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Tamoxifen Inhibits the Progression of Trauma-Induced Heterotopic Ossification in Mice

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Background: Heterotopic ossification (HO) is a kind of abnormal mineralized bone which usually occurs in muscle, tendon, or ligament. There are currently no effective drugs for the treatment and prevention of HO. Developing effective drugs that can inhibit HO is of profound significance and would provide new strategies for clinical treatment of this disease. The present investigation evaluated the inhibitory effect of tamoxifen against HO.

Material/Methods: Using an Achilles tendon trauma-induced HO female mice model, we screened different doses of tamoxifen (1, 3, and 9 mg/kg) in mice to determine the optimal dosage on the inhibition of the HO formation. The curative effect of tamoxifen was also illustrated at different HO progression stages including inflammation, chondrogenesis, osteogenesis, and HO maturation.

Results: Heterotopic bone was formed with typical endochondral ossification in Achilles tendons 6 weeks after surgery and continued to enlarge up to 12 weeks. The formation of HO was significantly inhibited with the treatment of tamoxifen at the dosage of 9 mg/kg, whereas 1 mg/kg and 3 mg/kg did not reduce HO bone volume remarkably. The progression of HO was both attenuated by tamoxifen from Day 1 and Week 4 post-surgery, whereas no inhibitory effect was shown at the osteogenesis and maturation stages treated with tamoxifen.

Conclusions: Tamoxifen exerts an inhibitory effect on the heterotopic bone progression at inflammation and chondrogenesis stages, with the TGF- β signaling pathway suppressed following the increase in estrogen receptor α activity.

MeSH Keywords: **Achilles Tendon • Estrogen Receptor • Ossification, Heterotopic • Tamoxifen**

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Background

Heterotopic ossification (HO) is a bone formation at extra-skeletal sites where bone normally does not exist. It is classified as acquired and hereditary [1]. Acquired HO often occurs after trauma or orthopedic surgeries, such as bone fractures, severe burns, central nervous system injury, combat blast wounds, and total hip arthroplasty. Inherited genetic HO is clinically characterized by progressive and extensive HO that occur in children, with gradual immobilization afflicting individuals [2–5]. Until now, there are no effective treatments for HO therapeutic strategies which are limited to systemic administration of non-steroidal anti-inflammatory drugs (NSAIDs), prophylactic low-dose radiation therapy, and surgical excision [6–11]. These treatments usually have little effect and are followed by extensive side effects and high recurrence rate [12]. To research targeted drugs that significantly inhibit HO with minor side effects and low rate of recurrence, there remains many key issues to be clarified.

An increasing number of studies have shown that the incidence of HO has a remarkable correlation with gender. Lee et al. systematic reviewed 55 articles investigating 384 patients with elbows complicated by HO which were treated with surgical resection. The ratio of male to female was 65: 35, indicating the incidence of HO in elbow joint injuries in male patients is nearly 2 times that of female patients [13]. Another article about the effectiveness of celecoxib assessed by Lavernia et al. in the prevention of HO following total hip replacement found that 1 year after total hip replacement, compared to women (48%), men had a significantly greater incidence of HO (69%) [14]. Our previous research also confirmed a similar conclusion, 9 months after arthrolysis, the incidence of HO in male and female patients was 59.5% and 24.4%, respectively [15]. Based on these research studies, the male gender is determined as a risk factor for HO, whereas women predominance in the groups should have favored a lower incidence [13–18]. This gender difference giving rise to diverse incidence manifests that the formation of HO may be related to sex hormones such as estrogen or androgen, and treated with agonist or antagonist of sex hormones is likely to attenuate the progression of HO.

Estrogen can regulate the secretion of cytokines, such as transforming growth factor (TGF)- β , tumor necrosis factor (TNF)- α , playing important roles in the metabolism of bone, cartilage, and other extragonadal tissues [19]. As a selective estrogen receptor modulator (SERM), tamoxifen is used for the prophylaxis and treatment of estrogen receptor (ER)-positive breast cancer in high-risk women [20]. It exerts species-specific pharmacologic impacts in target tissues, such as antiestrogenic effects in breast cancer and agonist effects on bone homeostasis [20,21]. TGF- β has been shown to induce the migration of bone mesenchymal stem cells to the bone resorptive sites and play a positive role in the initiation of HO through a Smad

signaling pathway [22,23]. The purpose of this research is to unravel the potential relationship between tamoxifen and HO. We investigate whether tamoxifen can inhibit the formation of HO by suppressing TGF- β /Smad pathway in a trauma-induced HO mouse model. The results in this paper provide strong support for our hypothesis and supply an effective clinical therapeutic strategy for HO, especially to female patients.

Material and Methods

Mice HO models

All animal procedures in this protocol were performed following institutional regulations and approved by the Wuxi 9th People's Hospital Institutional Review Board.

The 7-month-old C57BL/6 female mice were purchased from Yangzhou University (Yangzhou, China). We built an Achilles tendon HO model using the trauma-inducing method [24]. Briefly, after the mice were anesthetized, we punctured the Achilles tendon using a 27-gauge needle from the lateral surface with 6 repetitions at different positions. The mice impaled through the skin but without damage to the Achilles tendon were used as the sham group.

