

A higher expression of P2X7 receptor at the site of heterotopic ossification promotes osteogenesis

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Research article

Keywords: ATP-signal, BBG, Heterotopic ossification, inflammation condition, osteogenesis, P2X7R

Posted Date: May 28th, 2021

DOI: <https://doi.org/10.21203/rs.3.rs-561162/v1>

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Abstract

Background

Heterotopic ossification (HO) refers to a painful and complex disease. Adenosine triphosphate (ATP), as a key modulator of inflammation, is verified to promote the development of HO. However, the mechanism remains to be illustrated. The ionotropic P2X7 receptor (P2X7R) is an ATP-gated ion channel expressed in the majority of stem cells. Here, this paper hypothesizes that P2X7R may be activated by extracellular ATP and promote osteogenesis of stem cells under inflammatory condition, ending up in the formation of ectopic bone.

Methods

The tenotomy puncture and burn injury-induced HO model was constructed. The expression of P2X7R was found increasing at the site of injured Achilles tendon where HO occurs. Mesenchymal stem cells (MSCs) were cultivated under an inflammatory condition plus Bz-ATP treatment which mimicked a microenvironment of HO site. An induction in P2X7R expression was also observed along with an enhancement of osteogenesis. In addition, an inhibition of P2X7R expression by its specific antagonist successfully reversed the above process.

Results

P2X7R expression of the Achilles tendon and osteogenic capability of SCs is higher in HOG than in other two groups. Bz-ATP promoted osteogenesis under inflammation condition. BBG impeded the heterotopic bone formation in animal model.

Conclusions

P2X7R is a crucial mediator in ATP-signaling promotion of HO, blocking which may represent a potential therapeutic target for HO.

Background

Heterotopic ossification (HO) refers to a complex, reactive, musculoskeletal condition characterized by ectopic bone formation in muscles, tendons, or other soft tissues^[1]. HO tends to be accompanied by trauma, burns, and orthopedic surgeries etc. There are many theories explaining why it develops. However, the mechanism remains largely unknown. Therefore, there are no completely effective treatment methods that can be taken to prevent this clinically devastating pathological condition.

Tissues vulnerable to HO act out a dysregulated inflammatory response to injury. Dwelling in a prolonged or abnormally heightened inflammatory microenvironment ^[2–3], stem cells including mesenchymal stem cells (MSCs) that attempt to repair tissue differentiate into osteogenic cells instead of its original fate. Moreover, such osteogenic cells have witnessed the intensifying formation of bone which has been recognized as a fundamental process for HO. Adenosine triphosphate (ATP) is a key modulator of inflammation ^[4]. Prior research has illustrated that extracellular ATP is of importance to inflammation-induced HO, as it interacts with bone morphogenetic protein–mediated canonical SMAD signaling ^[5]. Moreover, with the application of apyrase, an enzyme that hydrolyzes ATP to adenosine monophosphate (AMP), sees a rise in intracellular adenylate cyclase activity and cyclic AMP (cAMP). The reduction of bone formation was achieved at the site of HO ^[5].

The ionotropic P2X7R is an ATP-gated ion channel expressed in most stem cells. Activated by extracellular ATP, P2X7R plays a wide range of physiological and pathological roles in inflammation and inflammation-related condition^[6]. It is noteworthy that P2X7R expression on bone marrow MSCs has been found to regulate the formation of osteoblasts at different stages to orchestrate bone metabolism ^[7]. Also, recent researches have confirmed the crucial role for P2X7R in periodontal ligament stem cells (PDLSC) osteogenesis under inflammatory condition ^[8]. Nonetheless, the expression pattern of P2X7R on MSCs at the HO site and whether it is essential to the mediation of ATP signaling-related bone formation remain unknown.

Here, this paper applied a tendon puncture and burn injury-induced HO model. The increasing expression of P2X7R emerged at the site of injured Achilles tendon where HO occurs. Subsequently, *in vitro* model mimicking an inflammatory microenvironment with ATP intervention around MSCs was establishment. An induction in P2X7R expression was also observed along with an enhancement of osteogenesis. In addition, an inhibition of P2X7R expression by its specific antagonist successfully reversed the above process, which may suggest that it could be a potential therapeutic target for HO.

Method

Animal model

The animal experiment was grouped into three cohorts, sham group(Sham), Achilles tendon puncture group (Tendon puncture group, TPG), 30% total body surface area (TBSA) partial thickness burn injury combined Achilles tendon puncture group (HOG), respectively. Partial thickness scald injury was performed as Peterson JR et al. described^[9]. In brief, 8 weeks, male, C57BL/6 mice in sham group, the Achilles tendons were exposed alone. In TPG, after Achilles tendon exposed, a 27G needle was punctured into the Achilles tendon body perpendicularly at different parts from proximal to distal and this process was repeated three times. In HOG, apart from the Achilles tendon punctured, mice also received about 30% TBSA burn injury with exposure to 60°C for 18 s (Fig. 1a) ^[10].

Cell harvest and in vitro culture assays

Achilles tendon stem cells were harvested from the different groups. Stem cells (SCs) from Achilles tendon were isolated based on the research Xu et al. reported^[8], and modified slightly. In short, freshly harvested tendon was washed with sterile phosphate-buffered saline (PBS; Gibco, New York, USA), and then tendon tissue was placed into 30mm dish. The tissue was cut into fragments and digested in 3 mg/ml type I collagenase (Sigma-Aldrich, St. Louis, USA) for 45 minutes. Then, the digested fragments were cultivated in 35 mm dish (Corning, New York, USA) in α -minimum essential medium (α -MEM, Gibco) containing 100 U/mL penicillin (Gibco), 100 μ g/mL streptomycin (Gibco), and 10% fetal bovine serum (FBS; Gibco, Australian). In *in vitro* experiment, mesenchymal stem cells (MSCs) were purchased from the Peking Union Medical College Hospital cell bank. The complete culture medium was changed every 3 days. Cell passage was performed until the cell fusion rate reached about 80 ~ 90%. MSCs at passage 2–5(P2-P5) were used for further experiment. For osteogenic differentiation, complete osteo-inductive medium was replaced every 2 days when the percentage of cell fusion dipped to 60–70%. Osteo-inductive medium contained α -MEM and 10% FBS supplemented with 100 U/mL penicillin, 100 μ g/mL streptomycin, 10 mM β -glycerophosphate, 50 μ g/mL L-ascorbic-2-phosphate and 10 nM dexamethasone (all from Cyagen). TNF- α (10 ng/ml) and IL-1 β 5 ng/ml was added to the medium of MSCs to simulate inflammation environment *in vitro*^[11].

Micro CT

Micro CT imaging of HO of Achilles tendon was obtained from all animals by means of the high-resolution Micro CT equipment (Siemens, Munich, Germany). CT scan settings: 80 kV, 500 μ A, and 1100-ms exposure. The bone mineral in the soft tissues was quantified for HO bone volume using standard protocols as Peterson et al. described^[12].

