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Extracorporeal Shock-Wave Therapy in the Experimental Rat Osteoarthritis with Osteoporosis

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Abstract: **Purpose:** Ustilizing the osteoarthritis(OA) model rats with osteoporosis(OP) evaluate and anticipate the effect of extracorporeal shockwave therapy (ESWT) in various aspects to coexisting of OA and OP in elderly women. **Methods:** Thirty-six female Sprague-Dawley rats were selected to make model of OPOA through surgery. Firstly, OP model were marked by the surgery of OVX, and were selected through micro-CT. Then, these OP model rats were treated by the surgery of ACL+MM. Finally, OPOA models were comfirmed by the hologiexamination. These model rats were divided into OPOA and OPOA + ESWT (n=12/group). Rats in the sham rats (n=12) were randomly selection from the group that were without treated by surgery. After ESWT, biomechanical analysis, micro-CT analysis, hologiexamination and immunohistochemical assay were utilized to evaluate the therapeutic effect. Mineralisation and alkaline phosphatase (ALP) in osteoblasts was analyzed to speculate the possible therapeutic mechanism of ESWT to OPOA. **Results:** Results of biomechanical analysis and hologiexamination show that ESWT could prevent further degeneration of articular cartilage. Micro-CT analysis and immunohistochemical assay demonstrate that the treatment could improve the microstructure of subchondral bone. Meanwhile, ESWT may improve the proliferation ability or activity of osteoblasts including in subchondral bone of OPOA rats in vivo. **Conclusion:** Our research suggests that ESWT prevented cartilage damage progression in the development of OPOA through intervening to degradation of subchondral bone.

Keywords*:* Osteoarthritis; Osteoporosis; Extracorporeal Shockwave Therapy; Subchondral Bone

Introduction

Osteoarthritis (OA) is the most common joint disease of affecting all joint tissues, particularly articular cartilage and subchondral bone[1][2]. Osteoporosis(OP) is a chronic bone metabolic disease with the character of reduceing of bone mass and increasing fracture risk[1]. Incidence rate of OA and OP extremely rise along with age, expecially in women after menopause[3]OA and OP associated dyskinesia even disability badly impact on life quality among elderly people. Since 1972, Foss and Byers continuedly study the relationship between OP and OA and have found that the incidence of hip osteoarthritis was with high proximal femoral fractures in these patients[4]. Recent advances in osteoimmunology elucidated that bone loss occurs not only in OP but in the early stage of OA[3]. In addition, OP can result in decrease of the bone quality, and fracture of microarchitectural in subchondral bone[5]. Some studies manifest that OA was induced by varied microarchitectural and mechanical property of the subchondral bone[6][7][8]. Increasing investigative results of clinic shows that osteophyte formation and osteosclerosis, common features of pathological change of subchondral bone, go with cartilage degradation. The subchondral bone are nutrition supplier and metabolic adjuster of articular cartilage except for mechanical supporter, therefore, the tissue plays a critical role for the development and healthy form of articular cartilage[9][10]. It has been proposed that integrity of the articular cartilage depend on the biomechanical properties of the subchondral bone[11]. The stabilization of

biomechanical properties was maintained referring to balance of bone metabolism. Therefore, except for repairing of damaged articular cartilage directly, a new target and new breakthrough point in the treatment of osteoarthritis was to improve bone metabolism and to induce bone remodeling of subchondral bone, thus to improve the structure of varied articular cartilage in OA lesion[12][13]. Through the targeted treatment, the degradation of bone can be controlled and subchondral bone microstructure is reconstituted, so as to improve osteoarthritis.

Conventional therapies to OA or OPOA were oral administrate(non-steroidal drugs and other analgesics), or intra-articular injection(sodium hyaluronate). However, a lot of adverse reactions from patients through these treatments were reported[14], ultimately, surgical treatment was the only choice to these patients. For OPOA, purpose of the treatment not only is to relieve the pain and improve the function, but also is to inhibit the progression of cartilage degeneration through improving the microstructure and biomechanical properties of subchondral bone. Excepting medicines, ESWT offer a kind of mechanical wave with acoustic, optical and mechanical properties, as a very becomingly physical stimulating to be usted to treat OPOA depending on highly sensitive of musculoskeletal tissues to mechanical stimulation[10][15]. Compared with the conventional treatment, ESWT has these advantages of short treatment period, low cost, slight injury and quick recovery. ESWT of different energy acting on the body to improve lesion were through mechanical effect, cavitation effect, acoustic effect and thermal effect, and was without side effect. In other hand, patients to ESWT appear with good tolerability and compliance. In clinic, ESWT has been applied to treat musculoskeletal system disease, including nonunion of bone, tendinopathy and osteonecrosis of the femoral head (ONFH)[16][17][18], and has been widely proved for wound, OP and OA were curative[19][20][21]. The treatment is through stimulating neovascularization, relieving pain, controling inflammation and modulating differentiation of mesenchymal stem cell in subchondral bone, therefore, protecting and preventing chondro degenerating of OA[12][22]. Osteoblasts play an important role in the bone formation and microarchitectural reconstruction and were with high sensitive to mechanical stimulation[23]. Further researchers found that ESWT stimulates releasing of VEGF, BMP-2, osterix and osteocalcin to induce the development of bone and cartilage with osteogenic differentiation of mesenchymal stem cell[24]. In the process, cooperated with elevating of nitric oxide (NO), promotes proliferation of human osteoblasts[25], and further to promote the development of bone and cartilage.