Drug treatments

For the dosage-screening experiments, mice were assigned into 5 groups with 8 mice in each group. From the day of puncture, mice were administered intragastrically with tamoxifen (1, 3, and 9 mg/kg) in 0.1% dimethyl sulfoxide (DMSO); the sham and vehicle groups were orally gavaged with the same volume of vehicle. The treatment was conducted every other day for a total of 8 weeks. All the mice were euthanized 9 weeks post puncture.

To further illustrate the curative effect of tamoxifen at different HO progression stages, mice were assigned into 6 groups with 8 mice in each group and administrated with tamoxifen (9 mg/kg) or vehicle every other day at Day 1, Week 4, Week 7, and Week 10 for a total of 3 weeks after puncture. The sham group and vehicle group were treated with an identical volume for a total of 3 weeks. All the mice were euthanized 3 months post puncture.

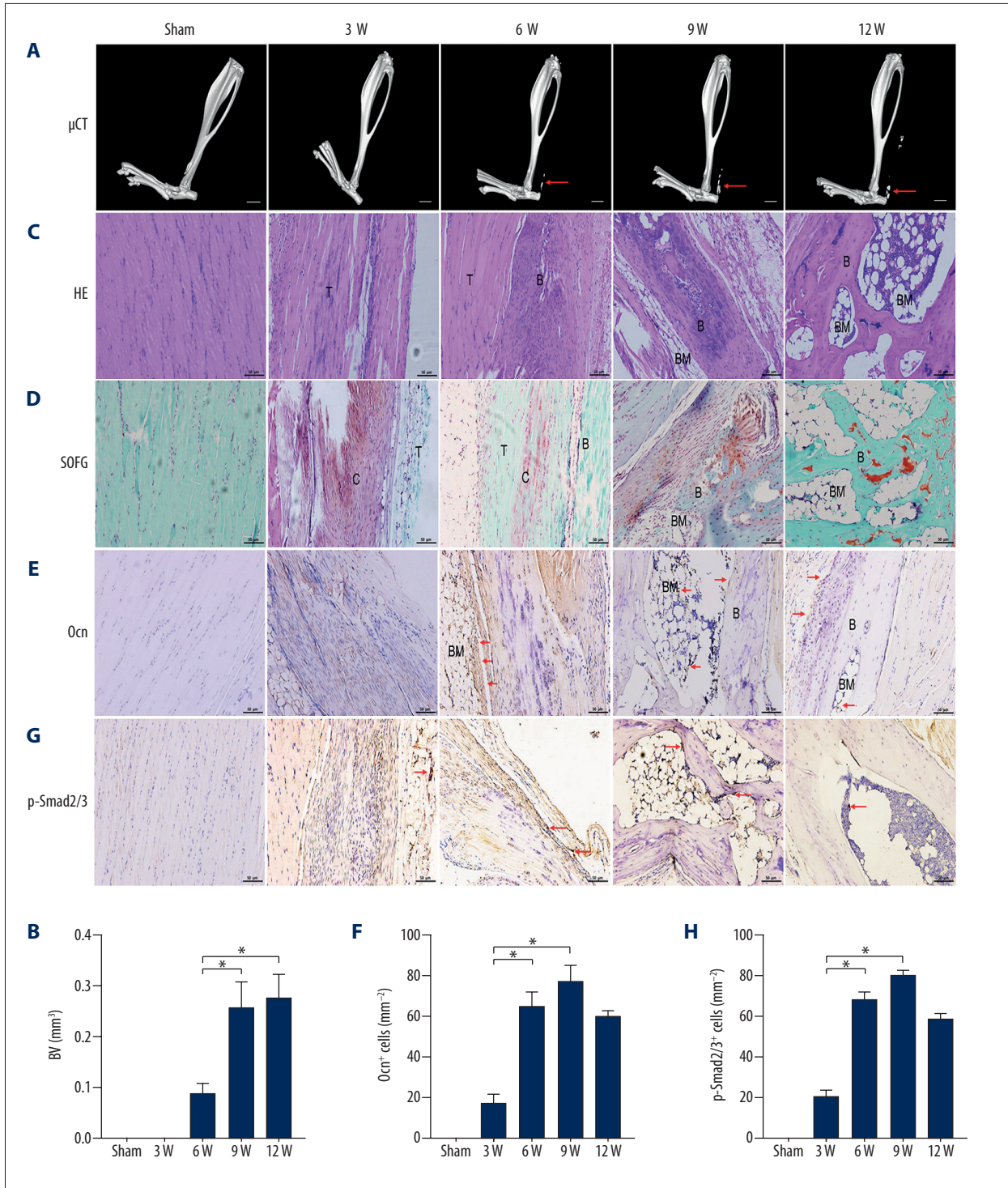
Microcomputed tomography (μ CT) analysis

Samples with tibia and Achilles tendon were dissected and fixed in 10% neutral buffered formalin for 48 hours. A high-resolution Skyscan 1176 (Skyscan, Aartselaar, Belgium) scanner was used to analyze the bone formation. This equipment was set with 50 kV voltage and a resolution of 25 μ m per pixel. HO bone volume (BV) was analyzed by CTVol v2.0.

