

Mechanistic Insight Into the Efficient Osteogenic Potential of Dihydrotestosterone: Exploring Sequential Expression of Bone-Related Protein Biomarkers

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Abstract- Numerous *in vitro*, *in vivo* and clinical studies have evidenced the outstanding potential of dihydrotestosterone (DHT) in the treatment of male osteoporosis. Despite of promising clinical efficacy of DHT in regulating the skeletal growth and homeostasis, the exact molecular and translational mechanism is yet to be explored. This study was aimed to investigate the bone-forming molecular mechanism of DHT using MC3T3-E1 cell line as *in vitro* model. The mechanism of bone-forming ability of DHT was assessed by evaluating the time-mannered expression of bone-related biomarkers such as bone morphogenic protein-2 (BMP-2), alkaline phosphatase (ALP), runt-related transcription factor 2 (Runx-2), osteocalcin (OCN), type I collagen, osteopontin (OPN), transforming growth factor- β 1 (TGF- β 1) and androgen receptor (AR). Results demonstrated a remarkable efficacy of DHT (at a dose of 0.1 ng/mL) in promoting the expression of these vital bone-forming mediators. The resulting analysis revealed that the DHT-0.1 group showed higher expression of BMP-2 (106 \pm 9 pg/mL), ALP (381 \pm 16 pg/mL), Runx-2 (664 \pm 32 pg/mL), OCN (2265 \pm 111 pg/mL), type I collagen (276 \pm 16 pg/mL), TGF- β 1 (81 \pm 7 pg/mL) and AR (411 \pm 21 pg/mL) compared to the control (CN) and other DHT-treated groups. These findings provide an *in vitro* evidence for the bone-forming capacity of DHT and its therapeutic significance for the treatment of male osteoporosis.

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Introduction

Osteogenesis entails a series of intricate events that collectively contribute to the proliferation and differentiation of osteoprogenitor cells. Bone formation requires actively differentiated osteoblasts to synthesize extracellular matrix (ECM) and for mineralization process (1). Osteogenic differentiation is a highly regulated developmental process and continues during the turnover and repair of mature bone. The synthesis and chronological expression of bone matrix proteins (type I collagen and alkaline phosphatase (ALP)) and the accumulation of minerals (calcium and phosphate) are potential markers of osteogenic differentiation (2,3).

Like all metabolically active cells, osteoblasts require endocrine players or hormonal guidance to

execute their metabolic activities (4). It is well-established that sex hormones (estrogen, progesterone, and androgen) are among the vital modulators of bone health, particularly in protecting bones from weakness and regulating minerals to their optimum levels (5-7). Of these sex hormones, androgen evidenced prime role in regulating osteoblasts and osteogenesis (8-10). 5 α -dihydrotestosterone (5 α -DHT) (5 α -androstane-17 β -ol-3-one) is a sex steroid and androgen hormone (11,12). The enzyme 5 α -reductase synthesizes 5 α -DHT from testosterone in the prostate, testes, hair follicles and adrenal glands (13,14). Relative to testosterone, 5 α -DHT is a much more potent agonist of androgen receptors (15). In addition, 5 α -DHT was found to have greater effects on inhibiting cells growth, arresting cell cycle, and inducing apoptosis in many cancer cells (16). 5 α -

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DHT has an affinity for bone, and its influence on bone metabolism has been researched since the 1980's (17). Numerous studies have reported that 5 α -DHT stimulates osteoblast function by promoting proliferation and differentiation of osteoblast-like cells in culture media (18,19). These studies had also shown that 5 α -DHT positively affects bone metabolism to promote bone formation and decrease bone resorption, leading to normalization of bone density (18,19). 5 α -DHT is being used as an effective treatment for osteoporosis in elderly male patients (20). Clinical trials have confirmed that 5 α -DHT could decrease bone resorption and stimulate osteoblasts activity to form new bone in male osteoporosis (21,22). The effects of 5 α -DHT on osteoblastic in vitro activities are dependent on the pharmacological concentrations of the hormone used, time and duration of exposure to the drugs (23). Although numerous in vitro, in vivo, and clinical studies indicated the significance of 5 α -DHT to regulate bone formation, the time-mannered regulations and mechanisms of osteogenic differentiation of 5 α -DHT have not been extensively studied.