Immunofluorescent Staining

MSCs were seeded into a 96-well culture plate designed for confocal microscopy and cultivated in α -MEM containing 10% FBS. When the confluence of cells reached 80 ~ 90% (day 0), different condition was added respectively for intervention. After 7d culture or 14d osteogenesis inducing, adherent cells were fixed with 4% paraformaldehyde, permeabilized with 0.3% Triton X-100 for 15 min and blocked with 10% goat serum for 40 min at room temperature. The cells were then incubated at 4°C with an antibody against P2X7R (1:200, Abcam, ab48871) overnight. The samples were washed with PBST, and then incubated in the dark place at 25°C with Alexa Fluor 488 goat anti-rabbit IgG secondary antibody (1:500; Abcam, ab150077) for 2 h. Finally, the cells were stained in a dark room at 25°C with DAPI for 30 min. Images were captured by a confocal laser scanning microscopy (A1R S1; Nikon, Tokyo, Japan).

ALP staining

ALP activity in MSCs was analyzed with ALP staining. In brief, cells were seeded at 1×10^4 cells/well in 96-well plates. ALP staining was performed after 14 days of osteo-inductive culture using an ALP staining kit (Beyotime Biotechnology, China) according to the instructions. The quantitative analysis of

ALP staining was performed with the help of an ALP assay kit (Nanjing Jiancheng Bioengineering Institute, China).

ARS staining

According to the manual instructions, Alizarin red staining (ARS, Cyagen, Suzhou, China) was completed which was used to evaluate the osteogenic differentiation of cells. To quantify the staining for statistical analysis, the mineralized nodes were dissolved in 2% cetylpyridinium chloride for 20 min, and then OD values were measured at 560 nm wavelength^[13].

Protein assays

The protein expression profiles of cells or tissue (prepared for analysis) were measured by ProteinSimple Jess Capillary electrophoresis instrument. In brief, total proteins were extracted using RIPA lysis (Proteintech, PR20001). After cell lysis of 30 minutes, the supernatant was collected by centrifugation with 13,000g. Protein concentration was determined by BCA kit (Keygen BioTECH, Jiangsu, China). Supernatant were run on a ProteinSimple Jess Capillary electrophoresis instrument using instrument default settings and manufacturer's standard protocol^[14]. Approximately 3µg (1µg/µl) total protein were loaded per capillary. Quantification of the final images was carried out using compass for SW software (Version 5.0.1).

qRT-PCR assays

Total RNA was extracted using RNAeasy Kit (Vazyme, RC112-01) and 1 µg of total RNA was used to generate cDNA using HiScript II Q RT SuperMix (Vazyme, R223-01) for qPCR. Quantitative Real time PCR (qRT-PCR) was performed on cDNA samples diluted twentyfold in double-distilled water using SYBR Green Master Mix (Vazyme, Q711). The CFX Connect™ Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA) was used. To amplify them under the following conditions: denaturation at 95°C for 5 min; 40 cycles at 95°C for 10 s, 55°C for 20 s, and 72°C for 20 s, with melt curve analysis from 65 to 95°C in increments of 0.5°C. The gene-specific qRT-PCR primers were specific for mouse. Relative mRNA gene expression levels were measured by qRT-PCR. Relative quantification for qRT-PCR was calculated using the $2^{-\Delta\Delta CT}$ method. The sequences of primers used in the present study are shown in supplementary *Table. 1*.

Statistical Analysis

All results are calculated by the mean and standard deviation (mean ± S.D.). There are more than three samples. Experiments for each cell were performed independently for three times. GraphPad Prism 9 software was utilized for statistical tests. The Student's t-test was used for analysis of two unpaired groups, and one-way ANOVA followed by Tukey's posttest for analysis of multiple groups. Statistical significance was established at $p < 0.05$.

Ethics statement

The ethics committee of the general hospital of People Liberate Army of China and Harbin Medical University approved this experiment.

Results

P2X7R expression of the Achilles tendon and osteogenic capability of SCs is higher in HOG than in other two groups

In this study, TPG and HOG all significantly had given rise to the HO. The largest size of heterotopic ossification formation was in HOG, and the no HO was observed in the sham group (Fig. 1b). Then, this paper compared the bone volume of HO in different groups. These disparities were of statistical significance. Bone volume (BV) of HO in HOG and TPG outstripped that of Sham, p values were less than 0.0001 and 0.0001, respectively. There was a significantly higher BV in the HOG than in the TPG ($p < 0.01$) (Fig. 1b). Burn injury enhanced bone formation as previous reports^[15-16].

After the establishment of the model, at 7 days, the Achilles tendon was resected for further study. Then, the Achilles tendon tissues of different groups received pathological analysis. Among three groups (HOG, TPG and Sham), the fluorescence intensity of P2X7R in Achilles tendon was considerably different from each other ($p < 0.05$) (Fig. 1c). In HOG and TPG, the immunofluorescence (IF) intensity of P2X7R was significantly higher compared with sham group ($p < 0.001$) (Fig. 1d). The expression of P2X7R protein also differed in three groups. HOG and TPG had higher expression level compared to sham group. However, there was no significant difference between HOG and TPG ($p > 0.05$) (Fig. 1e).

Then, the osteogenic capabilities of SCs extracted from Achilles tendon in different groups were examined. Osteogenic differentiation was evaluated by alkaline phosphatase (ALP) staining. After 14 days of the osteogenic induction, osteogenesis differentiation of the SCs was assessed by ALP (Fig. 1f). The results of the ALP positive cells suggested the difference among the different groups. In HOG, SCs had better osteogenesis compared with the other groups. The software of Image J was employed to quantify ALP staining. The ability of osteogenesis of SCs from HOG was higher than that of the TPG and sham on account of a higher cell ALP staining positive area rate (Fig. 1g) ($p < 0.0001, 0.001$, respectively). The result indicates that the higher expression of P2X7R, the higher ability of osteogenesis of SCs. Therefore, the ATP and inflammation condition plays an important role in the formation of HO.

P2X7R agonist promoted the expression of P2X7R of MSCs under inflammation condition

During the HO formation, inflammation environment is an essential part^[10]. To exclude other purinergic receptors' interference, Bz-ATP, a P2X7R specific agonist was used. This investigated the effect of Bz-ATP (100 μ M) under inflammation condition on P2X7R of MSCs. MSCs grown in normal growth medium with the Bz-ATP added served as the control. MSCs grown in inflammation condition medium with the Bz-ATP served as the experimental group. IF analysis illustrated that under inflammation condition, Bz-ATP

promoted the expression of P2X7R (Fig.2a). Relative fluorescence intensity of P2X7R between two groups had significant difference. In the Bz-ATP combined inflammation group was higher than the other group significantly ($p<0.001$) (Fig.2b). Similarly, the results of protein assays and qRT-PCR analysis supported the outcome of IF (Fig.2c, d). These results confirm that under inflammation condition, Bz-ATP stimulates the overexpression of P2X7R.