Histological and immunohistochemical analyses

For histological and immunohistochemical analysis, Achilles tendons with calcaneus were separated and fixed with 10% neutral buffered formalin for 48 hours, then put in 10% ethylenediaminetetraacetic acid (EDTA; pH 7.6) to decalcify for

2 weeks, dehydrated with graded concentrations of ethanol and paraffin embedding. The blocks were sectioned with 4- μ m thick slices longitudinally using a Paraffin Microtome and processed for hematoxylin and eosin (H&E) staining, Safranin O and fast green (SOFG, Solarbio, Beijing, China) staining, osteocalcin (Ocn), and p-Smad2/3 immunohistochemistry staining



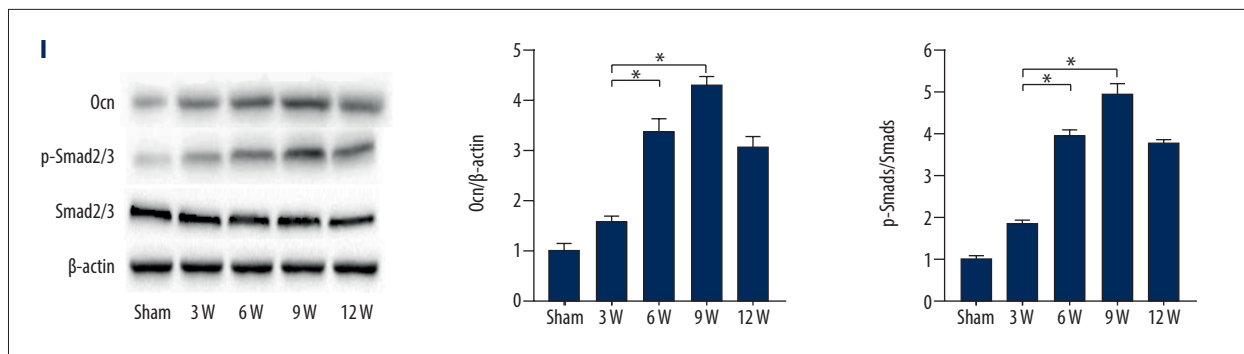


Figure 1. The progression of HO in trauma-induced mice model. (A) Representative imageological examination of Achilles tendons by μ CT. (B) Quantitation of BV at different stages. (C, D) H&E and SOFG staining of Achilles tendons were performed at different stages to observe the osteogenesis process, respectively. (E, F) Representative immunohistochemical staining and quantitative analysis of Ocn-positive cells. (G, H) Immunohistochemical staining and quantitative analysis of p-Smad2/3 positive cells. (I) Representative western blot assay and relative quantification of Ocn and p-Smad2/3 protein levels in Achilles tendons. Scale bars: A, 2 mm; C, D, E, G, 50 μ m. HO – heterotopic ossification; μ CT – microcomputed tomography; BV – bone volume; H&E – hematoxylin and eosin; SOFG – Safranin O and fast green; B – bone; BM – bone marrow; T – tendon; C – cartilage. n=8 per group. All data are present as means \pm standard deviation. * $P<0.05$.

as described previously [22]. We used an optical microscope (Olympus, Japan) for imaging samples. The paraffin mass of each mouse was cut into 3 serial slices, and the number of positive cells was counted in 3 random visual fields. Ocn antibody, p-Smad2/3 antibody, and other related antibodies were all purchased from Abcam company.

Western blot

For Western blot assay, cell lysates were taken from tendons with RIPA lysis buffer (Beijing Labgic Technology Co., Ltd., Beijing, China), and the concentration of protein was quantified by NanoDROD 2000 (Thermo, Waltham, MA, USA). The total proteins (30 μ g) were separated on 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene difluoride (PVDF) membranes (Amersham Corp., Arlington Heights, IL, USA). The membranes were incubated with primary antibodies overnight at 4°C, and secondary antibodies were subsequently used. Proteins were analyzed with antibodies recognizing Ocn, Smad2/3, p-Smad2/3, ER α , and β -actin. All these antibodies were obtained from Abcam. Signals were detected using ECL and exposed to Bio-Rad ChemiDocTM (Bio-Rad, Hercules, CA, USA). The results were analyzed using ImageJ.

