doi.org/10.36721/PJPS/2026.40.15637.1

Danxiong huoxue tablets attenuate experimental heterotopic ossification after direct anterior approach total hip arthroplasty by modulating inflammation and oxidative stress

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Abstract: **Background**: Direct anterior approach total hip arthroplasty (DAA-THA) is associated with postoperative heterotopic ossification (HO), which may impair joint function. Inflammation and oxidative stress are important contributors to HO development. Danxiong Huoxue Tablets (DHT) possess potential anti-inflammatory and antioxidant activities, but their effects on postoperative HO remain insufficiently investigated. **Objectives**: This preclinical study aimed to evaluate the effects of DHT on experimental HO and to explore its possible association with inflammatory and oxidative stress-related signaling using a rat DAA-induced HO model and an osteogenic differentiation model of human bone marrow mesenchymal stem cells (hBMSCs). **Methods**: A rat DAA-induced HO model was established and treated with low- and high-dose DHT for 28 days. X-ray and histological analyses were used to evaluate ectopic bone formation. ELISA and Western blotting were employed to assess inflammatory cytokines (TNF-α, IL-6, IL-1β), oxidative stress biomarkers (SOD, GSH-Px, MDA) and related signaling proteins (NF-κB p65, NLRP3, Nrf2, HO-1). In parallel, hBMSCs were used to examine the effects of DHT on cell viability, osteogenic differentiation and related molecular changes. **Results**: DHT administration reduced ectopic bone formation in the rat HO model, as confirmed by imaging and histological analyses. DHT also decreased pro-inflammatory cytokine levels, improved oxidative stress-related markers, reduced NF-κB/NLRP3 pathway-related protein expression and increased Nrf2/HO-1 expression. *In-vitro*, DHT suppressed hBMSC osteogenic differentiation and downregulated Runx2, Osterix, BMP-2 and OCN expression. **Conclusion**: DHT attenuated experimental DAA-induced HO in rats and inhibited osteogenic differentiation of hBMSCs, possibly by reducing inflammatory responses and oxidative stress. These findings provide preclinical evidence supporting further investigation of DHT as a potential adjunctive strategy for preventing postoperative HO.

Keywords: Danxiong Huoxue tablets; Heterotopic ossification; Inflammation; Oxidative stress; Total hip arthroplasty

Submitted 15-11-2025 – Revised 27-05-2026 – Accepted 22-06-2026

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INTRODUCTION

Total hip arthroplasty (THA) represents a well-established and efficacious treatment modality for advanced hip disorders, such as osteoarthritis, rheumatoid arthritis and femoral head osteonecrosis (Grant, 2021, Kim, *et al*., 2019). THA effectively relieves joint pain and improves patients' functional outcomes and quality of life. Advances in surgical technique have led to the development of direct anterior approach total hip arthroplasty (DAA-THA), which has been reported to offer several benefits. These include the use of the intermuscular approach, which minimizes soft-tissue injury and facilitates rapid postoperative recovery (Wang, *et al*., 2018). This approach is gaining popularity among a growing number of surgeons. Several investigations have shown that DAA-THA can reduce intraoperative blood loss, shorten hospital stay and improve early functional recovery compared with conventional posterolateral or lateral approaches.

However, Heterotopic ossification (HO) commonly occurs after THA and can significantly impact postoperative rehabilitation and joint function (Herman, *et al*., 2021, Łęgosz, *et al*., 2019). The term "HO" is used to denote the process of maturation of the lamellar bone and bone marrow in the surrounding soft tissue of the joint. This condition can result in symptoms such as hip pain, joint impingement and, in more severe cases, hip stiffness (Sui, *et al*., 2023, Wang, *et al*., 2024, Mujtaba, *et al*., 2019). Research has demonstrated that the prevalence of HO ranges from 5% to 90% in patients undergoing open reduction and internal fixation (ORIF), 46% in patients undergoing hip arthroscopy (Zhou, *et al*., 2022) and 7.8% to 45.2% in patients undergoing THA (Hürlimann *et al*., 2017). When HO progresses to Brooker grade IV, it will significantly limit hip joint range of motion, offset the clinical benefits of joint replacement and even require further surgical intervention (Pohl, *et al*., 2005). Consequently, the effective prevention of HO after THA is of great clinical significance.

Presently, the primary focus of HO prevention is on the utilisation of non-steroidal anti-inflammatory drugs (NSAIDs) and local radiotherapy (Joice, *et al*., 2018, Migliorini, *et al*., 2022). Although it exerts a certain effect, its application is limited due to side effects and

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safety concerns (Kan, *et al*., 2015). Consequently, the development of new, safe and efficient prevention and control strategies has become a major research focus.

As demonstrated in the extant literature, there is a strong correlation between inflammation and oxidative stress in HO (Convente, *et al*., 2015). Surgical injury activates local immunity, causing the production of inflammatory mediators (e.g., TNF-α, IL-6, IL-1β) and promoting abnormal osteogenic differentiation in bone marrow mesenchymal stem cells (BMSCs) (Agarwal, *et al*., 2017). The NF-κB/NLRP3 signaling cascade is activated, leading to amplification of inflammation and accumulation of reactive oxygen species (ROS), thereby causing oxidative stress. This is further exacerbated by inhibition of Nrf2/HO-1-mediated antioxidant mechanisms, thereby aggravating pathological osteogenesis. Consequently, regulating inflammatory and oxidative stress pathways is a pivotal target for HO prevention and therapy.

Danxiong Huoxue Tablets (DHT), formulated by Professor Cai Limin, are composed of multiple traditional Chinese medicinal herbs, including Salvia miltiorrhiza and Ligusticum chuanxiong. These herbs have been selected based on years of clinical experience and syndrome differentiation. DHT exerts a plethora of pharmacological effects, including promoting blood circulation, eliminating blood stasis, enhancing microcirculation and augmenting body resistance. From the perspective of traditional Chinese medicine, postoperative HO may be associated with local tissue injury, blood stasis, impaired microcirculation and persistent inflammatory stimulation. Therefore, DHT was selected for investigation because its blood-activating and stasis-resolving properties are theoretically consistent with the pathological features of postoperative HO. In addition, several components of DHT, such as Salvia miltiorrhiza and Ligusticum chuanxiong, have been reported to exert anti-inflammatory, antioxidant and bone-metabolism-regulating effects, suggesting that DHT may have potential value in modulating the inflammatory and oxidative microenvironment involved in HO formation.