The aim of the present study was to investigate and establish the mechanistic pathway for the proliferative and osteogenic effects of 5 α -DHT using MC3T3-E1 cells as in vitro osteoblastic model. To gain insight into the molecular mechanism of the anti-osteoporotic effects of 5 α -DHT, a variety of bone-related protein markers which include bone morphogenic protein-2 (BMP-2), alkaline phosphatase (ALP), Runx-2, osteocalcin (OCN), type I collagen, osteopontin (OPN), transforming growth factor- β 1 (TGF- β 1) and androgen receptor (AR) were evaluated in 5 α -DHT-treated MC3T3-E1 cells.

Materials and Methods

Materials

Mouse calvariae origin osteoblastic cell line subclone 4 (CRL-2594) was purchased from American Type Culture Collection (ATCC) Cell Bank (Manassas, VA, USA). Cell culture reagents (Alpha modified minimal essential medium (α -MEM), penicillin, streptomycin and fetal bovine serum were sourced from Gibco Laboratories (Grand Island, New York, USA). Ascorbic acid, β -glycerophosphate, and MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H tetrazolium) dyes were purchased from Sigma-Aldrich, USA. 5 α -Dihydrotestosterone (5 α -DHT) and crystal violet powder were purchased from Sigma Aldrich, Germany. ELISA kits for Runx2, AR,

OPN, OCN, BMP2, ALP, TGF- β 1, and collagen type I were purchased from Elabscience Biotechnology Co., Ltd. (Wuhan, China). All other chemicals used were of analytical grade and were sourced from pharmacology and cell culture laboratories of Universiti Kebangsaan Malaysia (Malaysia).

Cell culture

In this study, highly differentiating MC3T3-E1 subclone 4 (MC-4) cells were used. These cells exhibit optimum levels of expression of all the characteristics of molecular markers and form extensively mineralized ECM when grown in ascorbic acid containing medium for several days. The cell culturing and sub-culturing were performed by growing active MC3T3-E1 cells in a growth medium consisting of α -MEM supplemented with 10% heat-inactivated FCS and 1% penicillin/streptomycin (Antibiotic/Antimycotic). The cells were then incubated in a humidified chamber (95% air and 5% CO₂) at 37° C until they reached 80% confluence. The adhered cells were then released from the flask using an aqueous solution of 0.2% trypsin and 0.02% EDTA (ethylene-diamine tetra acetic acid). The cells were counted using a hemocytometer and were seeded at a density of 1 \times 10³ cells/cm² in 96-well plate and were cultured under the same incubation circumstances. For experiments, cells were cultured for 24 h to obtain monolayers containing α -MEM with 10% FCS to promote cell survival, division and metabolism. Prior to initiating osteogenic differentiation, cells were sparsely seeded into 96-well plate and were cultured in an incubator overnight. For cell differentiation experiments, MC3T3-E1 cells were cultured in osteogenic differentiation medium which contains additional 50 μ g/L ascorbate analog which can resist hydrolysis (ascorbate-2-phosphate) to permit collagen type I fibril assembly and 10 mM β -glycerophosphate to promote mineralization of collagen fibrils. During the pre-determined experimental period, cultured cells were typically fed twice weekly over a 2 to 3-week period with an osteogenic differentiation medium. Cells were not used beyond passage-15. Treatments commenced 24 h later when the cultures become confluent.

Treatment

Prior to starting drug treatment, a stock solution (1 ng/mL) of 5 α -DHT was prepared using either α -MEM or differentiation media. After that, three concentrations (0.1, 0.01, and 0.001 ng/mL) of 5 α -DHT were prepared using the stock solution and were sterilized using 0.2 μ m syringe filter (Sartorius, Germany). MC3T3-E1 cells were

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then treated with these three different concentrations of 5 α -DHT and culture media was replaced every three days throughout the experimental period.