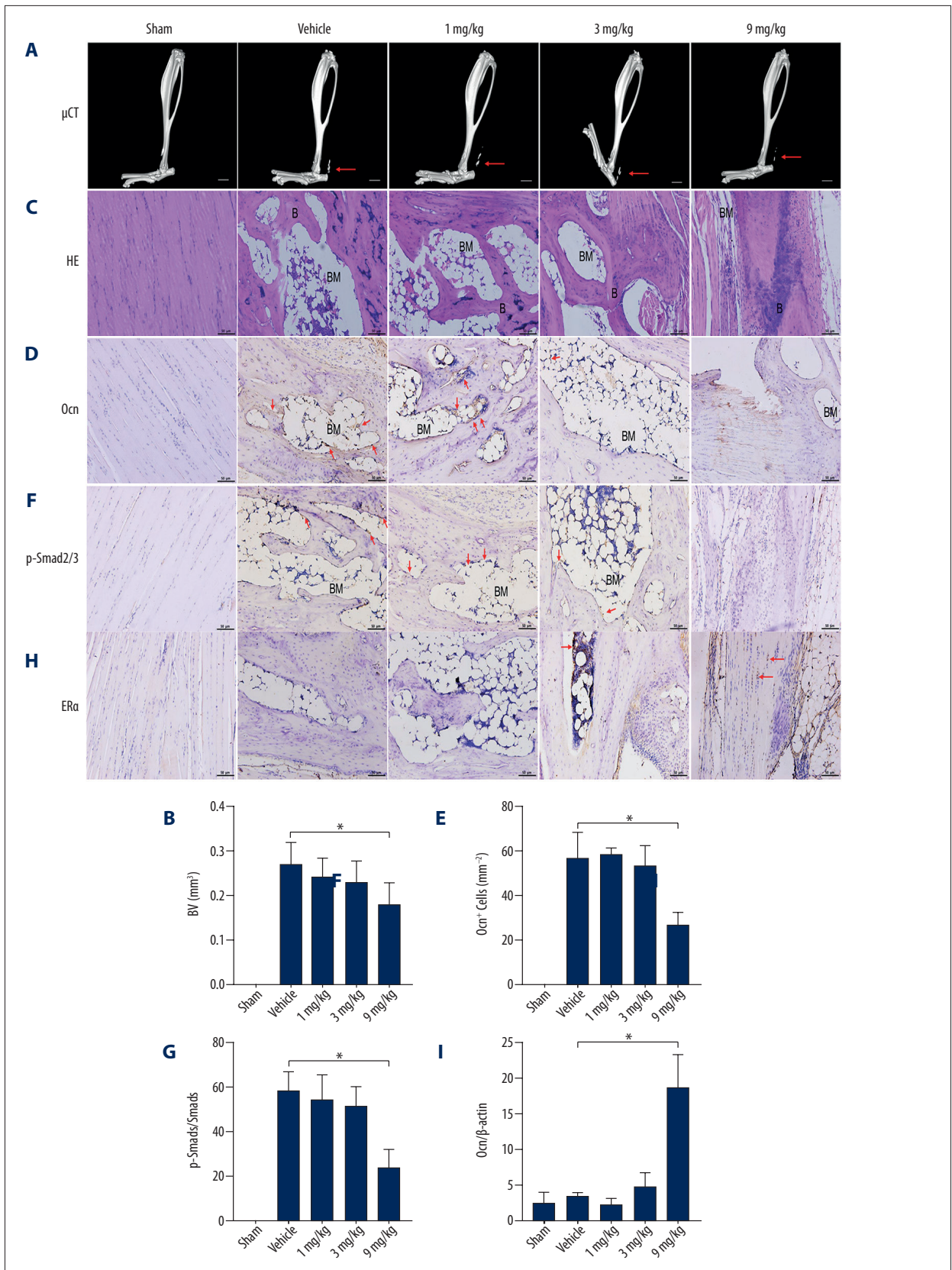
Statistical analysis

All results were expressed as means \pm standard deviation (SD) and performed with SPSS 25.0 software (SPSS Inc. Chicago, IL, USA) for statistical analysis. Differences among groups were determined by one-way ANOVA and the value of $P<0.05$ was confirmed statistically significant and marked as *.

Results

The progression of HO in traumatic Achilles tendons model

In order to study the formation of HO, a trauma-induced HO model was utilized. We compared HO in traumatic Achilles tendons at different periods. Six weeks post puncture, a few ectopic bones became visible in the puncture position of Achilles tendon by μ CT analysis (Figure 1A). With the development of HO, the ectopic bone volume (BV) was enlarging up to 9 weeks and then remains stable to 12 weeks (Figure 1B). H&E and SOFG staining showed typical endochondral osteogenesis in the progression of HO (Figure 1C, 1D). Immature fibrous bones and mature cancellous bones with more bone marrow cavities were shown after 6 weeks and 9 weeks, respectively (Figure 1C). Abundant cartilage tissues around the perimeter of the trauma area were found 3 weeks after puncture and progressively extended to the outside of the HO center where calcified cartilage was newly formed from 3 weeks to 9 weeks (Figure 1D). Osteoblasts with specific expression of osteocalcin (Ocn) were present for bone formation 6 weeks after puncture and decreased at 12 weeks (Figure 1E, 1F). Smad2/3, which transfer intracellular signal as downstream effectors in the TGF- β pathway, are phosphorylated and activated by TGF- β receptors. Similar to Ocn $^+$ cells, p-Smad2/3 positive cells are most numerous at week 9 (Figure 1G, 1H), signifying that the TGF- β pathway is involved in the formation of heterotopic ossification. The protein levels of Ocn and p-Smad2/3 by western blot assay were consistent with the immunohistochemistry results (Figure 1I). All those staining results indicated that the ectopic bone formation after trauma triggered by TGF- β was following the highly orchestrated mechanism of endochondral