Bz-ATP promoted osteogenesis under inflammation condition

To further investigate the role of increased expression of P2X7R on osteogenesis under inflammatory condition, Bz-ATP was added in medium. To test the osteogenic differentiation variances among the different groups, ALP staining and Alizarin Red staining(ARS) were performed, and then ALP activity and ARS solution absorbance was measured. Under inflammation condition, Bz-ATP intervention brought about a remarkable surge of ALP activity and the mineralized nodes formation, compared with no inflammation condition intervention. ALP activity and staining revealed higher ALP activity in inflammation condition intervention group than in the no inflammation condition intervention group ($p<0.05$). The results of ARS staining was also similar to ALP ($p<0.05$) (Fig.3a). According to the qRT-PCR analysis, under the same Bz-ATP intervention, in the group of inflammation condition intervention, mRNA expression levels of osteogenic-associated genes such as *ALP*, *RUNX2*, *OPN* were significantly higher compared with the other group ($p<0.001$, $p<0.05$, $p<0.0001$, respectively) (Fig.3b). Similarly, protein analysis maintained that under the same Bz-ATP intervention, the expression of ALP, RUNX2 and OPN protein were significantly higher in the inflammation condition intervention than that of without the inflammation condition ($p<0.001$, $p<0.001$, $p<0.001$, respectively) (Fig.3c). Based on the above data, when Bz-ATP was added into osteo-inductive medium, the expression of P2X7R and the ability of osteogenesis of MSCs were enhanced under inflammation condition.

Brilliant Blue G (BBG) decreased the osteogenetic ability of MSCs promoted by Bz-ATP under inflammation condition

To diminish the effect between overexpression of P2X7R and the enhancement of osteogenesis of MSCs under Bz-ATP and inflammatory condition, the P2X7R antagonist BBG was employed. When BBG (10 μ M) was added into osteo-inductive medium under Bz-ATP and inflammation condition, it significantly crippled the expression of P2X7R proved by the IF analyse (Fig4. a). Relative fluorescence intensity of P2X7R between two groups had significant difference (Fig4. b). Similarly, qRT-PCR analysis showed that the inhibition of P2X7R with BBG in MSCs significantly curtailed P2X7R *mRNA* expression (Fig4. c). Then, the ALP and ARS staining were conducted to explore the intervention of BBG on MSCs osteogenesis under inflammation and Bz-ATP condition. The results of ALP activity and staining revealed that ALP activity in no BBG intervention group was higher than that of BBG intervention (Fig. 4d) ($p<0.05$). The results of ARS staining also had the similar result as ALP (Fig. 4d) ($p<0.01$).

To further investigate the mechanism of BBG on osteogenesis of MSCs under Bz-ATP and inflammation condition, the methods of qRT-PCR and protein assays were used. According to the qRT-PCR, with the BBG intervention, the *mRNA* expression of osteogenic-related genes, such as *ALP*, *RUNX2*, *OPN*, deceased

significantly ($p < 0.0001$, $p < 0.0001$, $p < 0.01$, respectively) (Fig. 4e). Similarly, the results of protein assays confirmed that BBG also decreased the expression of ALP, RUNX2, OPN of MSCs in comparison to the no BBG intervention ($p < 0.01$, $p < 0.05$, $p < 0.01$, respectively). Taken together, under Bz-ATP and inflammation condition, the intervention of BBG could control the osteogenesis of MSCs. Cytological results of BBG intervention may provide a novel treatment for HO.

Intervention of BBG reduced heterotopic bone formation in animal model

To further confirm whether the therapeutic effects of intervention of BBG in animal model, animal experiments were carried out. HO animal model of the 30% TBSA partial thickness burn injury combined Achilles tendon puncture was used. 6 hours after the modeling, the intervention of BBG (50 mg/kg) by intraperitoneal administration was employed. The BBG administered once daily for 14 consecutive days, and the mice were watched for 12 weeks. Saline solution was injected as the control. Micro CT analysis was conducted to evaluate heterotopic bone formation at 1st, 6th and 12week (Fig. 5a). At the 1st week, there was no heterotopic bone formation between two groups. At the 6th week, there was heterotopic bone formation in two groups. The difference of bone volume (BV) of heterotopic bone between two groups was statistically significant ($p < 0.05$). At the 12th week, the difference of BV was more significant between the two groups ($p < 0.0001$) (Fig. 5b). Based on the result of animal experiment, treatment of BBG reduced heterotopic bone formation in HO model.

Discussion

Inflammation has been confirmed to be a key driver of HO [17]. A commonality across many of the conditions that predispose to HO formation such as autoimmune diseases, trauma, burn etc. is that they are more or less related to inflammation [15]. Clinical application of NSAIDs for HO prophylaxis rests on the theory that reducing postoperative inflammation will likewise reduce HO formation albeit that no robust evidence has been provided [1]. Prior review by Benjamin Levi et al. summarized the crucial role of inflammation in development of HO [18]. Among which, processes involved in innate immunity, adaptive immunity, secretion of inflammatory cytokines had all been demonstrated to be of importance underlying the mechanism of HO [18].

As a key inflammatory mediator, ATP acts as not only an energy supply molecule but also a signal transducer released upon stimulation or due to passive leakage of damaged or dying cells. The role of extracellular ATP in inflammation is supported by the direct in vivo demonstration that inflammatory sites contain high (hundred micromolar) extracellular ATP concentration [4]. In HO model, prior research has shown that extracellular ATP acted as a signal transducer, which interacts with bone morphogenetic protein mediated canonical SMAD signaling to promote HO development [5]. Moreover, with application of apyrase, a rise occurs in intracellular adenylate cyclase activity and cAMP and a reduced bone formation was achieved at the site of HO [5]. In this study, when MSCs were cultivated under Bz-ATP and inflammation condition, the result showed a significant enhancement of osteogenesis compared with

those without inflammation condition, which also confirmed the crucial role of inflammation condition in HO formation.

Almost all living organisms, from protozoa to higher mammals, have evolved a sophisticated receptor set for extracellular nucleotides. In higher mammals, the nucleotide receptor family (P2 receptors, P2Rs) is comprised of two subfamilies: G protein- coupled metabotropic P2Y (P2YR) and ligand (ATP)-gated ionotropic P2X receptors (P2XR)^[19]. Among these family, P2X7R is the most studied and outstanding as the single member of the P2XR family with a firmly established role in multiple inflammatory and immune responses^[6]. Prior studies have shown that P2X7R plays a crucial role of bone homeostasis with controversial conclusions^[19–20]. The P2X7R has previously been shown that it plays a crucial role in the bone anabolic under the mechanical loading^[21]. P2X7R involves bone formation through Wnt/ β -catenin signaling^[22]. But another study points out that at low concentrations of Bz-ATP (from 0.5 to 5.0 mM), Bz-ATP not only decreases the mRNA expression of Wnt3a, but also reduces mineralization without effect on proliferation^[23]. In this study, *in vivo* model showed an intensified expression of P2X7R in the injured Achilles tendons together with a formation of exotic bone. Using *in vitro* model mimicking an inflammatory microenvironment with BZ-ATP, an induction in P2X7R expression was also observed. As exposed to an osteogenesis condition medium, an enhancement in osteogenesis was achieved in MSCs and they expressed higher P2X7R. Moreover, this enhancement was attenuated when BBG was added into the medium. These results suggested that P2X7R plays a crucial role in the mechanism underlying inflammation-induced HO.