Cell viability

Prior to the establishment of screening and evaluation of pharmacological effects of 5 α -DHT, cell viability was assessed. For that, MC3T3-E1 cells were incubated with three different concentrations (0.1, 0.01, and 0.001 ng/mL) of 5 α -DHT for six days using MTS assay. Briefly, MC3T3-E1 cells were seeded at a density of 1×10^3 cells/well in 96-well plate and maintained in growth media for 24 h at 5% CO₂ at 37° C. After 24 h incubation; the media was replaced with fresh medium until day-6. The cells were segregated into four groups; 1) cells treated with fresh growth media (without adding 5 α -DHT) were termed as negative control (NC) group, 2) cells treated with 0.1 ng/mL of 5 α -DHT were termed as DHT-0.1 group, 3) cells treated with 0.01 ng/mL of 5 α -DHT were termed as DHT-0.01 group and the cells treated with 0.001 ng/mL of 5 α -DHT were termed as DHT-0.001 group. At the end of the treatment period, 20 μ L of diluted MTS solution was introduced to each well, and the cells were incubated at 37° C in the dark while being covered with aluminum foil for 2 h. The absorbance was recorded using at 490 nm using microplate reader.

Sequential expression of bone-related protein markers

In this study, we have investigated the potential influence of three different concentrations of 5 α -DHT on the sequential expression and regulation of various bone formation protein markers which include Runx2, BMP2, AR, ALP, OCN, OPN, TGF- β 1 and collagen type I. These proteins are major phenotypic markers for pre-osteoblast differentiation during bone formation. During early stages of osteoblast differentiation, osteoblasts synthesize Col1 α 1 and other matrix proteins, followed by production of ALP and other osteoblastic biomarkers that ultimately lead to induction of ECM calcification.

The expression of each bone-formation specific protein markers was estimated using the sandwich-ELISA technique. In this method, 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.

Statistical analysis

Data analysis was performed using one-way analysis of variance (ANOVA) using SPSS version 21.0. All the experiments were performed independently three times with quadruplicate sampling (n=4). Data were given as mean and standard deviation (mean \pm S.D). A significant difference (*) was recognized by $P < 0.05$.

Results

Cells viability

Results showed that MC3T3-E1 cells treated with different concentrations of 5 α -DHT showed significantly higher cell growth ($P < 0.05$, ANOVA) compared to the CN group (Figure 1). The increase in cell viability was more obvious in the DHT-0.1 group that was treated with 0.1 ng/mL of 5 α -DHT compared to other treatment and CN groups.

Effect of 5 α -DHT on bone-related protein biomarkers

The expression pattern of specific bone-forming proteins was also investigated in 5 α -DHT treated MC3T3-E1 cells in comparison to the CN group. The major phenotypic bone-related protein markers responsible for regulation of osteoblastic differentiation such as BMP-2, ALP, type I collagen, Runx2, AR, OCN, OPN and TGF- β were evaluated in the present study.

Expression of BMP-2

The present study was attempted to evaluate the time-mannered expression of BMP-2 in MC3T3-E1 cells treated with three different concentrations of 5 α -DHT compared to the CN group using the sandwich ELISA technique (Figure 2a). A consistent increase in the levels of BMP-2 was observed in all the test groups from days 3 to 15; however, the increase was more obvious ($P < 0.05$, ANOVA) on day-9 and day-15. Results also showed that the expression levels of BMP-2 were significantly ($P < 0.05$, ANOVA) higher in MC3T3-E1 cells treated with different concentrations of 5 α -DHT particularly in DHT-0.1 group (181 \pm 9 pg/ml) compared to the DHT-0.01 (158 \pm 7 pg/ml), DHT-0.001 (143 \pm 5 pg/ml), and CN groups (69 \pm 4 pg/ml) at day-15. Data also showed that the levels of BMP-2 were reduced in all the test groups at day-21; however, the concentration of this bone-related specific protein marker was comparatively higher in the DHT-0.1 group (Figure 2a).

ALP activity

Figure 2b showed that ALP activity was lowest at day-3 in the all the tested groups; however, a consistent increase in ALP activity was observed from days-3 to 15. Results showed that increase in ALP activity was significantly higher ($P<0.05$, ANOVA) at days-9 and 15 in the all tested groups (Figure 2b). Results also showed that ALP levels for all the experimental groups were detected at the early stage (day-3) and increased concomitantly until day-15 but subsequently declined to day-21 (Figure 2b). Comparative analysis revealed that among all the test groups, the expression levels of ALP activity was significantly higher ($P<0.05$, ANOVA) in MC3T3E1 cells treated with different concentrations of 5α -DHT, particularly at a dose of 0.1 ng/mL (434 ± 32 pg/ml) compared to those treated with 0.01 ng/mL (401 ± 17 pg/ml), 0.01 ng/mL (387 ± 14 pg/ml), and NC group (311 ± 11 pg/ml) groups (Figure 2b).