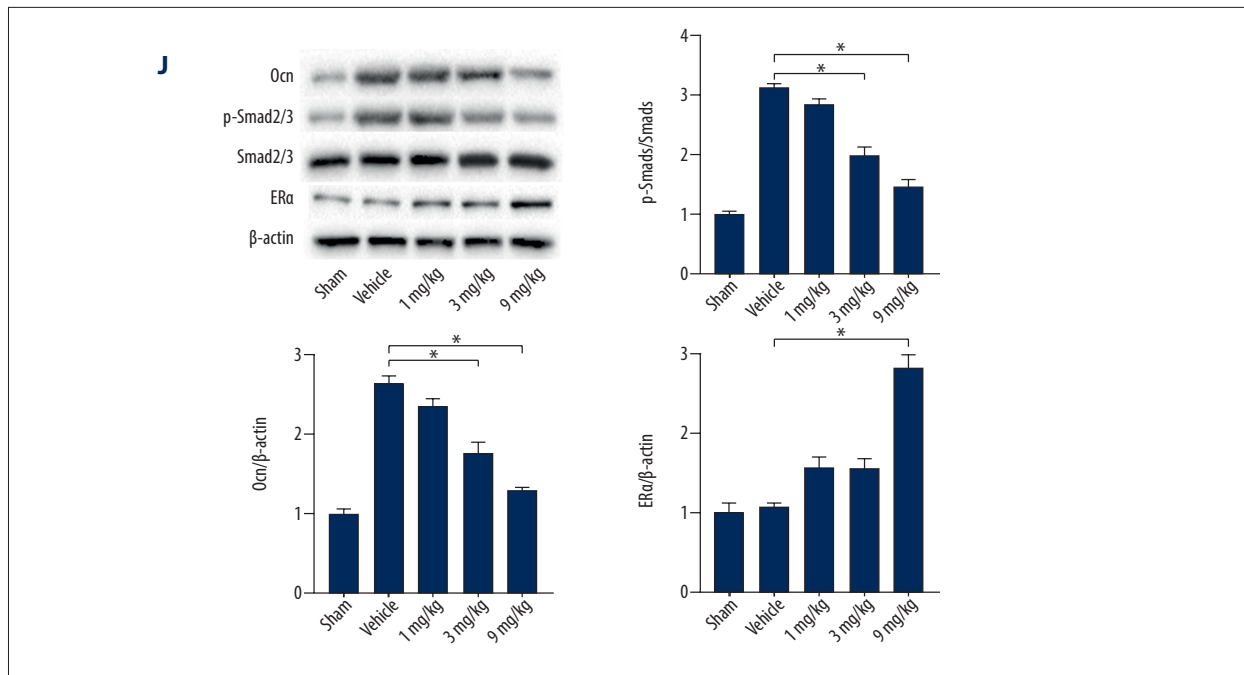


Figure 2. Tamoxifen inhibits HO with different dosages. (A) Representative imageological examination of Achilles tendons by μ CT. (B) Quantitation of BV at different groups. (C) H&E staining of Achilles tendons. (D, E) Representative immunohistochemical staining and quantitative analysis of Ocn-positive cells. (F, G) Immunohistochemical staining and quantitative analysis of p-Smad2/3 positive cells. (H, I) Immunohistochemical staining and quantitative analysis of ER α positive cells. (J) Representative western blot assay and relative quantification of Ocn, p-Smad2/3 and ER α protein levels in Achilles tendons. Scale bars: A, 2mm; C, D, F, H, 50 μ m. HO – heterotopic ossification; μ CT – microcomputed tomography; BV – bone volume; H&E – hematoxylin and eosin; SOFG – Safranin O and fast green; ER – estrogen receptor; B – bone; BM – bone marrow. n=8 per group. All data are present as means \pm standard deviation. * $P<0.05$.

ossification which is identical to the phenomenon of long bones embryonic development.