BBG is a P2X7R antagonist of low toxicity and high selectivity. Previous studies have reported that BBG treatment reduces tissue injury and promotes motor function recovery after spinal cord injury (SCI)^[24–25]. There are some limitations in this research. Although the treatment of BBG reduces progression of heterotopic ossification in animal model, the role of BBG in the initiation occurrence of heterotopic ossification is uncertain. In clinical research, progression of heterotopic ossification in tendon leads to restricted joint mobility and pain^[26]. Nevertheless, there are differences in current HO prophylaxis. Taken together, the treatment of reducing the rate of surgery is a key for HO prevention and management. Because BBG inhibits osteogenesis of MSCs under inflammation and ATP condition, it may provide a new therapeutic target for HO in the future.

Conclusion

The expression of P2X7R was found increasing in tendon puncture and burn injury- induced HO. A combination of inflammatory condition and ATP may be the trigger of this up-expression of P2X7R. An induction in P2X7R expression led to an enhancement of osteogenesis while an inhibition of P2X7R attenuated it, which may suggest that P2X7R is a crucial mediator underlying the mechanism of HO. This study may bring forward a novel therapy for the HO.

Abbreviations

AMP	Adenosine Monophosphate
ARS	Alizarin Red Staining
ATP	Adenosine Triphosphate
BBG	Brilliant Blue G
BV	Bone Volume
cAMP	Cyclic AMP
HO	Heterotopic Ossification
HOG	Heterotopic Ossification Group
IF	Immunofluorescence
MSCs	Mesenchymal Stem Cells
P2 receptors	P2Rs
P2X7R	P2X7 Receptor
P2XR	P2X Receptors
PDLSC	Periodontal Ligament Stem Cells
SCI	Spinal Cord Injury
SCs	Stem Cells
TBSA	Total Body Surface Area
TPG	Tendon Puncture Group

Declarations

Ethics approval

The ethics committee of the general hospital of People Liberate Army of China and Harbin Medical University approved this experiment.

Consent to participate

Not applicable

Consent for publication

Written informed consent for publication was obtained from all participants.

Availability of data and material

The results of data and material included in this study may be available on request, with a clear statement of purpose.

Competing interests

The authors declare that they have no competing interests.

Funding

This work was supported by the National Natural Science Foundation of China (No. 81972115, No. 81772369, No. 82002330, No. 82072472), and Natural Science Foundation of Heilongjiang Province (ZD2020H003).

Authors' contributions

The study was designed by Shi Cheng, Pengbin Yin, Jinglong Yan, Songcen Lv, Licheng Zhang, and Peifu Tang. Acquisition of the data was performed by Yi Li, Ming Chen, Siqi Zhang, Duanyang Wang, Yuan Lin, Shi Cheng, Pengbin Yin, Jinglong Yan, Songcen Lv, Licheng Zhang, and Peifu Tang. Analysis and interpretation of the data were performed by Haikuan Yu, Ruijing Chen, Mingming Zhang, Shi Cheng, Pengbin Yin, Jinglong Yan, Songcen Lv, Licheng Zhang, and Peifu Tang. Drafting of the manuscript, including critical revision, was performed by all authors. All authors accept responsibility for the integrity of the data analysis.

Acknowledgements

we are grateful for all other people involved in this study. All authors would like to thank to the Translational Medicine Research Center Laboratory of the general hospital of People Liberate Army of China.

Code availability

The code included in this study may be available on request, with a clear statement of purpose.

References

1. Meyers C, Lisiecki J, Miller S, Levin A, Fayad L, Ding C, Sono T, McCarthy E, Levi B, James AW. Heterotopic Ossification: A Comprehensive Review. *JBMR Plus* 2019, **3**(4): e10172.
2. Vanden Bossche L, Vanderstraeten G. Heterotopic ossification: a review. *J REHABIL MED* 2005, **37**(3): 129-136.
3. Dilling CF, Wada AM, Lazard ZW, Salisbury EA, Gannon FH, Vadakkan TJ, Gao L, Hirschi K, Dickinson ME, Davis AR, Olmsted-Davis EA. Vessel Formation is Induced Prior to the Appearance of Cartilage in BMP2-Mediated Heterotopic Ossification. *J BONE MINER RES* 2009, **25**(5): 243505207-243505232.

4. Linden J, Koch-Nolte F, Dahl G. Purine Release, Metabolism, and Signaling in the Inflammatory Response. *ANNU REV IMMUNOL* 2019, **37**(1): 325-347.
5. Peterson JR, De La Rosa S, Eboda O, Cilwa KE, Agarwal S, Buchman SR, Cederna PS, Xi C, Morris MD, Herndon DN, Xiao W, Tompkins RG, Krebsbach PH, Wang SC, Levi B. Treatment of heterotopic ossification through remote ATP hydrolysis. *SCI TRANSL MED* 2014, **6**(255): 132r-255r.
6. Di Virgilio F, Dal Ben D, Sarti AC, Giuliani AL, Falzoni S. The P2X7 Receptor in Infection and Inflammation. *IMMUNITY* 2017, **47**(1): 15-31.
7. Jørgensen NR. The purinergic P2X7 ion channel receptor – a ‘repair’ receptor in bone. *CURR OPIN IMMUNOL* 2018, **52**: 32-38.
8. Xu X, He X, Wang J, Li X, Xia Y, Tan Y, Chen F. Role of the P2X7 receptor in inflammation-mediated changes in the osteogenesis of periodontal ligament stem cells. *CELL DEATH DIS* 2019, **10**(1): 20.
9. Peterson JR, Okagbare PI, De La Rosa S, Cilwa KE, Perosky JE, Eboda ON, Donneys A, Su GL, Buchman SR, Cederna PS, Wang SC, Kozloff KM, Morris MD, Levi B. Early detection of burn induced heterotopic ossification using transcutaneous Raman spectroscopy. *BONE* 2013, **54**(1): 28-34.
10. Wang X, Li F, Xie L, Crane J, Zhen G, Mishina Y, Deng R, Gao B, Chen H, Liu S, Yang P, Gao M, Tu M, Wang Y, Wan M, Fan C, Cao X. Inhibition of overactive TGF- β attenuates progression of heterotopic ossification in mice. *NAT COMMUN* 2018, **9**(1).
11. Yu Y, Bi C, Wu R, Yin Y, Zhang X, Lan P, Chen F. Effects of short-term inflammatory and/or hypoxic pretreatments on periodontal ligament stem cells: in vitro and in vivo studies. *CELL TISSUE RES* 2016, **366**(2): 311-328.
12. Peterson JR, Okagbare PI, De La Rosa S, Cilwa KE, Perosky JE, Eboda ON, Donneys A, Su GL, Buchman SR, Cederna PS, Wang SC, Kozloff KM, Morris MD, Levi B. Early detection of burn induced heterotopic ossification using transcutaneous Raman spectroscopy. *BONE* 2013, **54**(1): 28-34.
13. Wu R, Bi C, Yu Y, Zhang L, Chen F. Age-related decline in the matrix contents and functional properties of human periodontal ligament stem cell sheets. *ACTA BIOMATER* 2015, **22**: 70-82.
14. Prudner BC, Rathore R, Robinson AM, Godec A, Chang SF, Hawkins WG, Hirbe AC, Van Tine BA. Arginine Starvation and Docetaxel Induce c-Myc–Driven hENT1 Surface Expression to Overcome Gemcitabine Resistance in ASS1-Negative Tumors. *CLIN CANCER RES* 2019, **25**(16): 5122-5134.
15. Peterson JR, De La Rosa S, Sun H, Eboda O, Cilwa KE, Donneys A, Morris M, Buchman SR, Cederna PS, Krebsbach PH, Wang SC, Levi B. Burn Injury Enhances Bone Formation in Heterotopic Ossification Model. *ANN SURG* 2014, **259**(5): 993-998.
16. Peterson JR, Agarwal S, Brownley RC, Loder SJ, Ranganathan K, Cederna PS, Mishina Y, Wang SC, Levi B. Direct Mouse Trauma/Burn Model of Heterotopic Ossification. *Journal of Visualized Experiments* 2015(102): e52880.
17. Rosina M, Langone F, Giuliani G, Cerquone Perpetuini A, Reggio A, Calderone A, Fuoco C, Castagnoli L, Gargioli C, Cesareni G. Osteogenic differentiation of skeletal muscle progenitor cells is activated by the DNA damage response. *SCI REP-UK* 2019, **9**(1): 5447.