A previous study (Xiang, *et al*., 2022) employed network pharmacology and molecular docking approaches to investigate the potential mechanisms of DHT in HO prevention and therapy. The identification process yielded 39 active compounds and 328 associated targets, which were predominantly enriched in pathways such as PI3K/Akt and MAPK signaling. Molecular docking further validated strong binding affinities between core targets and active compounds. However, these previous findings were mainly predictive and lacked experimental validation in animal or cellular models of postoperative HO. Moreover, whether DHT can directly attenuate ectopic bone formation after THA-related surgical injury and whether its effects are associated with the regulation of inflammation, oxidative stress and osteogenic differentiation, remain unclear. Therefore, an important knowledge gap exists between network pharmacology-based prediction and biological verification of DHT in HO prevention.

The present study systematically evaluated the intervention effect of DHT on HO formation. To this end, a rat HO model and an osteogenic differentiation model of human bone marrow mesenchymal stem cells (hBMSCs) were established. Unlike previous studies that mainly focused on computational prediction or the general pharmacological effects of traditional Chinese medicine compounds, this study combined in vivo and in vitro experiments to assess the anti-HO effect of DHT and to explore its possible association with changes in inflammatory, oxidative stress and osteogenic signaling. Furthermore, the mechanism by which the tablets regulate the NF-κB/NLRP3 inflammatory and Nrf2/HO-1 antioxidant pathways was explored. This study aimed to provide experimental evidence and theoretical support for further investigation of DHT as a potential preclinical strategy for preventing postoperative HO.

MATERIALS AND METHODS

Drugs and reagents

DHT was provided by Dongguan Zhongyi Hospital (Batch Number: 20210223). The formula consists of 15 g each of Salvia miltiorrhiza, Lycopus lucidus, Morus alba twigs and Liquidambar orientalis fruit; 10 g each of Angelica sinensis tail, Ligusticum chuanxiong, Caesalpinia sappan and Stephania tetrandra; 3 g of Eupolyphaga sinensis; and 1 g of Daemonorops draco. All herbal materials were authenticated and prepared according to the internal quality control standards of Dongguan Zhongyi Hospital. The formulation was produced as a single batch to minimize batch-to-batch variation and the appearance, weight uniformity and preparation process were checked before use. The tablets were ground into powder and suspended in normal saline to prepare solutions of different concentrations before use. The suspension was freshly prepared before administration or cell treatment and mixed thoroughly to ensure homogeneity.

Animals and experimental design

Male Sprague–Dawley (SD) rats (250–300 g) were obtained from the Guangdong Medical Experimental Animal Center. They were maintained under specific pathogen-free conditions in a controlled environment (22 ± 2 °C, 12 h light/dark cycle) and had free access to food and water.

Rats were randomly divided into four groups (n = 10 per group): a) Sham group (sham surgery); b) Model group

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(DAA-THA-induced HO model); c) DHT low-dose group (DHT-L, 200 mg/kg); d) DHT high-dose group (DHT-H, 400 mg/kg). Randomization was performed using a computer-generated random number table after acclimatization. Group allocation was conducted by an investigator who was not involved in subsequent imaging, histological evaluation, or data analysis. During X-ray assessment, HE staining evaluation and ImageJ-based quantification, the investigators were blinded to group allocation to reduce potential measurement bias. Rats in the DHT-L and DHT-H groups received daily oral gavage of DHT starting on postoperative day 1 for 28 consecutive days. Rats in the Sham and Model groups were administered an equal volume of normal saline. The sample size of 10 rats per group was selected based on previous studies using rodent HO models and was considered sufficient to detect treatment-related differences in ectopic bone formation while minimizing animal use (Kan and Kessler, 2011, Davis, *et al*., 2016).

Establishment of the heterotopic ossification (HO) model

The rat HO model was established using a modified method previously described (Kan and Kessler, 2011). After anesthesia with 2–3% isoflurane, a longitudinal incision (~2 cm) was made over the left hip along the direct anterior approach. The skin and muscle layers were separated to expose the femoral head and acetabular region. The joint capsule was excised and the periosteum was carefully debrided with a sterile curette, resulting in a localized bone injury. A polyethylene implant (8 mm length, 1 mm diameter) was inserted into the surgical site to enhance ectopic ossification. The wound was irrigated with sterile saline and sutured in layers. Postoperatively, rats were monitored daily for wound healing and general behavior.

X-ray evaluation of heterotopic ossification

At 28 days post-intervention, all rats were anesthetized with 2% isoflurane, after which the operated hip regions were scanned with a high-resolution digital X-ray imaging system (Philips DigitalDiagnost, Netherlands) to assess ectopic bone formation. Imaging parameters were standardized for all samples (40 kV, 10 mA, exposure time 10 ms, source-to-object distance 40 cm). Anteroposterior radiographs were acquired to visualize the periarticular region of the left hip while maintaining consistent positioning to minimize projection bias.

Hematoxylin and eosin (HE) staining

HE staining was performed using a commercial kit (Servicebio, G1005, Wuhan, China) to evaluate bone trabecular formation, cartilage structure and tissue morphology. Sections were examined under a Leica DM4000B light microscope (Leica Microsystems, DM2700M, Germany) and representative images were captured. Quantitative analysis of the newly formed bone area was conducted using ImageJ software (NIH, USA).

Cell culture

Human bone marrow–derived mesenchymal stem cells (hBMSCs) were purchased from Procell Life Science and Technology Co., Ltd. (Wuhan, China; Cat. No. CP-H166). Cells were maintained in α-Minimum Essential Medium supplemented with 10% fetal bovine serum and 1% penicillin–streptomycin. Cultures were incubated in a humidified 5% CO_2 atmosphere at 37 °C and the medium was changed every 2–3 days until cells reached 80–90% confluence for passage.