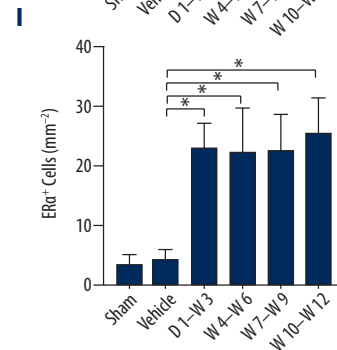
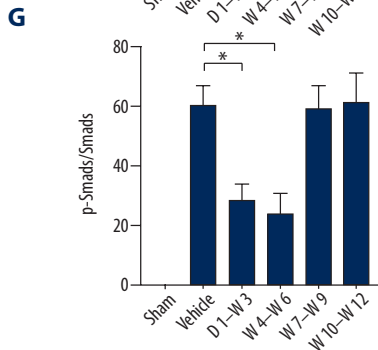
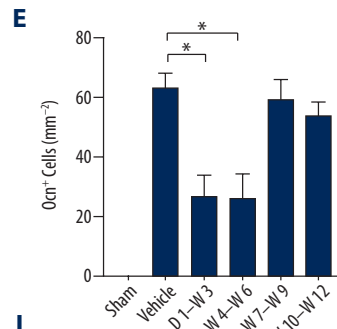
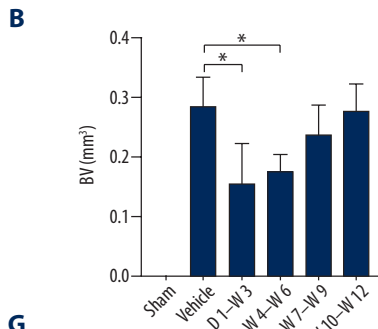
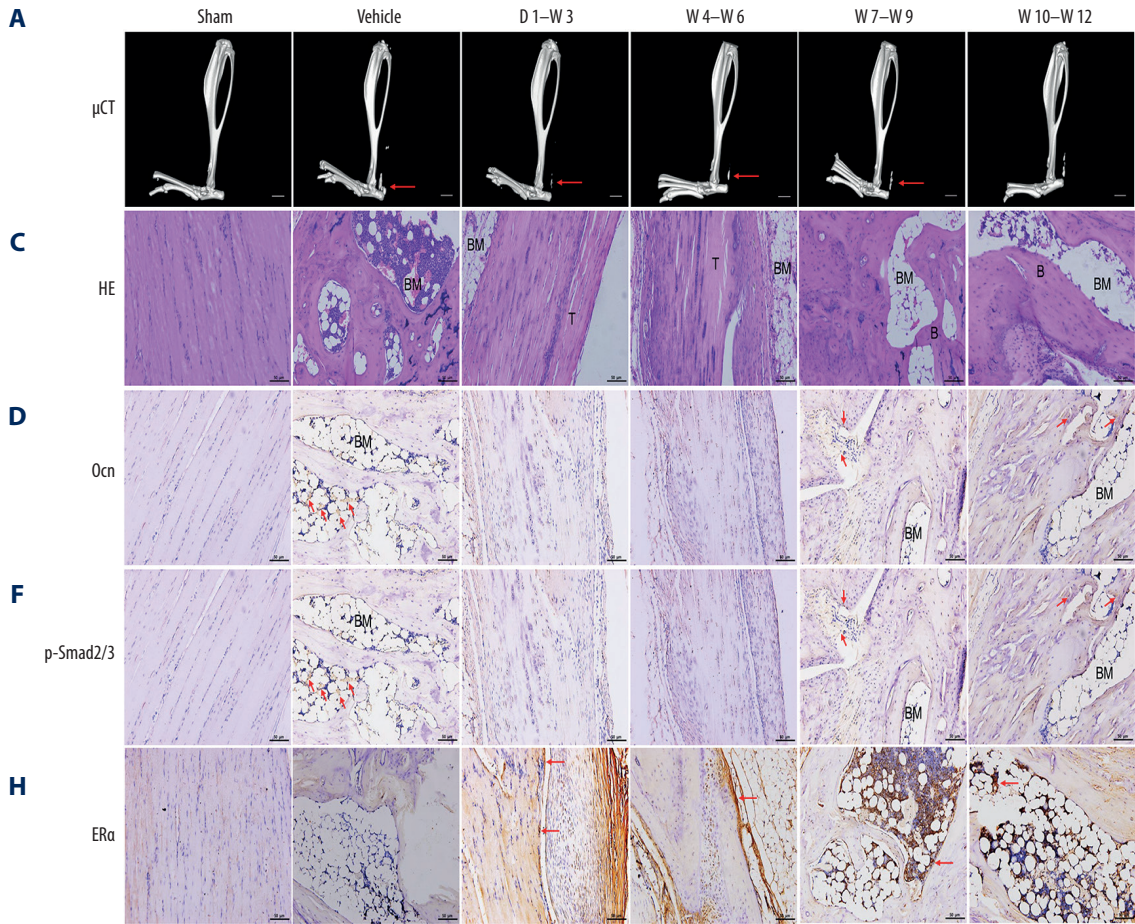
Effect of different doses of tamoxifen on bone formation in HO

To test the effects of tamoxifen on ectopic bone formation in HO, we treated the mice with tamoxifen at 3 different dosages (1, 3, and 9 mg/kg) once every 2 days for a total of 8 weeks and gathered samples 9 weeks after puncture. As shown by the μ CT analysis, compared to vehicle-treated mice, the propagation of HO was significantly attenuated by tamoxifen at 9 mg/kg dosage ($P<0.05$) (Figure 2A, 2B). However, oral gavage of tamoxifen at the dosage of 1 mg/kg and 3 mg/kg did not significantly inhibit HO formation (Figure 2B). H&E staining showed mature bone marrow in the vehicle group, while high dose tamoxifen treatment prevented this ectopic bone formation (Figure 2C). Ocn⁺ osteoblasts by immunohistochemistry staining exhibited a mild reduction of osteogenic activity in 3 mg/kg group but effectively decreased when the mice were treated with tamoxifen at the dose of 9 mg/kg ($P<0.05$) (Figure 2D, 2E). Results of staining for ER α showed that the number of ER α -positive cells was prominently

increased at 9 mg/kg dosage ($p<0.05$) (Figure 2H, 2I), in contrast, the p-Smad2/3-positive cells were diminished significantly at this dosage (Figure 2F, 2G), indicating that ER α activity has an inhibitory effect on the TGF pathway. As shown in Figure 2J, the protein expression levels of Ocn and p-Smad2/3 are significantly reduced with an increasing tamoxifen dose, whereas the level of ER α was raised as feedback on tamoxifen. Taken together, these results manifested an inhibition effect of tamoxifen on the trauma-induced HO model in a dose-dependent fashion, and TGF- β signaling pathway was affected with increasing ER α activity by the stimulation of tamoxifen.