18. Sorkin M, Huber AK, Hwang C, Carson WF, Menon R, Li J, Vasquez K, Pagani C, Patel N, Li S, Visser ND, Niknafs Y, Loder S, Scola M, Nycz D, Gallagher K, McCauley LK, Xu J, James AW, Agarwal S, Kunkel S, Mishina Y, Levi B. Regulation of heterotopic ossification by monocytes in a mouse model of aberrant wound healing. *NAT COMMUN* 2020, **11**(1).
19. Burnstock G. Physiology and pathophysiology of purinergic neurotransmission. *PHYSIOL REV* 2007, **87**(2): 659-797.
20. Adinolfi E, Giuliani AL, De Marchi E, Pegoraro A, Orioli E, Di Virgilio F. The P2X7 receptor: A main player in inflammation. *BIOCHEM PHARMACOL* 2018, **151**: 234-244.
21. Li J, Liu D, Ke HZ, Duncan RL, Turner CH. The P2X7 Nucleotide Receptor Mediates Skeletal Mechanotransduction. *The Journal of biological chemistry* 2005, **280**(52): 42952-42959.
22. Grol MW, Brooks PJ, Pereverzev A, Dixon SJ. P2X7 nucleotide receptor signaling potentiates the Wnt/ β -catenin pathway in cells of the osteoblast lineage. *PURINERG SIGNAL* 2016, **12**(3): 509-520.
23. Sindhavajiva PR, Sastravaha P, Arksornnukit M, Pavasant P. Purinergic 2X7 receptor activation regulates WNT signaling in human mandibular-derived osteoblasts. *ARCH ORAL BIOL* 2017, **81**: 167-174.
24. Zhou X, Yang Y, Wu L, Wang Y, Du C, Li C, Wang Z, Wang Y. Brilliant Blue G Inhibits Inflammasome Activation and Reduces Disruption of Blood–Spinal Cord Barrier Induced by Spinal Cord Injury in Rats. *MED SCI MONITOR* 2019, **25**: 6359-6366.
25. Marcillo A, Frydel B, Bramlett HM, Dietrich WD. A reassessment of P2X7 receptor inhibition as a neuroprotective strategy in rat models of contusion injury. *EXP NEUROL* 2012, **233**(2): 687-692.
26. Xu L, Xu K, Wu Z, Chen Z, He Y, Ma C, Moqbel SAA, Ran J, Zhang C, Wu L, Xiong Y. Pioglitazone attenuates advanced glycation end products-induced apoptosis and calcification by modulating autophagy in tendon-derived stem cells. *J CELL MOL MED* 2020, **24**(3): 2240-2251.

Tables

Table 1 Primers used in polymerase chain reactions (PCR).

Genes	Primer sequence (both 5' - 3')
For semi-quantitative methods	
GAPDH	Forward: AGGTCGGTGTGAACGGATTTG Reverse: TGTAGACCATGTAGTTGAGGTCA
P2X7R	Forward: GACAAACAAAGTCACCCGGAT Reverse: CGCTCACCAAAGCAAAGCTAAT
ALP	Forward: CCAACTCTTTTGTGCCAGAGA Reverse: GGCTACATTGGTGTTGAGCTTTT
RUNX2	Forward: ATGCTTCATTGCGCTCACAAA Reverse: GCACTCACTGACTCGGTTGG
OPN	Forward: AGCAAGAACTCTTCCAAGCAA Reverse: GTGAGATTCGTCAGATTCATCCG