Cell viability assay (Cell Counting Kit-8, CCK-8)

The cytotoxic effect of DHT on hBMSCs was evaluated using the Cell Counting Kit-8 (CCK-8; C0037, Guangzhou Yujia Biotechnology Co., Ltd.) to determine the safe and effective concentration range for subsequent experiments. Cells were plated in 96-well plates (5×10^3 cells/well) and cultured for 24 h to allow adhesion. Thereafter, various DHT concentrations (0, 50, 100, 200 and 400 μg/mL) were applied for 24, 48 and 72 h. This concentration range was designed to cover an untreated control and low-, moderate- and high-concentration exposure levels, thereby allowing evaluation of cytotoxicity and the concentration window suitable for subsequent functional experiments. Based on the CCK-8 results, concentrations that did not markedly affect hBMSC viability were selected for subsequent osteogenic differentiation experiments to minimize nonspecific cytotoxic interference. At each interval, 10 μL of CCK-8 reagent was introduced and left at 37 °C for 2 h. Optical density (OD) at 450 nm was determined by a BioTek Synergy HTX reader (Agilent Technologies, USA).

Alkaline phosphatase (ALP) activity assay

To evaluate early osteogenic differentiation, ALP activity was assessed in hBMSCs after 7 days of osteogenic induction. Cells were rinsed twice with PBS and lysed in 0.2% Triton X-100, after which the lysate was centrifuged (12,000 rpm, 10 min, 4 °C) to obtain the supernatant. ALP activity was measured using an ALP assay kit (Aladin, A1491772-100T) following the manufacturer's instructions. In brief, equal volumes (50 μL) of cell lysate and substrate solution were combined and incubated at 37 °C for 15 min. The reaction was stopped with NaOH and OD was recorded with a BioTek Synergy HTX reader. ALP activity was adjusted to protein levels determined by the BCA method and expressed as U/mg protein. For qualitative visualization, ALP staining was performed using a BCIP/NBT kit (Aladdin, B273090) and stained areas were imaged under an inverted microscope (Olympus IX73, Olympus, Japan).

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Alizarin red S staining

Calcium nodule formation, an indicator of late osteogenic differentiation, was assessed by Alizarin Red S (ARS) staining on day 21 after osteogenic induction. hBMSCs were fixed with 4% paraformaldehyde for 30 min, washed three times with PBS and stained with 2% Alizarin Red S solution (pH 4.2, Solarbio, Beijing, China; Cat. No. G1450) for 30 min at room temperature. Following staining, cells were rinsed thoroughly with distilled water to remove unbound dye and photomicrographs were acquired with a phase-contrast microscope (Olympus IX73, Japan). For quantification, bound dye was extracted with 10% cetylpyridinium chloride (Sigma-Aldrich, 6004-24-6, USA) for 30 min and the OD at 562 nm was determined using a microplate reader. The amount of mineralized nodules was normalized by total protein content and all assays were performed in triplicate.

Enzyme-linked immunosorbent assay (ELISA)

Serum and periarticular tissue homogenates were collected after 28 days of treatment. Tissue samples were processed in chilled PBS (1:9, w/v), followed by centrifugation (12,000 rpm, 10 min, 4°C). The supernatant and serum samples were used to measure TNF-α, IL-6, IL-1β, SOD, GSH-Px and MDA levels using ELISA kits (ml106471, ml106838, ml107050, ml106903, ml107105, ml454321, MLBIO, Shanghai Enzyme-linked Biotechnology Co., Ltd., China) according to the supplier's protocol. OD at 450 nm were recorded on a BioTek Synergy HTX reader and results were calculated from standard curves. Each assay was conducted three times.

Western blotting

HO tissue lysates were prepared with RIPA buffer containing protease and phosphatase inhibitors (Beyotime, China). Protein amounts were quantified with the bicinchoninic acid (BCA) method. Equal protein amounts (30 μg) were subjected to SDS-PAGE followed by PVDF membrane transfer (Millipore, USA). After blocking with 5% non-fat milk for 1 h, membranes were incubated overnight at 4°C with the following primary antibodies: NF-κB p65 (ab207297, Abcam)，NLRP3 (ab263899, Abcam)，Nrf2 (ab62352, Abcam)，HO-1 (ab52947, Abcam)，Runx2 (ab192256, Abcam), Osterix (ab209484, Abcam), BMP-2 (ab214821, Abcam), OCN (ab93876, Abcam) and β-actin (ab8227, Abcam) After washing, membranes were incubated with HRP-conjugated secondary antibodies, including anti-rabbit or mouse IgG (ab6721, ab97040, Abcam) for 1 h at room temperature. Immunoreactive signals were detected via enhanced chemiluminescence and analyzed using ImageJ, with expression levels normalized to β-actin.

Statistical analysis

Data are presented as mean ± standard deviation (SD). Statistical analysis was conducted with SPSS 23.0 software (IBM Corp., Armonk, NY, USA). Before intergroup comparisons, the normality of data distribution was assessed using the Shapiro–Wilk test and homogeneity of variance was examined using Levene's test. For data that met the assumptions of normality and equal variance, differences among groups were analyzed using one-way analysis of variance (ANOVA), followed by Tukey's post hoc test. If these assumptions were not met, appropriate nonparametric tests were considered. A value of $p < 0.05$ was regarded as statistically significant.

RESULTS

DHT attenuates ectopic bone formation after DAA surgery

Radiographic analysis revealed substantial heterotopic ossification in the model group relative to the control group, characterized by dense ectopic bone surrounding the surgical site (Fig. 1A). In contrast, administration of DHT markedly reduced the extent and density of ectopic bone formation, with the effect increasing with dose. The high-dose DHT group exhibited almost complete suppression of ectopic bone development, indicating a robust inhibitory effect.

Histological examination further supported the radiographic findings. Hematoxylin and eosin (HE) staining showed extensive trabecular bone structures and disorganized fibro-osseous tissue in the model group, while control samples displayed normal muscle and connective tissue morphology (Fig. 1B). Quantitative analysis showed that the HO-related new bone area was markedly increased in the model group and was reduced by approximately 54% and 82% in the DHT-L and DHT-H groups, respectively, compared with the model group ($p < 0.001$). These results indicate that DHT effectively attenuated DAA-induced ectopic ossification.

DHT suppresses inflammatory response and oxidative stress after DAA surgery

ELISA analysis showed that DAA surgery markedly increased serum levels of inflammatory cytokines compared with the control group (Figs. 2A–2C). Compared with the model group, DHT-L and DHT-H reduced TNF-α levels by approximately 49% and 78%, respectively. Similar downward trends were observed for IL-6 and IL-1β, with stronger suppression in the DHT-H group ($p < 0.001$). These findings suggest that DHT alleviated the inflammatory response after DAA surgery. Oxidative stress markers were also significantly altered in the model group, as reflected by decreased SOD and GSH-Px activities and increased MDA levels (Figs. 2D–2F). DHT treatment partially restored antioxidant enzyme activity and reduced lipid peroxidation.