Expression of Runx-2

Results showed that expression pattern of Runx-2 was gradually increased from days 3 to 15 and subsequently declined on day 21 in all the experimental groups; however, the increased expression was more

obvious on days 9 and 15 (Figure 3a). Results also showed that the expression intensity of Runx-2 in DHT-0.1 group (832 ± 32 pg/mL) was significantly higher ($P<0.05$, ANOVA) than DHT-0.01 (801 ± 21 pg/mL), DHT-0.001 (711 ± 15 pg/mL) and CN group (467 ± 26 pg/mL) at day 15 (Figure 3a). The expression levels of Runx-2 were declined in all the experimental groups on day 21; however, the levels were still higher in DHT-treated groups compared to the CN group.

Expression of OCN

The resulting data showed that the level of OCN was not detected in the CN group until day-9; however, in case of DHT-0.1, DHT-0.01 and DHT-0.001 groups, low levels of OCN were detected at days 3, 6, and 9 (Figure 3b). Thereafter, the expression of OCN was progressively increased from days 9 to 21 in all the tested groups (Figure 3b). The comparative analysis of the trend in OCN expression indicated that although the levels of OCN were progressively increased in all the test groups, the OCN level on day 21 of DHT-0.1 group was significantly higher ($P<0.05$, ANOVA) (2265 ± 111 pg/mL) than the DHT-0.01 (1787 ± 76 pg/mL), DHT-0.001 (1643 ± 43 pg/mL), and NC (1255 ± 54 pg/mL) groups (Figure 3b).

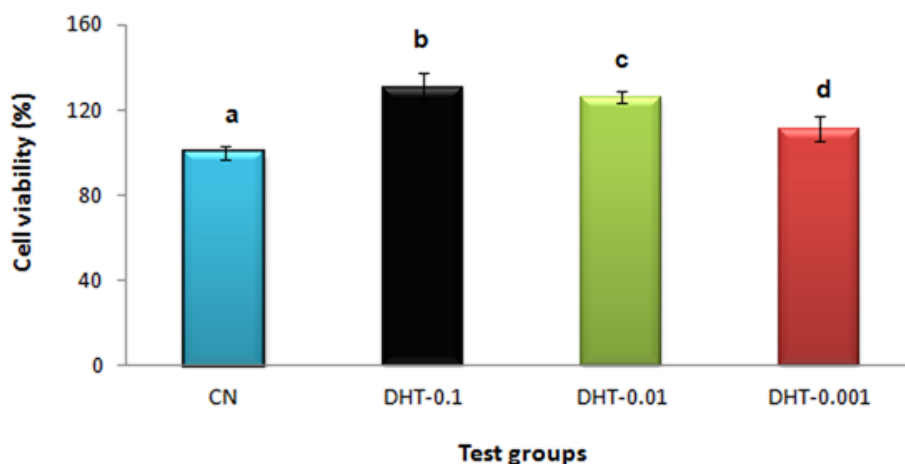


Figure 1. Cell viability of MC3T3-E1 cells treated with different concentrations (0.1, 0.01, and 0.001 ng/mL) of 5α -DHT compared to the CN group

Results were obtained from three independent experiments in quadruplicate ($n=4$) and were expressed as mean \pm S.D. Different letters indicate significant difference ($P<0.05$, ANOVA)