Figures

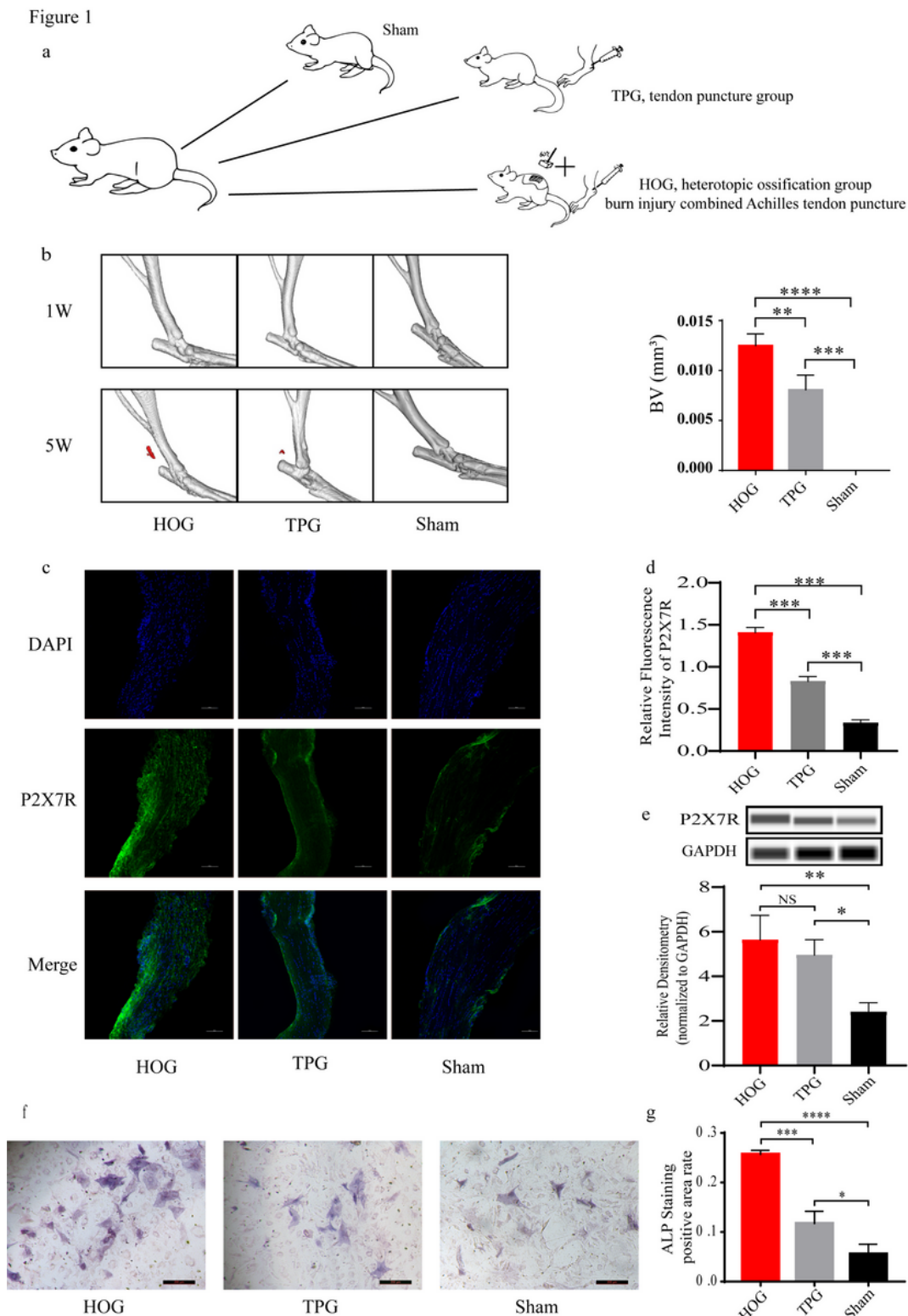


Figure 1

In HOG, apart from the Achilles tendon punctured, mice also received about 30% TBSA burn injury with exposure to 60°C for 18 s (Fig. 1a). The largest size of heterotopic ossification formation was in HOG, and the no HO was observed in the sham group (Fig. 1b). Then, this paper compared the bone volume of HO in different groups. These disparities were of statistical significance. Bone volume (BV) of HO in HOG and TPG outstripped that of Sham, p values were less than 0.0001 and 0.0001, respectively. There was a

significantly higher BV in the HOG than in the TPG ($p < 0.01$) (Fig. 1b). Burn injury enhanced bone formation as previous reports [15-16]. After the establishment of the model, at 7 days, the Achilles tendon was resected for further study. Then, the Achilles tendon tissues of different groups received pathological analysis. Among three groups (HOG, TPG and Sham), the fluorescence intensity of P2X7R in Achilles tendon was considerably different from each other ($p < 0.05$) (Fig. 1c). In HOG and TPG, the immunofluorescence (IF) intensity of P2X7R was significantly higher compared with sham group ($p < 0.001$) (Fig. 1d). The expression of P2X7R protein also differed in three groups. HOG and TPG had higher expression level compared to sham group. However, there was no significant difference between HOG and TPG ($p > 0.05$) (Fig. 1e).

Figure 2.

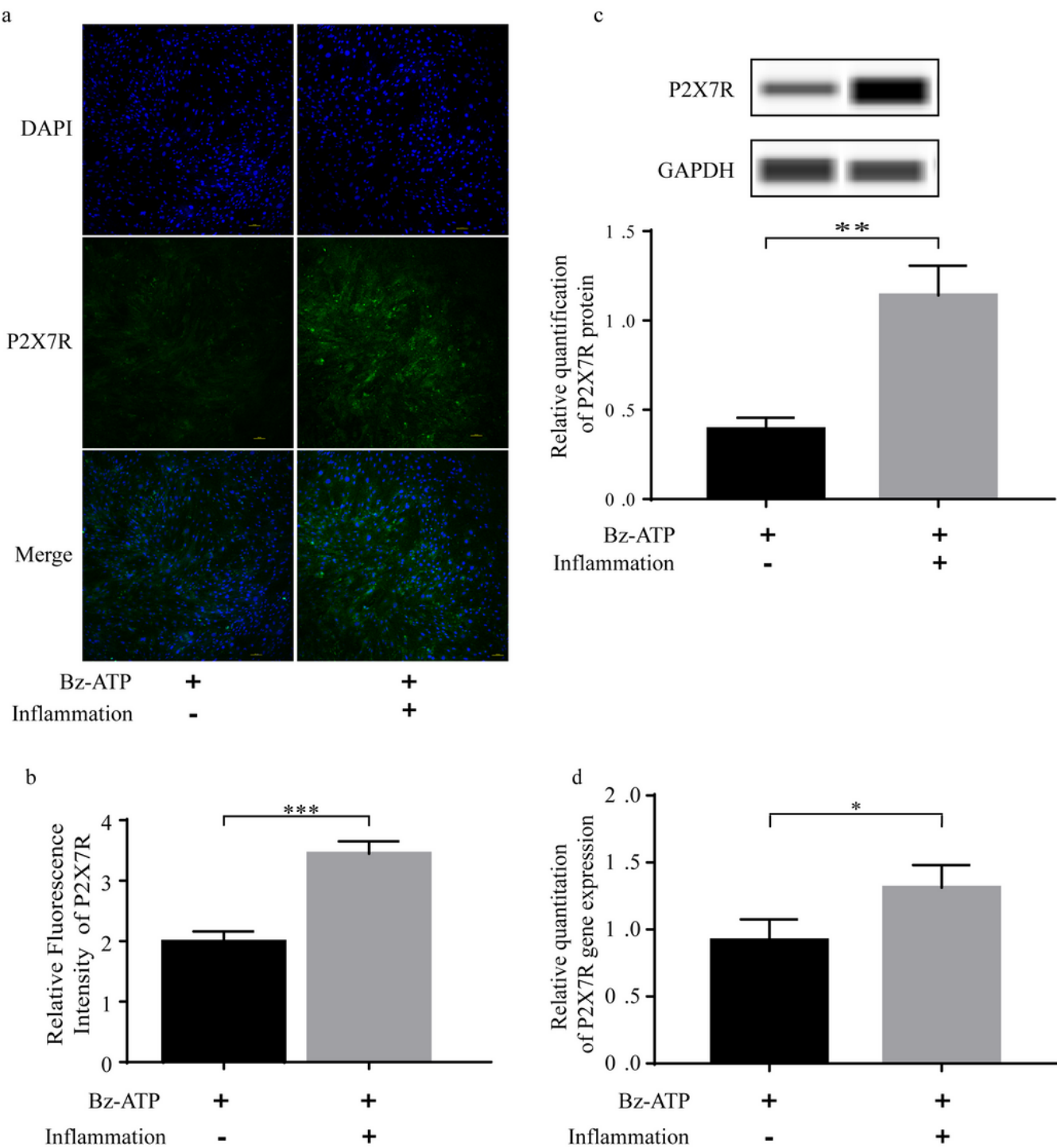


Figure 2

IF analysis illustrated that under inflammation condition, Bz-ATP promoted the expression of P2X7R (Fig.2a). Relative fluorescence intensity of P2X7R between two groups had significant difference. In the Bz-ATP combined inflammation group was higher than the other group significantly ($p < 0.001$) (Fig.2b). Similarly, the results of protein assays and qRT-PCR analysis supported the outcome of IF (Fig.2c, d).