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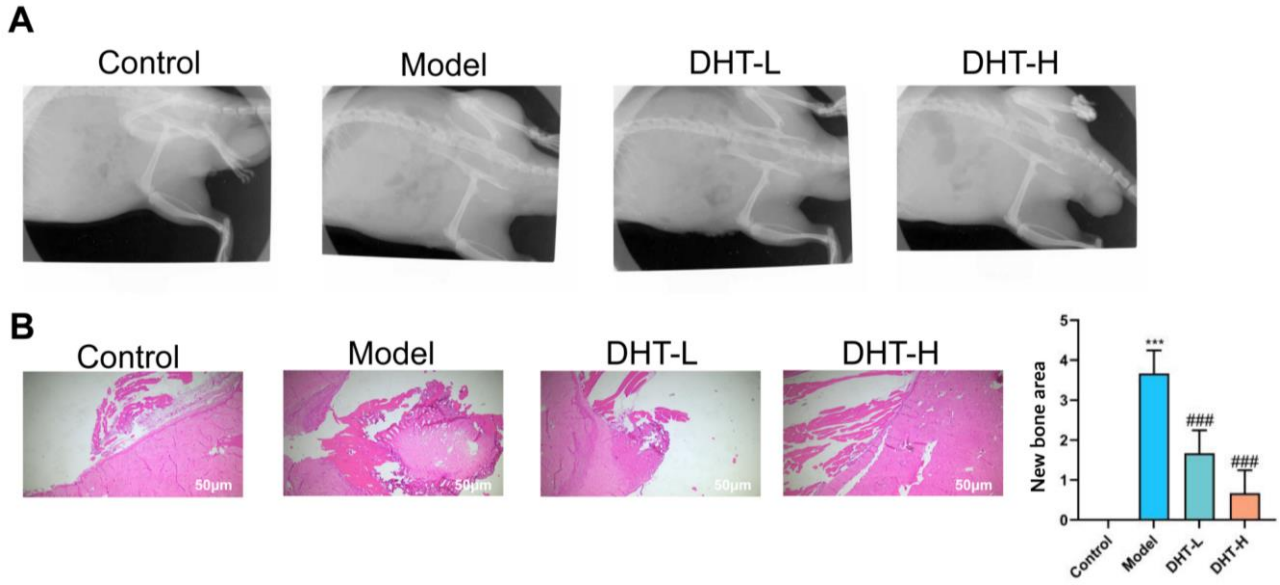


Fig. 1: X-ray and histological evaluation of heterotopic ossification following DAA surgery. (A) Representative radiographs of rat hip joints from each group after 28 days of treatment; (B) Representative HE staining of periarticular tissues showing histological features of heterotopic ossification. Scale bar: 50 μm. Quantification depicted in the right panel. n=10, ***$p < 0.001$ vs. Control; ###$p < 0.001$ vs. Model.

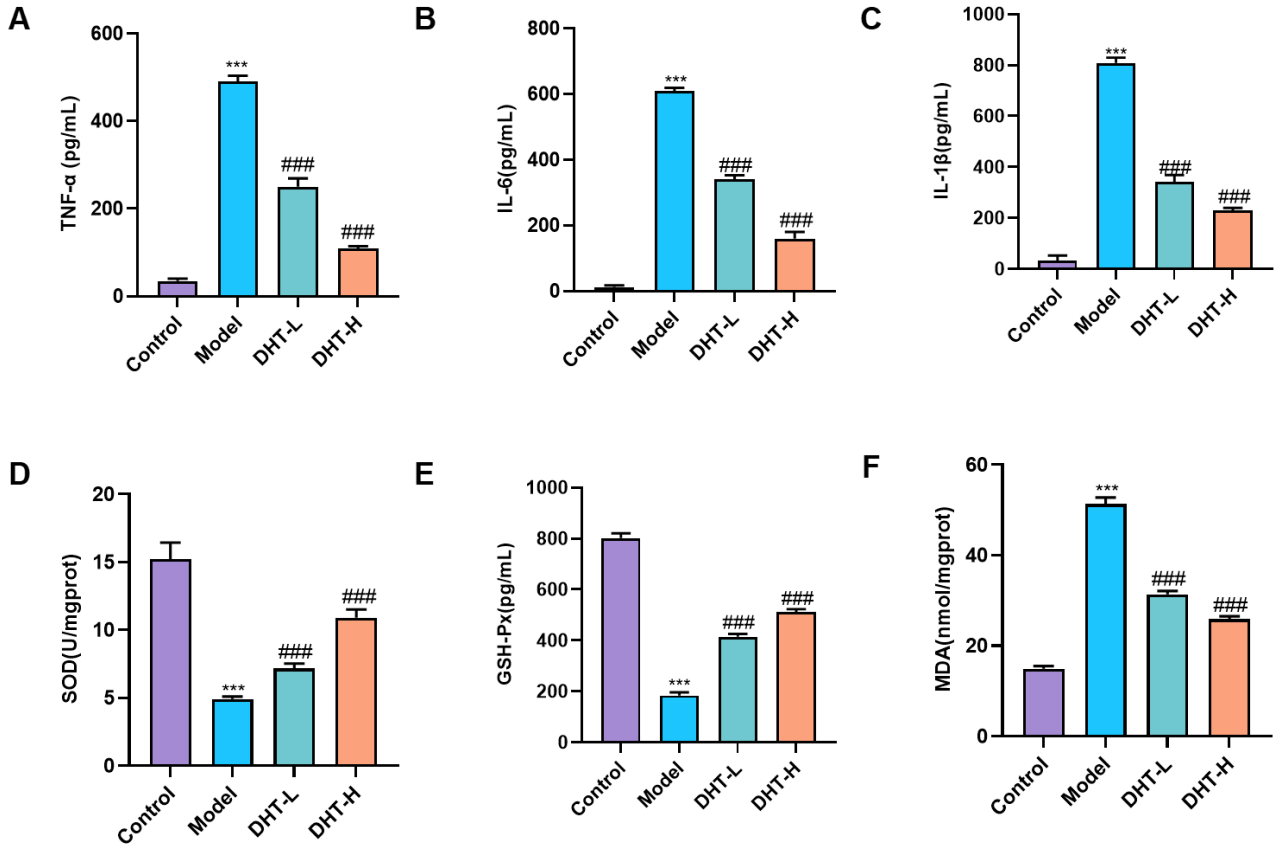


Fig. 2: ELISA analysis of inflammation- and oxidative stress–related markers after DAA surgery. (A–C) Serum concentrations of TNF-α, IL-6 and IL-1β; (D–F) Serum activities of SOD and GSH-Px and levels of MDA. n=10, ***$p < 0.001$ vs. Control; ###$p < 0.001$ vs. Model.