Effect of tamoxifen at different phases of HO progression

To further illustrate the curative effect of tamoxifen for different HO progression stages, mice were administered with tamoxifen (9 mg/kg) every other day for a total of 3 weeks post puncture at different stages of HO progression mainly including inflammatory stage (Day 1–Week 3), chondrogenesis stage (Week 4–Week 6), osteogenesis stage (Week 7–Week 9) and maturation stage (Week 10–Week 12) [25]. Analysis of samples scanned by μ CT revealed that the bone volume of HO was both significantly decreased in mice with the treatment



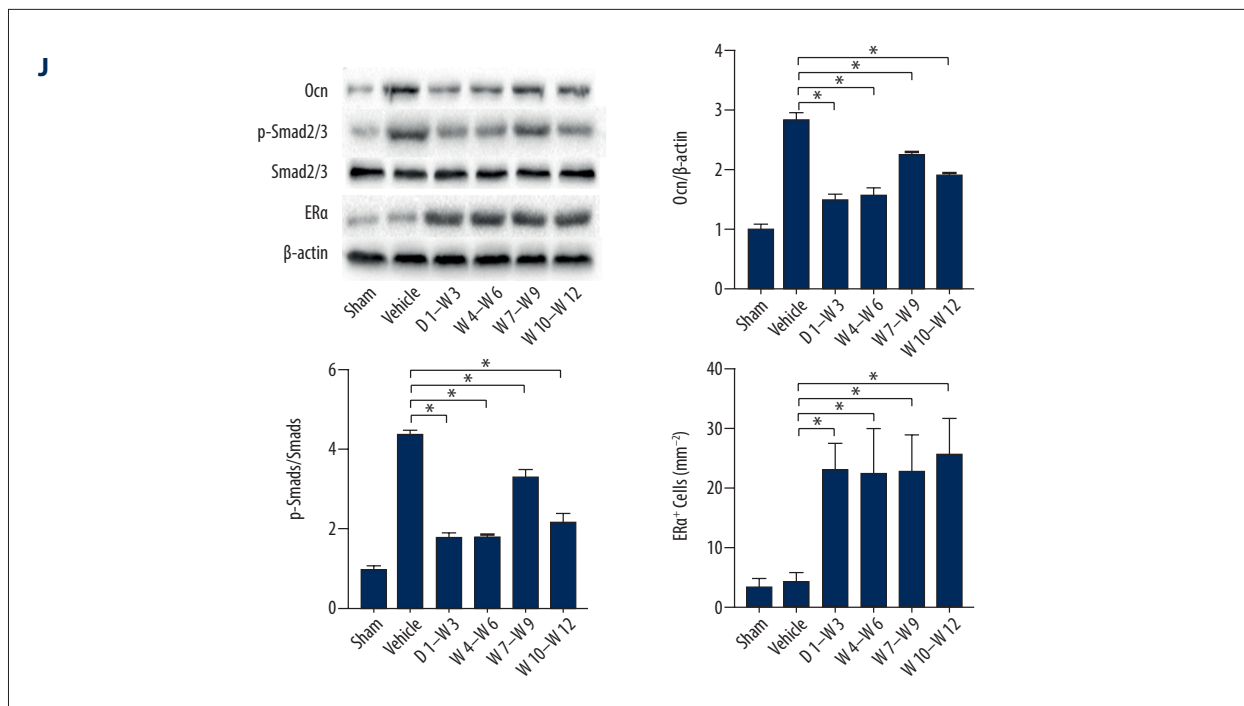


Figure 3. Tamoxifen inhibits HO at different phases of progression. (A) Representative imageological examination of Achilles tendons by μ CT. (B) Quantitation of BV at different groups. (C) H&E staining of Achilles tendons. (D, E) Representative immunohistochemical staining and quantitative analysis of Ocn-positive cells. (F, G) Immunohistochemical staining and quantitative analysis of p-Smad2/3 positive cells. (H, I) Immunohistochemical staining and quantitative analysis of ER α positive cells. (J) Representative Western blot assay and relative quantification of Ocn, p-Smad2/3 and ER α protein levels in Achilles tendons. Scale bars: A, 2 mm; C, D, F, H, 50 μ m. HO – heterotopic ossification; μ CT – microcomputed tomography; BV – bone volume; H&E – hematoxylin and eosin; ER – estrogen receptor; T – tendon; B – bone; BM – bone marrow. n=8 per group. All data are present as means \pm standard deviation. * $P < 0.05$.

of tamoxifen from day 1 and week 4, respectively ($p < 0.05$) (Figure 3A, 3B), whereas no significant reduction was found at week 7 and week 10 groups compared to vehicle-treated mice (Figure 3B). The bone marrow cavity showed by H&E staining was diminished with the treatment of tamoxifen from day 1 and week 4 relative to week 7, week 10 and vehicle groups (Figure 3C). In addition, the number of Ocn⁺ osteoblasts (Figure 3D, 3E) and p-Smad2/3⁺ cells (Figure 3F–3I) were both significantly reduced in mice treated with tamoxifen at inflammatory and chondrogenesis stages relative to vehicle-administered mice ($p < 0.05$), showed an inhibitory bone propagation. The downtrends in Ocn and p-Smad2/3 protein levels are consistent with immunohistochemistry results, and administration of tamoxifen at different periods can significantly improve the activity of ER α (Figure 3J). Collectively, all these results demonstrated that HO propagation could be attenuated by tamoxifen at the early stages of inflammation and chondrogenesis accompanied with the TGF- β signaling pathway was suppressed by ER α .