These results confirm that under inflammation condition, Bz-ATP stimulates the overexpression of P2X7R.

Figure 3

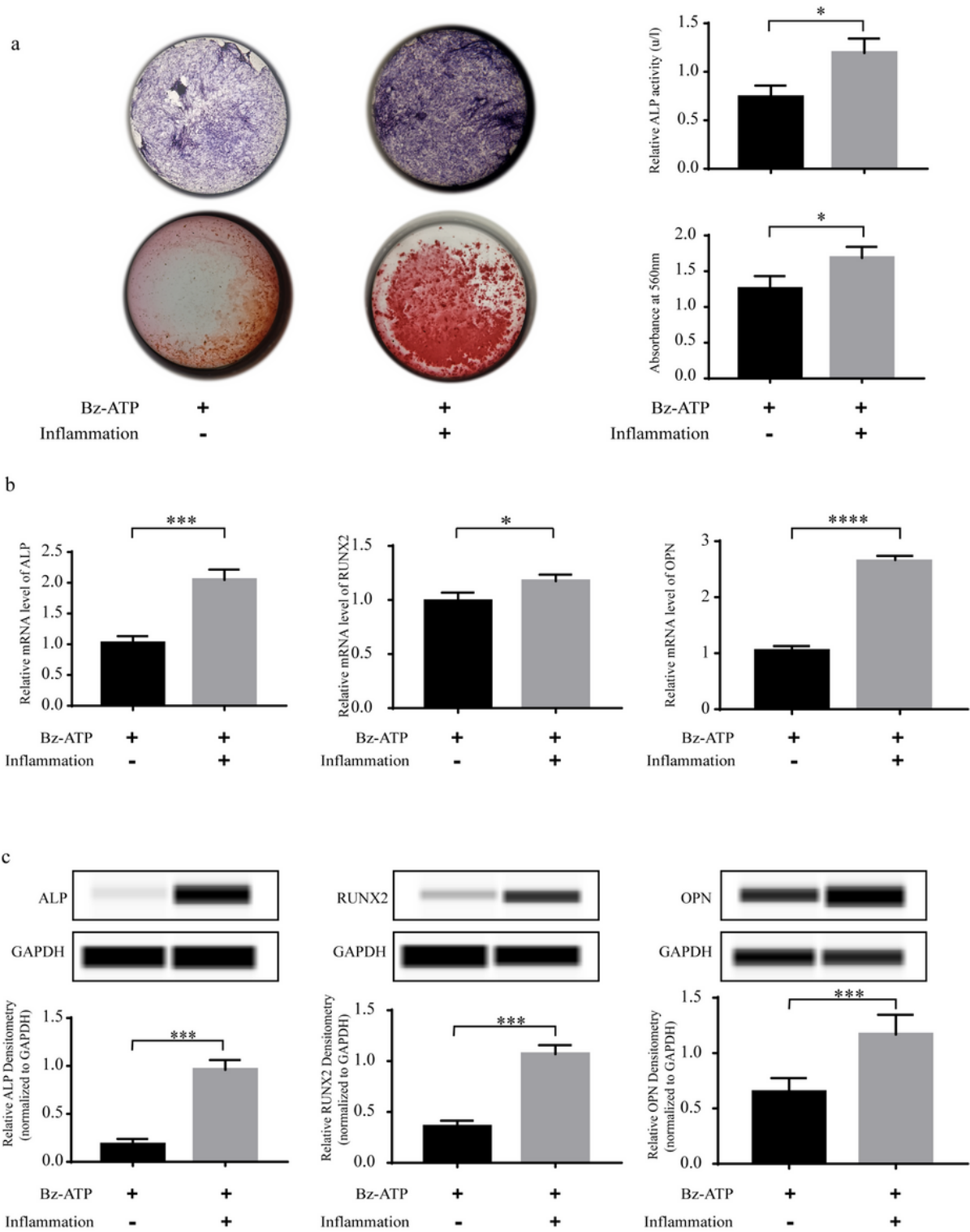


Figure 3

ALP activity and staining revealed higher ALP activity in inflammation condition intervention group than in the no inflammation condition intervention group ($p<0.05$). The results of ARS staining was also similar to ALP ($p<0.05$) (Fig.3a).