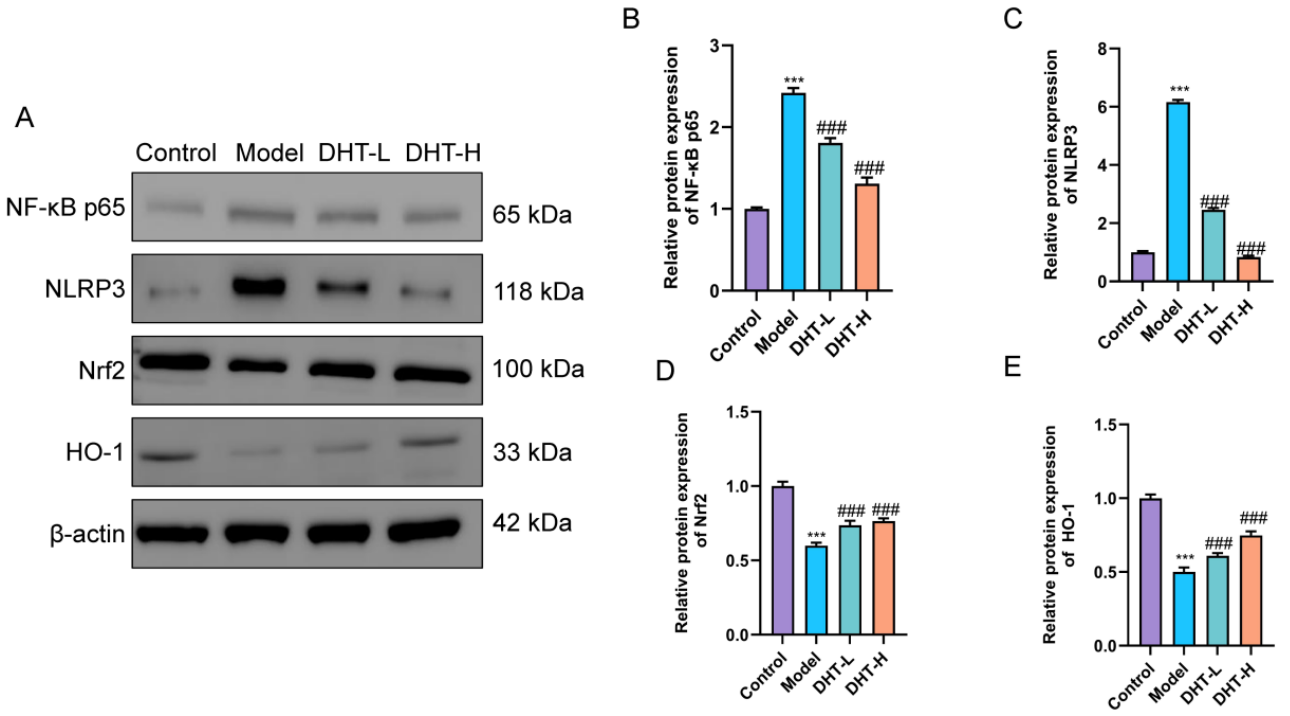


Fig. 3: Western blot detection of inflammatory and oxidative stress–related signaling in HO tissue. (A) Representative protein bands of NF-κB p65, NLRP3, Nrf2 and HO-1 in each group. β-actin served as an internal control; (B–E) Quantitative expression profiles for NF-κB p65, NLRP3, Nrf2 and HO-1. n=10, ***$p < 0.001$ vs. Control; ###$p < 0.001$ vs. Model.

In particular, SOD activity increased by approximately 50% in the DHT-L group and more than doubled in the DHT-H group compared with the model group, while MDA levels were reduced by approximately 39% and 49%, respectively ($p < 0.001$). These results indicate that DHT improved the imbalance in oxidative stress in the DAA-induced HO model.

DHT modulates NF-κB/NLRP3 and Nrf2/HO-1 signaling pathways in HO tissue

Western blot analysis revealed that DAA-induced heterotopic ossification was accompanied by a marked activation of the NF-κB/NLRP3 signaling cascade and suppression of the Nrf2/HO-1 antioxidant axis (Fig. 3A). Compared with control rats, model animals displayed pronounced increases in NF-κB p65 and NLRP3 protein levels ($p < 0.001$), along with markedly decreased Nrf2 and HO-1 expression ($p < 0.001$, Figs. 3B–3E).

DHT treatment significantly reversed these molecular changes. Among these markers, NLRP3 showed the most obvious reduction, decreasing from approximately 6.1-fold in the model group to 2.4-fold and 0.8-fold in the DHT-L and DHT-H groups, respectively. NF-κB p65 expression also decreased after DHT treatment, whereas Nrf2 and HO-1 expression increased, with a stronger effect observed in the DHT-H group (all $p < 0.001$). These findings suggest that DHT-mediated inhibition of HO may be associated with reduced inflammatory signaling and enhanced antioxidant responses.

DHT inhibits osteogenic differentiation of hBMSCs

The CCK-8 assay was used to assess DHT cytotoxicity in hBMSCs. As shown in Fig. 4A, treatment with 100 μg/mL DHT did not affect cell viability within 72 h ($p > 0.05$), whereas higher concentrations (≥200 μg/mL) markedly decreased viability ($p < 0.001$), indicating that 50–100 μg/mL was a safe and effective concentration range for subsequent experiments.