Discussion

HO was first reported by Patin in children with myositis ossificans progressive more than 300 years ago [26]. As of now, there are no effective medical treatments for HO, because the potential cellular and molecular pathogenesis and mechanisms have not been fully elucidated, resulting in surgical resection as the preferred treatment for already formed HO. However, this is not recommended as surgery can enlarge the size of the lesion, inducing wound healing complications, and recurrent heterotopic ossification [27]. Developing new drugs with minimal side effects will provide additional approaches for the prevention and treatment of trauma-induced or genetic HO. Our data in the present study demonstrated an inhibitory effect of tamoxifen in a dose dependence manner on the inchoate progression of HO in a female mice model. Using an Achilles tendon trauma-induced HO mice model, we found HO in the Achilles tendon body was typically endochondral in structure and laid down via a cartilaginous matrix which reflects the cartilaginous metaplasia similar to human patients. Ossification is a common complication of Achilles tendon injury especially observed in athletes [28]. The incidence of HO after repair

of Achilles tendon rupture was reported to be 14–62% [29]. We can use this model to mimic the formation of HO in human Achilles tendon, although most HO is induced by either musculoskeletal trauma or neurogenic injuries. This experimental paradigm also serves as a powerful tool to examine the efficacy of drugs which particularly target the molecular signal pathways underlying heterotopic ossification in tendons or ligaments.

A series of retrospective studies and original articles found that men have higher incidence rates and are more susceptible than women in HO after trauma and articular surgeries [13,14,18,30]. Nowadays, researchers are paying more attention to the correlation between morbidity of HO and gender difference, which is inferred as a risk factor, to substantially illuminate the mechanism of ectopic bone formation. As a kind of steroidal sex hormone, estrogen can pass through the cytomembrane freely and migrate into the nucleus then bind to its estrogen receptors (ER) α and β , and modify gene expression. ER α and ER β were both expressed in human ligaments, tendons, and osteoblasts [31–33], elevating estrogen levels is beneficial to protect tendons and ligaments health [34–37]. Beyond its central role in the sexual gland, estrogen has an important function in the differentiation and development of the skeleton and connective tissues via cross-talking with other cytokines, such as TNF- α and TGF- β [38–40]. A recent article showed that overactive TGF- β that was elevated by inflammation initiated HO formation in trauma-induced and BMP-induced mice models, and this progression were remarkably attenuated with the inhibition of TGF- β [22]. The aberrant TGF- β signaling may become a potential therapeutic target for HO in the future.

As an anti-cancer drug, tamoxifen has been proven by preclinical studies to exert species-specific pharmacologic impacts in different species, acting as a partial antagonist in rats, chickens, and humans, whereas as an estrogen agonist in mice [41]. From the results of this study, we can conclude that the prevention of HO mediated by tamoxifen is predominantly via the interaction between the estrogen receptor and TGF- β /Smad signaling pathway. As the activity of ER α was stimulated by tamoxifen in mice, the expression of p-Smad2/3 (which are downstream transcription factors of TGF- β signaling pathway

and essential in the progression of acquired and congenital HO) was decreased in a dose-dependent response to tamoxifen, following the overactive TGF- β signaling which was interrupted in the inflammation and chondrogenesis phases and subsequently the ectopic bone formation was retarded. This cross-regulation between ER α and TGF- β is consistent with a recent study of Peng et al. [42]. It showed that estrogen has a potential inhibitory effect on TGF- β 1 signaling pathway by reducing the expression of Smad2/3 after inflammation. Likewise, by inhibiting TGF- β /Smad pathway through ERs binding to Smad2/3, estrogen attenuated elastogenesis was induced by TGF- β 1 in rat urethral smooth muscle cells [43]. In addition, ER α could inhibit the TGF- β signaling pathway via forming a complex with Smads and ubiquitin-protein ligases, and induced Smads ubiquitination and subsequent degradation in an estrogen-dependent manner [39]. Although a lot of research has been performed on the cross-talk between estrogen/ERs and TGF- β /Smad pathway, however, the underlying molecular mechanisms of tamoxifen on the inhibition of HO, or in contrast, whether androgen has a positive role in the aggravation of HO formation, and whether tamoxifen can inhibit BMP-induced or congenital HO still need to be further studied thoroughly.

In summary, we showed that tamoxifen as an estrogen receptor agonist in mice could effectively inhibit the initiating heterotopic bone formation in female mice HO model by mediating the cross-talk between estrogen receptors and TGF- β signaling pathway. Thus, it might shed light on the effective clinical therapeutic strategies of estrogen or the relevant hormones for the treatment of trauma-induced or genetic heterotopic ossification in humans, especially for women.

Conclusions

We revealed an unappreciated role of tamoxifen on the inhibition of trauma-induced heterotopic ossification in female mice.

Conflict of interests

None.

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