Figure 4

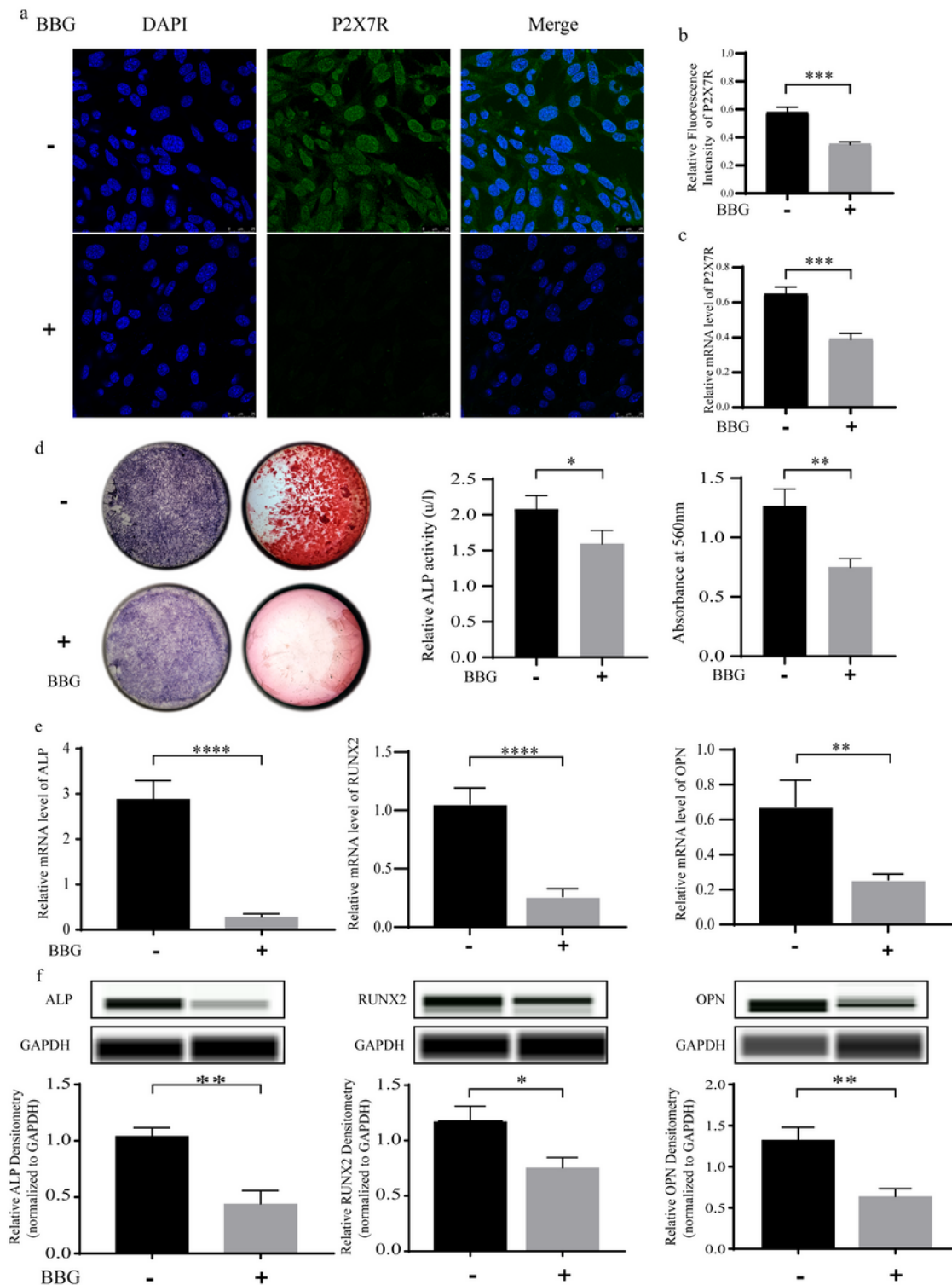


Figure 4

The results of ARS staining was also similar to ALP ($p < 0.05$) (Fig.3a). According to the qRT-PCR analysis, under the same Bz-ATP intervention, in the group of inflammation condition intervention, mRNA expression levels of osteogenic-associated genes such as ALP, RUNX2, OPN were significantly higher compared with the other group ($p < 0.001$, $p < 0.05$, $p < 0.0001$, respectively) (Fig.3b). Similarly, protein analysis maintained that under the same Bz-ATP intervention, the expression of ALP, RUNX2 and OPN

protein were significantly higher in the inflammation condition intervention than that of without the inflammation condition ($p<0.001$, $p<0.001$, $p<0.001$, respectively) (Fig.3c).

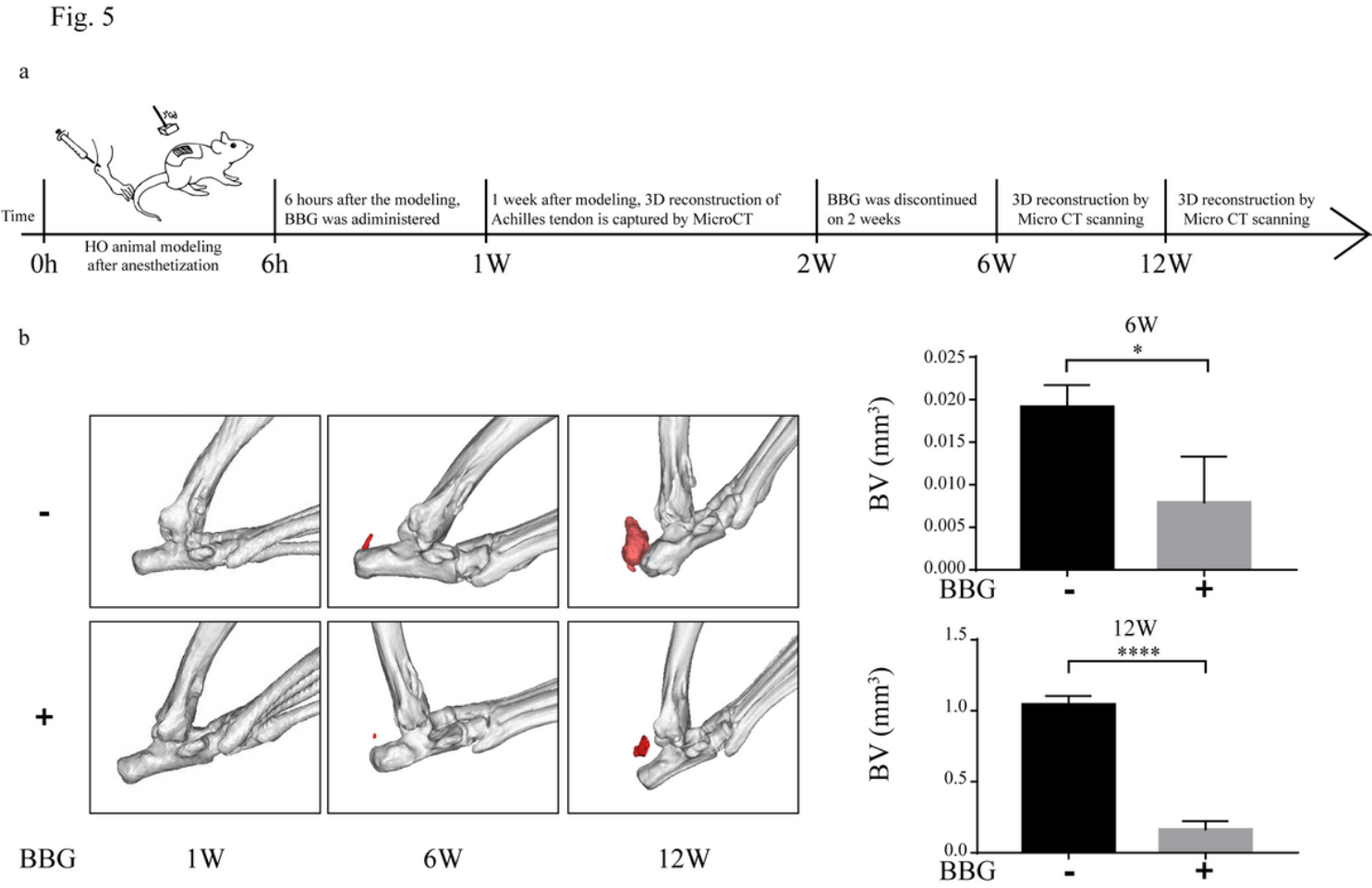


Figure 5

Saline solution was injected as the control. Micro CT analysis was conducted to evaluate heterotopic bone formation at 1st, 6th and 12week (Fig. 5a). At the 1st week, there was no heterotopic bone formation between two groups. At the 6th week, there was heterotopic bone formation in two groups. The difference of bone volume (BV) of heterotopic bone between two groups was statistically significant ($p < 0.05$). At the 12th week, the difference of BV was more significant between the two groups ($p < 0.0001$) (Fig. 5b).