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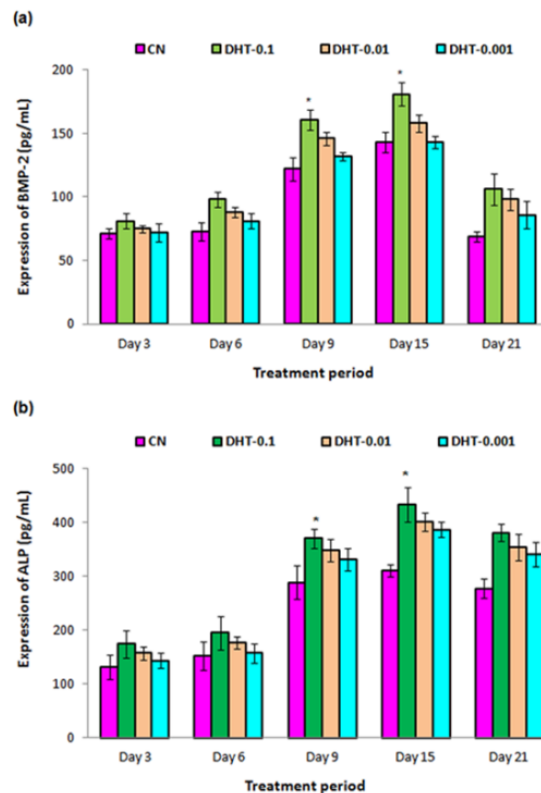


Figure 2. Effect of different concentrations of 5 α -DHT (0.1, 0.01, and 0.001 ng/mL) on the expressions of BMP2 (a) and ALP activity (b) compared to the negative control (CN) groups

Results were obtained from three independent experiments in quadruplicate (n=4) and were expressed as mean \pm S.D

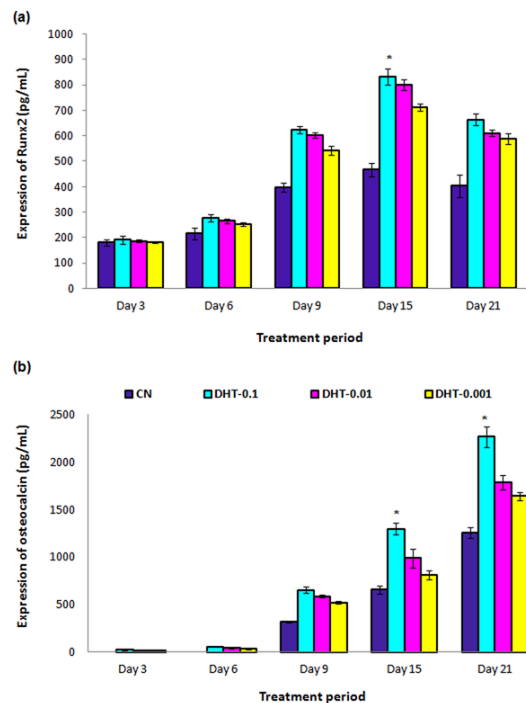


Figure 3. Effect of different concentrations (0.1, 0.01, and 0.001 ng/mL) of 5 α -DHT on the expressions of Runx-2 (a) and osteocalcin (c) compared to the negative control (NC) groups

Results were obtained from three independent experiments in quadruplicate (n=4) and were expressed as mean \pm S.D. Significant difference ($P<0.05$) was recognized (*)

Expression of Type I collagen

Results showed that 5 α -DHT-treated groups predominantly DHT-0.1 (276 \pm 16 pg/mL) showed significantly (P <0.05, ANOVA) higher magnitude of collagen synthesis compared to the DHT-0.01 (245 \pm 17 pg/mL), DHT-0.001 (233 \pm 5 pg/mL), and CN (211 \pm 16 pg/mL) groups on day 21 (Figure 4a).

Expression of OPN

The resulting data showed that OPN expression started to appear at day 3 and was increased gradually until day 15 in all the tested groups. A considerable drop in OPN expression was noticed on day 21 in all the experimental groups (Figure 4b). The amount of OPN detected in DHT-0.1 (72 \pm 8 pg/mL) was comparatively lower compared to the DHT-0.01 (98 \pm 7 pg/mL), DHT-0.001 (132 \pm 9 pg/mL) and CN (151 \pm 16 pg/mL) groups on day 21 (Figure 4b).