Osteogenic induction significantly increased ALP staining intensity and mineralized nodule formation compared with the control group (Figs. 4B–4D). Compared with the osteogenic group, DHT treatment reduced ALP staining intensity by approximately 46% and 52% at 50 and 100 μg/mL, respectively. Alizarin Red S staining intensity was also decreased, with the 100 μg/mL group showing a greater reduction than the 50 μg/mL group ($p < 0.001$). These results indicate that DHT inhibited both early and late stages of osteogenic differentiation in hBMSCs. Western blot analysis further supported these findings. Osteogenic induction markedly increased the expression of Runx2, Osterix, BMP-2 and OCN (Figs. 4E–4I). DHT treatment downregulated these osteogenic markers in a concentration-dependent manner, with the 100 μg/mL group showing a stronger inhibitory effect than the 50 μg/mL group ($p < 0.001$). These data suggest that DHT suppresses osteogenic differentiation at the molecular level.

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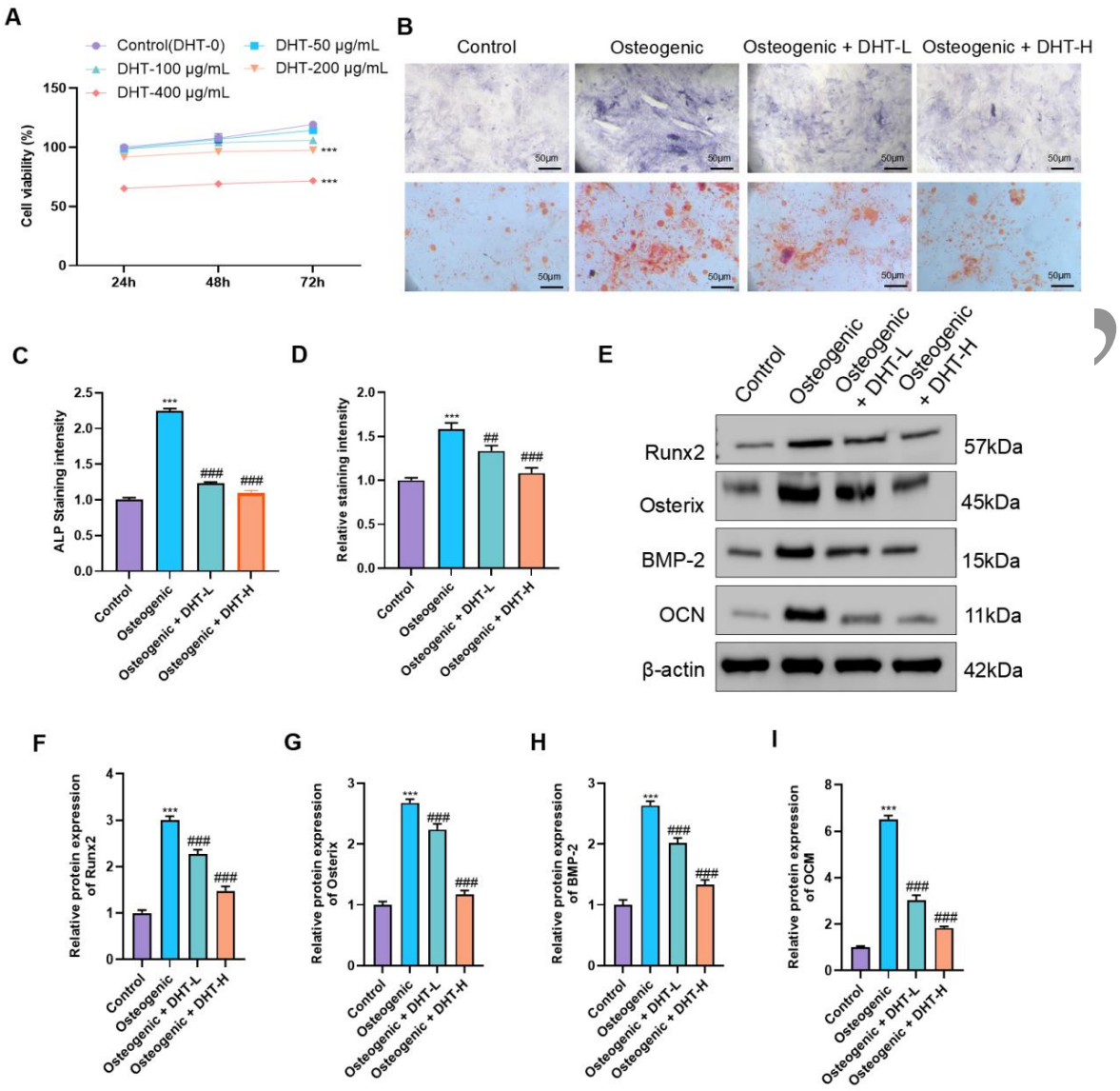


Fig. 4: Influence of DHT on osteogenic differentiation of hBMSCs. (A) CCK-8 assay showing the impact of different DHT concentrations (0–400 μg/mL) on hBMSC viability at 24, 48 and 72 h; (B) Representative staining images for ALP and Alizarin Red S after osteogenic induction. Scale bar: 50 μm; (C-D) Quantitative evaluation of ALP and Alizarin Red S staining intensity; (E) Representative Western blot bands of Runx2, Osterix, BMP-2 and OCN; (F–I) Quantitative analysis of corresponding protein expression. The experiment was conducted three times,***$p < 0.001$ vs. Control; ###$p < 0.001$ vs. Osteogenic group.

DHT regulates NF-κB/NLRP3 and Nrf2/HO-1 pathways in hBMSCs

Compared with the control group, osteogenic induction significantly increased the levels of TNF-α, IL-6 and IL-1β in the culture supernatant (Figs. 5A–5C). Compared with the osteogenic group, DHT-H reduced TNF-α and IL-1β levels by approximately 64% and 63%, respectively, while DHT-L produced moderate reductions. IL-6 showed a similar concentration-dependent decrease after DHT treatment ($p < 0.001$). These results indicate that DHT attenuated the inflammatory response during hBMSC osteogenic differentiation.

Western blotting showed that osteogenic induction increased NF-κB p65 and NLRP3 expression while decreasing Nrf2 and HO-1 expression (Figs. 5D–5H). DHT treatment significantly reversed these changes. Notably, NF-κB p65 and NLRP3 expression were reduced in a concentration-dependent manner, whereas Nrf2 and HO-1 expression increased after DHT treatment, with the strongest effect observed in the DHT-H group ($p < 0.001$). These findings suggest that the inhibitory effect of DHT on osteogenic differentiation may be associated with suppression of NF-κB/NLRP3 signaling and restoration of Nrf2/HO-1-related antioxidant responses.

