone (5α-DHT) on superoxide production (A) and superoxide dismutase (SOD) activity (B). RAW 264.7 cells were stimulated with RANKL and treated with different concentrations (0.1, 0.01, and 0.001 ng/ml) of 5α-DHT for 5 d. Superoxide production was measured by luminescence intensity assay. SOD activity was measured at 450 nm using a microplate reader. Data are representative of results from three independent experiments in quadruplicate sampling (n=12). Data are expressed as percentages of the value of the control cells (mean±S. D, n=12). *P<0.05 and **P<0.01 represent significant difference between tested and RANKL induced control group and ##P<0.01 represent significant difference between RANKL induced control and normal control, groups

DISCUSSION

Osteoporosis is a metabolic disease in which skeletal integrity is compromised due to decreased bone mass, leading to a significantly enhanced risk of skeletal fracture. Among the major pharmacological interventions used for the management of osteoporosis, estrogen replacement therapy, bisphosphonates [27], selective estrogen receptor modulators, and calcitonin are most commonly employed. However, these therapies are also associated with several adverse effects including breast cancer, hypercalcemia and hypertension. Numerous *in vitro*, *in vivo* and human clinical studies have evidenced the substantial anti-osteoporotic efficacy of 5α-DHT and their roles in the regulation of skeletal homeostasis [15–20]. Chen *et al.* [17] demonstrated that 5α-DHT inhibits osteoclast formation, differentiation and maturation by blocking androgen receptors. The involvement and prime importance of androgen receptors and estrogen receptors has also been critically discussed by Mohamed *et al.* [21]. They demonstrated that 5α-DHT regulates bone health via blocking androgen and estrogen receptors.

TRAPs are expressed particularly in osteoclasts and are commonly used as phenotype markers of osteoclasts; treatment of RAW 264.7 cells with RANKL has been shown to easily induce cell differentiation into osteoclasts, which are TRAP-positive cells [28]. The study of TRAP-positive cell formation and activity is a well-known method of determining osteoclast formation and function [29]. In this study, we evidenced that 5α-DHT significantly inhibited TRAP activity which indicates its promising inhibitory potential on osteoclastogenesis.

Moreover, we evaluated the effect of 5α-DHT on the regulation and expression of various key osteoclastogenesis-related protein biomarkers such as MMP-9, cathepsin-K, TRAP and NFATc1 in RANKL-induced RAW 264.7 cells. The resulting data revealed that 5α-DHT significantly downregulates the expression of all the protein markers responsible for osteoclastic activity and their maturation.

MMP-9 plays a critical role in the initiation of the osteoclast-mediated bone resorption process by removing the collagenous layer from the bone surface prior to demineralization [30]. Furthermore, MMP-9 is one of the most important phenotypic biomarkers to anticipate the bone resorption mechanism [31]. In this study, the resulting data revealed that the treatment of RAW 264.7 cells with 5α-DHT results in a dose-dependent decrease in the expression of MMP-9 which indicates that 5α-DHT exhibit promising potential to downregulate bone resorption. For digestion and solubilization of the bone matrix, the ruffled border secretes enzymes and protons after attachment to the bone. Cathepsin-K activity is required to start actin ring formation and, thus, activation and functioning of osteoclasts [32]. Our results clearly demonstrated that the treatment of RAW 264.7 cells with 5α-DHT resulted in a dose-dependent decrease in the expression of cathepsin-K *in vitro*.

Several studies have demonstrated that NFATc1 is an important transcription factor for RANKL-mediated osteoclast differentiation, fusion and activation [33,34]. It has also been noted that over-expression of NFATc1 induces differentiation into osteoclasts even in cases of RANKL deficiency [35]. Moreover, in NFATc1 knock-out mice, defective osteoclast differentiation and osteopetrosis have been noted. NFATc1 plays an important role in osteoclast activation through the release of osteoclastogenesis-related genes such as TRAP and MMP-9 and the expression of these mediators is mainly responsible for the degradation of bone mineral and collagen matrices [12-14]. In the present study, we noticed that 5α-DHT exerted significant inhibitory effects on the expression of NFATc1 in vitro.

ROS have been recognized as secondary messengers and play pivotal roles in regulating the differentiation of osteoclasts. Excessive ROS production results in an abnormal osteoclastogenesis and results in overproduction of osteoclasts [36]. By contrast, excessive ROS production results in an over-activation of osteoclast functions due to an increase in the number of osteoclasts [37]. Additionally, ROS production by osteoporotic bone tissue is significantly higher compared to normal bone tissue [38]. Several studies have reported that the ROS level increases during RANKL-induced osteoclast differentiation. Thus, to investigate whether 5α-DHT can inhibit superoxide generation during osteoclast differentiation, the production of superoxide was analyzed. Our resulting data evidenced that the treatment of RANKL-induced osteoclast with 5α-DHT significantly inhibited the production of ROS in a dose-dependent manner.

SOD is an important antioxidant defence in nearly all living cells including osteoclasts cells. This an enzyme alternately catalyzes the dismutation of the superoxide (O2−) radicals into either ordinary molecular oxygen (O2) or hydrogen peroxide (H2O2) and thus play an important role in maintaining and regulating homeostasis in bodily tissues including bone matrices. Hence, despite of the significant down-regulation of ROS production, we have also evaluated its effect on SOD levels. Our results indicated that 5α-DHT showed significant upregulation in SOD activity, suggesting that 5α-DHT has a positive effect on increasing antioxidant defence in RANKL induced RAW 264.7 cells.

CONCLUSION

In this study, the effect of dihydrotestosteron was systematically observed on the proliferation, differentiation, maturation, and activation of osteoclasts (RAW 264.7 cells). Our results evidenced that treatment of RAW 264.7 cells with 5α-DHT exhibits remarkable inhibitory effects on the bone resorption ability of osteoclasts. The inhibitory effects 5α-DHT on the differentiation and functioning of osteoclasts are regulated through mechanisms involving down-regulation of RANKL-induced TRAP activity and expression of various bone-resorption related protein biomarkers including MMP-9, cathepsin-K, TRAP and NFATc1. Taken together, suppression of superoxide generation and enhanced SOD activity also mediates pivotal role in alleviating the over-activation and functioning of osteoclasts. Our findings suggest that 5α-DHT exhibit promising potential for the treatment of bone diseases associated with excessive bone resorption.

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AUTHOR CONTRIBUTION

The experiments were conceived and designed by Ahmad Nazrun Shuid (ANS). The experiments were performed and executed by Hnin Ei Thu (HET). Manuscript was written by HET and ANS. Data were analysed by ANS, HET, Isa Naina Mohamed (INM) and Zahid Hussain (ZH). The reagents, materials and analytical tools were contributed by HET, ANS, INM and ZH.

CONFLICT OF INTERESTS

The authors declare that they have no competing interests

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