Expression of TGF- β 1

Results showed that the expression intensity of TGF- β 1 was not significantly (P >0.05, ANOVA) increased from days 3 to 6 and thereafter became markedly high in all the tested groups (Figure 5a) until day 15. At day 15,

amounts of TGF- β 1 detected in DHT-0.1 (182 \pm 12 pg/mL) was significantly higher (P <0.05, ANOVA) than the DHT-0.01 (171 \pm 9 pg/mL), DHT-0.001 (168 \pm 17 pg/mL) and CN (165 \pm 19 pg/mL) groups (Figure 5a). The expression intensities of TGF- β 1 were rapidly declined in all the experimental groups at day 21; however, the levels of this biomarker were relatively higher in 5 α -DHT-treated groups compared to the CN group.

Expression of AR

Results showed that the expression pattern of AR was up-regulated approximately ten-fold during the differentiation period from days 3 to 21 in all the experimental groups (Figure 5b). A comparative analysis of tested groups revealed that MC3T3-E1 cells treated with different concentrations of 5 α -DHT (particularly DHT-0.1) showed higher expression of AR compared to other experimental groups at all the predetermined time points. At day 21, the amounts of AR detected in DHT-0.1 (411 \pm 21 pg/mL) were significantly higher (P <0.05, ANOVA) than the DHT-0.01 (378 \pm 17 pg/mL), DHT-0.001 (352 \pm 28 pg/mL), and CN (312 \pm 12 pg/mL) groups (Figure 5b).

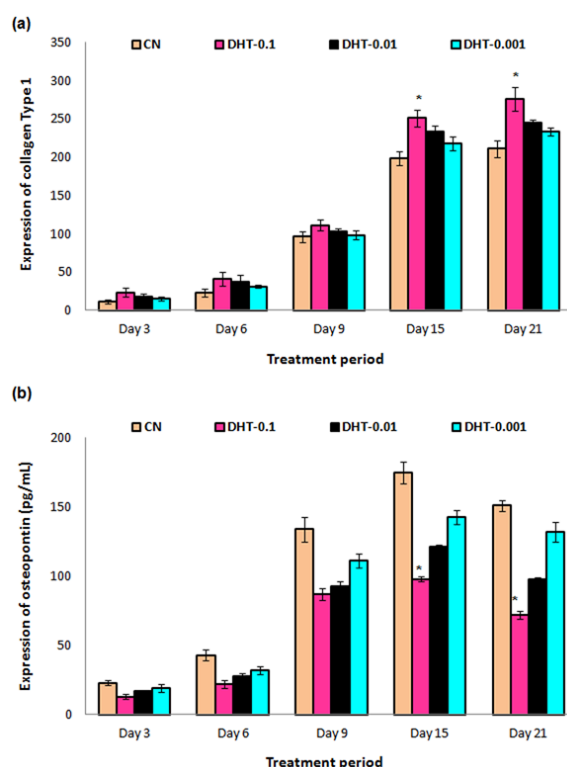


Figure 4. Effect of different concentrations (0.1, 0.01, and 0.001 ng/mL) of 5 α -DHT on the expression of collagen type I (a) and osteopontin (b) compared to the negative control (CN) group. Results were obtained from three independent experiments in quadruplicate (n=4) and were expressed as mean \pm S.D. Significant difference (P <0.05) was recognized (*)

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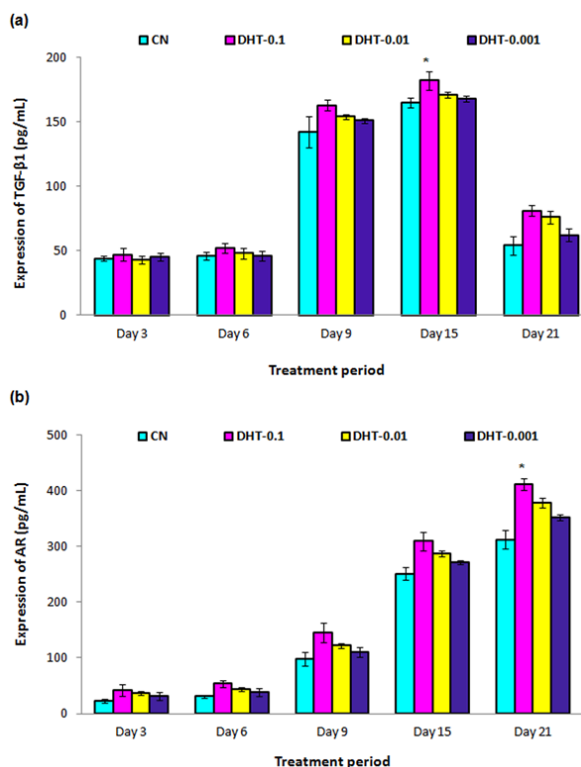