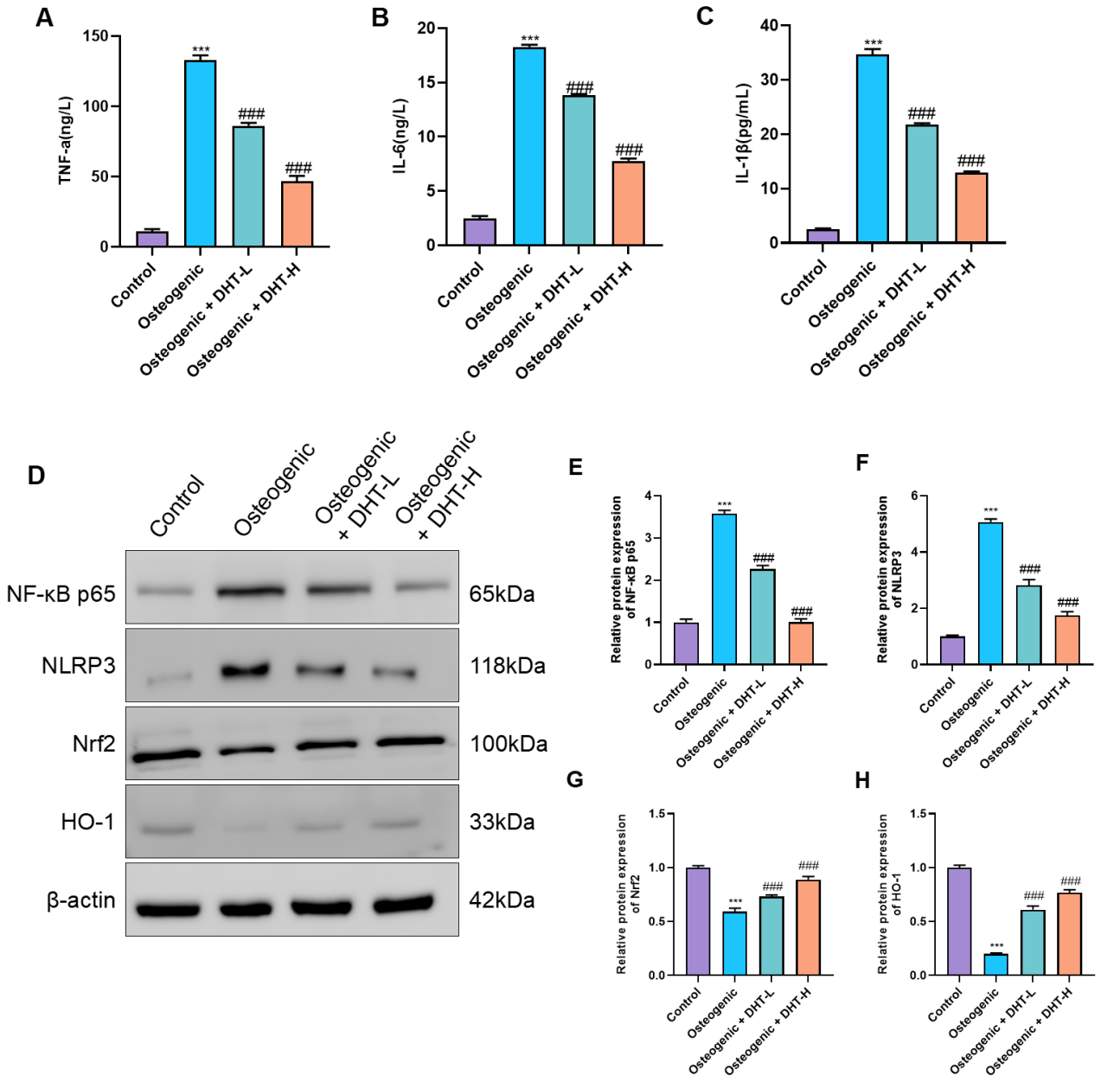


Fig. 5: Effects of DHT on inflammatory cytokine release and NF-κB/NLRP3/Nrf2/HO-1 signaling in hBMSCs. (A–C) ELISA analysis of TNF-α, IL-6 and IL-1β levels in the culture supernatant of each group; (D) Representative Western blot bands of NF-κB p65, NLRP3, Nrf2 and HO-1, with β-actin as a loading control; (E–H) Quantification of relative protein expression levels. The experiment was conducted in triplicate,***$p < 0.001$ vs. Control; ###$p < 0.001$ vs. Osteogenic group.

DISCUSSION

In this study, a rat model of HO following DAA-THA and an osteogenic differentiation model using hBMSCs were established to evaluate the inhibitory effect of DHT and explore their underlying mechanisms. The results showed that DHT intervention significantly reduced HO formation after DAA-THA, improved histological structure, decreased inflammatory cytokine levels in serum and cell supernatants, alleviated oxidative stress and inhibited osteogenic differentiation of hBMSCs. These effects were accompanied by reduced expression of NF-κB p65 and NLRP3 and increased expression of Nrf2 and HO-1, suggesting that changes in inflammatory and antioxidant signaling may be associated with the protective effects of DHT. However, because this study did not include pathway-specific rescue or inhibition experiments, these findings should be interpreted as associative rather than definitive evidence of direct causal regulation.

The formation mechanism of HO is complex, involving the synergistic effects of multiple factors, such as local tissue damage, inflammatory response, oxidative stress and abnormal osteogenesis (Juan, *et al*., 2024). Surgical injury has been shown to activate local immune cells, promote the massive release of TNF-α, IL-6, IL-1β and related inflammatory cytokines and induce the abnormal differentiation of BMSCs toward osteogenesis, thereby promoting the formation of ectopic bone tissue (Dey, *et al*., 2017). The NF-κB pathway, a pivotal regulatory axis of inflammatory response, is known to be significantly activated during the early phase of HO (Huang, *et al*., 2022). This activation may promote downstream NLRP3 inflammasome activation and the release of inflammatory cytokines. In the present study, NF-κB p65 and NLRP3 expression was increased in both the DAA-induced HO model and osteogenically induced hBMSCs, indicating enhanced inflammatory signaling. DHT administration reduced NF-κB p65 and NLRP3 expression and decreased TNF-α, IL-6 and IL-1β levels. These findings are consistent with the possibility that DHT attenuates HO-related inflammation, at least in part, by suppressing NF-κB/NLRP3-Mediated inflammatory signaling. Nevertheless, because specific NF-κB/NLRP3 pathway inhibitors, activators, knockdown models, or rescue experiments were not used, the current results cannot confirm that this pathway is indispensable for DHT's anti-HO effect.