Figure 5. Effect of different concentrations (0.1, 0.01, and 0.001 ng/mL) of 5 α -DHT on the expressions of TGF- β 1 (a) and androgen receptors (b) compared to the negative control (NC) groups

Results were obtained from three independent experiments in quadruplicate (n=4) and were expressed as mean \pm S.D. Significant difference ($P < 0.05$) was recognized (*).

Discussion

Bone remodeling is a lifelong process during which mature bone tissues are removed from the skeleton (a process called bone resorption), and new bone tissues are formed (a process called ossification or new bone formation). The balance between both of these processes is the key to maintaining the bone health. Osteoporosis is a seriously prevalent bone disorder characterized by fragile bones having higher susceptibility to fracturing. Among various management modalities, an enhanced bone formation activity of osteoblasts and down-regulated activation, maturation, and functioning of osteoclasts is anticipated to be crucial to improve bone density and maintain bone health.

A wide range of pharmacological modalities including estrogen replacement therapy (ERT), testosterone replacement therapy (TRT), bisphosphonates, selective estrogen receptor modulators (SERM), and calcitonin are currently being employed for the management of osteoporosis. Among various therapies, testosterone therapy is among most commonly employed; however, its molecular mechanism was yet to

elaborate. Therefore, to comprehend the molecular and translational mechanism of 5 α -DHT in enhancing the bone formation, we have executed a series of in vitro experiments to evaluate the time-mannered expression of bone-related protein biomarkers such as BMP-2, ALP, Runx-2, OCN, type I collagen, OPN, TGF- β 1, and AR.

Prior to evaluating the efficacy of 5 α -DHT in regulating various protein biomarkers, cell viability analysis was conducted to determine the safe dose of 5 α -DHT that could produce desirable effects on the functioning of bone-forming cells (MC3T3-E1). Our results indicated that 5 α -DHT was safe and could significantly ($P < 0.05$, ANOVA) enhance cell growth particularly at 0.1 ng/mL.

To gain further insight into the molecular mechanisms of 5 α -DHT involved in osteoblast differentiation, sequential expression of various protein biomarkers were quantitatively monitored in the absence or presence of 5 α -DHT. The major phenotypic bone-related protein markers responsible for regulation of osteoblast differentiation including BMP-2, ALP, type I collagen, Runx-2, AR, OCN, OPN and TGF- β were

evaluated in the present study. Among these proteins, BMP-2 is one of the most potent inducers of osteoblast's differentiation especially during the early stage of osteogenesis (24,25). Our results demonstrated a marked increase in BMP-2 levels in 5 α -DHT-treated cells. These findings suggested that 5 α -DHT may enhance osteoblastic proliferation and differentiation *via* the up-regulation of BMP-2 levels (a potent inducer of osteoblast differentiation) (26).

Upon reaching confluence, MC3T3-E1 cells initiate programmed multi-layering and expression of BMP-2 followed by the induction of ALP activity (27,28). ALP is among the key players that exhibit primary role in osteogenesis and thus, adequate levels of ALP are vital to induce and promote mineralization, differentiation and protein expression in MC3T3-E1 cells (29). Our results clearly evidenced a remarkable potential of 5 α -DHT in up-regulating the levels of ALP in MC3T3-E1 cells which demonstrates a physiological correlation between the expression pattern of ALP activity and osteoblastic differentiation. Our results were also in agreement with previous studies (30).