Conversely, oxidative stress has been identified as a significant contributor to the onset and progression of HO (Hu and Wang, 2021, Xie, *et al*., 2021, Guzik and Touyz, 2017). Elevated local ROS levels have been demonstrated to result in lipid peroxidation damage, inhibit the function of antioxidant enzyme systems and further amplify tissue inflammation and abnormal osteogenesis. The Nrf2/HO-1 signaling pathway is an important endogenous antioxidant pathway that regulates antioxidant enzymes, clears ROS and protects tissues. In this study, serum SOD and GSH-Px activities were markedly reduced in model rats, whereas MDA levels increased and Nrf2/HO-1 expression declined, suggesting that oxidative stress was aggravated after DAA-related surgical injury. DHT treatment restored SOD and GSH-Px activities, reduced MDA levels and increased Nrf2 and HO-1 protein expression. These results suggest that DHT may improve the microenvironment of oxidative stress associated with HO formation. However, Nrf2/HO-1 activation may represent one component of the antioxidant response induced by DHT rather than the sole mechanism responsible for its anti-HO effect. Other processes, such as changes in BMP-related osteogenic signaling, MAPK or PI3K/Akt signaling, macrophage polarization and local immune-cell infiltration, may also contribute to the observed effects.

Compared with established prophylactic strategies such as NSAIDs and local radiotherapy, which primarily target inflammatory prostaglandin signaling or local osteogenic activity, DHT may exert broader regulatory effects due to its multi-component nature. Previous pharmacological and molecular studies have suggested that HO formation can be attenuated by targeting inflammation, oxidative stress, mTOR signaling, NF-κB signaling, MAPK signaling, or macrophage-related responses. For example, rapamycin has been reported to partially prevent HO by inhibiting the mTOR pathway and reducing oxidative stress (Hu and Wang, 2021), while palovarotene has been shown to inhibit NF-κB signaling during HO prevention (Huang, *et al*., 2022). In addition, recent evidence indicates that modulation of macrophage polarization and MAPK signaling may influence traumatic HO formation (Wang, *et al*., 2024). In line with these studies, the present findings suggest that DHT may attenuate experimental HO by regulating inflammatory responses, oxidative stress status and osteogenic differentiation. Compared with single-target interventions, DHT may have potential multi-pathway regulatory characteristics. However, because DHT is a compound preparation, the precise active components, the direct molecular targets and the relative contributions of each pathway remain unclear. Therefore, DHT should be regarded as a promising preclinical candidate rather than a confirmed clinical alternative to existing HO prophylactic strategies.

It is important to note that this study has several limitations. Firstly, the relatively brief observation period did not permit evaluation of the long-term evolution, maturation, or recurrence of HO after DHT treatment. In addition, the sample size was relatively small and only one DAA-induced HO animal model was used; therefore, the generalizability of the findings should be further validated in larger cohorts and additional HO models. Secondly, although changes in NF-κB/NLRP3 and Nrf2/HO-1 pathway-related proteins were detected by Western blotting, the current experiments mainly demonstrate correlations between DHT treatment, pathway-associated protein expression and reduced HO formation. Pathway-specific functional validation, such as NF-κB/NLRP3 activation rescue, Nrf2 inhibition, gene knockdown/overexpression, or small-molecule agonist/inhibitor experiments, was not performed. Therefore, direct causal regulation of these pathways by DHT cannot be confirmed. Thirdly, DHT is a multi-component traditional Chinese medicine formulation and the active compounds responsible for the observed anti-inflammatory, antioxidant and anti-osteogenic effects were not identified. Pharmacokinetic analysis, compound characterization and target validation are needed to clarify the material basis and mechanistic specificity of DHT. Finally, alternative mechanisms, including BMP, MAPK and PI3K/Akt signaling, macrophage polarization and local immune-cell infiltration, were not investigated and may also contribute to DHT-mediated HO attenuation.

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In summary, DHT attenuated experimental HO formation after DAA-related surgical injury and inhibited osteogenic differentiation of hBMSCs. These effects were accompanied by reduced inflammatory responses, improved oxidative stress status, decreased NF-κB/NLRP3-associated protein expression and increased Nrf2/HO-1 expression. Although these findings suggest that inflammatory and antioxidant pathways may be involved, further mechanistic studies are required to confirm direct causal regulation. This study provides preclinical evidence supporting further investigation of DHT as a potential adjunctive strategy for preventing postoperative HO.

CONCLUSION

In this study, DHT significantly inhibited the formation of experimental HO following DAA-related surgical injury in rats. DHT exerted protective effects, possibly by attenuating inflammatory responses and oxidative stress, accompanied by reduced expression of NF-κB/NLRP3 pathway-related proteins and increased expression of Nrf2/HO-1 pathway-related proteins. Furthermore, DHT inhibited osteogenic differentiation of hBMSCs, further supporting its anti-HO potential. These findings provide preclinical evidence supporting further investigation of DHT as a potential adjunctive strategy for preventing postoperative HO. However, additional mechanistic and translational studies are required before its clinical application can be determined.

Acknowledgements
None.

Authors' contributions
Wenhui Luo and Xuhui Wu: Conception, study design, execution, data acquisition, analysis, interpretation, drafting, revising and critically reviewing the article. The authors agree to the final approval of the version to be published and to be accountable for all aspects of the work.

Funding
There was no funding.

Data availability statement
The data used to support the findings of this study are available from the corresponding author upon request.

Ethical approval
All animal experimental procedures were approved by the Institutional Animal Ethics Committee of Guangdong Medical Laboratory Animal Centre (Approval No.D202511-7) and conducted in strict accordance with the Regulations on the Administration of Laboratory Animals and the NIH Guide for the Care and Use of Laboratory Animals. This study was performed in adherence with the ARRIVE guidelines. See supplementary file for the ARRIVE checklist.

Conflict of interest
The authors declare no conflict of interest.

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