The expression level of Runx-2, a master regulatory protein which regulates osteoblastic differentiation and maturation during early stages, was also assessed in the present study. It was anticipated that the higher expression of Runx-2 observed in 5 α -DHT treated MC3T3-E1 cells could be a molecular basis for the higher differentiation, maturation, and mineralization of MC3T3-E1 cells (31). Komori and co-workers had also demonstrated that Runx-2 is involved in the regulation of type I collagen and OCN expressions, which ultimately resulted in higher differentiation and mineralization of bone forming cells. OCN, a non-collagenous and most abundant bone matrix protein synthesized by the bone cells, is among the most vital bone biomarkers responsible for the maintenance of bone mineralization. Our findings suggested that OCN was mainly expressed during the late stage of differentiation and thus could promote the formation of mineralized nodules in MC3T3-E1 cells (32). A remarkably higher expression of OCN in MC3T3-E1 cells treated with different concentrations of 5 α -DHT (particularly at 0.1 ng/mL) revealed that 5 α -DHT has strong potential to up-regulate and promote OCN expression in bone forming cells. This could provide a molecular basis for the higher mineralization and nodule formation in bone-forming cells that were also observed in a previous study on 5 α -DHT-treated MCET3-E1 cells (18).

Among the various characteristic features of

osteoblastic differentiation, collagen (predominantly collagen type-I) synthesis is the foremost and imperative cellular differentiation biomarker. Several studies have validated the biological significance of type-I collagen in regulating the bone matrix formation, osteoblastic differentiation and mineral deposition (33,34). Thus, in this study, time-mannered expression in collagen synthesis was observed from days 3 to 21. Results showed that the expression of collagen was low from days 3 to 6 in all the test groups; however, a progressive increase in the collagen levels was observed in all the experimental groups in a time-dependent manner from day 9 to 21. The higher intensity of collagen synthesis observed in 5 α -DHT-treated MC3T3-E1 cells clearly indicated the potential of 5 α -DHT in promoting osteoblastic maturation and differentiation as signs of cellular differentiation and bone matrix formation.

Another well-studied protein biomarker to regulate the homeostasis in bone remodeling is OPN (35). OPN is a negative regulator of cell proliferation, differentiation, and bone mineralization, most likely through inhibition of mineral crystal growth, at the later stages of osteogenic differentiation (36). Therefore, the effects of 5 α -DHT on OPN expression were examined during the different stages of differentiation. Our results demonstrated a significant down-regulation in the expression of OPN in 5 α -DHT treated cell culture which is anticipated to be one of the reasons to up-regulate the differentiation and mineralization of osteoblasts (37).

Among bone regulatory proteins, TGF- β displays imperative mitogenic functions in regulating the proliferation, early differentiation, and mineralization of bone cells (38). Thus, we have also examined the effects of different concentrations of 5 α -DHT on the expression of TGF- β 1, an important member of TGF- β superfamily. Based on our findings, higher expression of TGF- β 1 observed in 5 α -DHT-treated MC3T3-E1 cells could be the molecular basis for remarkably high proliferation, differentiation, and mineralization of MC3T3-E1 cells (39). AR is a single receptor protein that plays pivotal roles in regulating proliferation, differentiation, and mineralization in bone-forming cells (MC3T3-E1 cells). The expression of AR in active MC3T3-E1 cells has been well-documented. The comparative analysis of all the tested groups revealed that MC3T3-E1 cells treated with different concentrations of 5 α -DHT showed higher expression of AR compared to the control groups at all the predetermined time points. ARs are physiologically expressed under the influence of steroid hormone and thus promote osteoblastic proliferation, differentiation and mineralization in bone-forming cells (23,40). In this

study, higher expression of AR in 5 α -DHT-treated cell cultures and consequently higher proliferation, differentiation, and mineralization suggested that 5 α -DHT might bind to AR and consequently stimulate their cell proliferation and differentiation.

Conclusively, our results indicated that 5 α -DHT (particularly at a dose of 0.1 ng/mL) significantly up-regulated the proliferation of MC3T3-E1 cells. Analyses of various bone-related biomarkers including BMP-2, ALP, Runx-2, OCN, type I collagen, TGF- β 1 and AR revealed that cells treated with different concentrations of 5 α -DHT produced greater expressions of these bone-forming protein biomarkers and reduced expression of OPN compared to the control groups. These results provide the molecular basis of the osteogenic potential of 5 α -DHT in the prevention and treatment of osteoporosis. It was also noted that the expression patterns of these bone-related proteins were regulated in temporal manner during the successive developmental stages of proliferation, bone matrix formation/maturation, and mineralization. The present study strengthened the concept that 5 α -DHT remarkably promote bone mass homeostasis.

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