In here, the effect of that ESWT stimulate growing of subchondral bone by especial mechanical was tested in treated OPOA rats. We wish to ascertain whether ESWT improving the symptom of OPOA may be through regulating of bone metabolism and preventing of bone loss.

2. Materials and methods

2.1 Animals experiment

Thirty-six 8-week-old SPF female Sprague–Dawley (SD) rats (Ke Yu animal breeding centre, Beijing) with body weight about 280g were used in the experiment. The experimental protocol of the animal study was approved by the Animal Care Committee of Chinese Academy of Military Medical Sciences. The rats were housed at 24°C ± 1°C with a 12-hour light-and-dark cycle and given enough food and water. They moved freely in the cages and were kept at the Laboratory Animal Center for 1 week before the start of the experiment.

2.2 Experimental animal model

OP was experimentally induced in twenty-four rats by resecting bilateral ovariectomy (OVX) and kept for 12 weeks[26][27]. Twelve rats with age and gender-matched additional were used to control (Sham group) without resecting OVX. After 12 weeks, under general anesthesia using 25 mg/kg sodium pentobarbital (Pentotal, Abbott, Madrid, Spain), the rats of OP group were gone through anterior cruciate ligament(ACL) section and medial meniscectomy, inducing a surgical OA by destabilization[28]. For sham group, a sham operation was performed on the right knee joint using the same approach without(ACL+MM) transection.

2.3 ESW

Shock waves were applied by an EMS instrument (Swiss DolorClast, Switzerland). After surgery one week when the wounds healed , half of OPOA were subjected to extracorporal shock wave in vitro under the same anesthesia. The focus of shockwave treatment was the subchondral bone of the medial tibia condyle[29]. Each knee was treated with 1000 shocks of ESW (1.2 bar, 5 Hz) in a single session and other groups received no shockwave[30] The rats were euthanized using sodium pentobarbital (Pentotal, Abbott, Madrid, Spain) when the treatment was completely finished.

2.4 Biomechanical analysis

After harvesting knee joint from every group, the tibial plateau was dissected and then stored at −20°C until testing. Before testing, menisci was carefully removed from the tibial cartilage surface under a microscope. A micro-indentation test(Nano Indenter G200) at the center of the medial tibia plateau was performed, to determine the biomechanical properties of cartilage in each group. During the experiment, phosphate buffered saline (PBS) remains articular cartilage hydration at room temperature, and the diameter of 256 um flat pressing head contacts cartilage surface. All indentation creep testings were performed at the medial tibial plateau site. A preloading of 0.01N was applied and kept for 100s, followed by loading at the speed of 10 uN/s up to 0.1 N and maintaining for 300s. The stress-strain curve and creep(mm) behaviors were obtained from the indentation tests.

2.5 Micro-CT Scan

Prior to histological sectioning, the knee joints were scanned using micro-CT (Inveon CT, SIEMENS, Germany) with the following parameters: voltage of 70 kV, current of 142 A, exposure time 1475ms, 1mm aluminum filter, 0.5 degree of rotation steps, and 18 micron resolution. The reconstructed data sets were obtained and analyzed using three-dimensional data analysis software (Amira5.4, Visage, Berlin, Germany). All data sets were segmented with local threshold algorithm. The obtained projection of the image used the modified Feldkamp back projection to reconstruction. The separation of cortex and trabecular bone was realized by the built-in software. This experiment was mainly to measure trabecular bone volume fraction (Tb. BV/TV), trabecular bone number (Tb.N), trabecular bone thickness (Tb.Th), and trabecular spacing (Tb.Sp) which we selected the weight-bearing region in the medial tibia of subchondral bone according to previous repor[31].

2.6 Histopathologic and immunohistochemical

After Micro-CT, femur and tibia sections were rapidly took out, fixed in buffered formalin for 48h and then were decalcified in the 10% EDTA (PH 7.4) solution for four weeks. The tissues were dehydrated by the ASP200S hydroextractor (LEICA, Germany), and embedded with paraffin. Corresponding to the weight bearing area , the decalcified knee joints were cleaved 5 um thick sections using the microtome (Leica RM2235, Germany) in a sagittal plane. Then, the sections were stained with hematoxylin and eosin (HE) and toluidine blue. The degenerative changes of the cartilage were graded histologically using the mankin score and toluidine blue score. The scores were based on the most severe histologic changes within each cartilage section according to Osteoarthritis Research Society International (OARSI)[32]. For immunohistochemical, following xylene dewaxing, gradient ethanol hydration, hydrogen peroxide blocking, and antigen retrieval, the sections were incubated with a primary antibody to BMP-2 (Abcam, Cambridge, UK) and Osterix (Abcam, Cambridge, UK) for 12 hours. Then they were incubated with horseradish peroxidase (HRP)-labeled secondary antibody. Positive staining was observed and recorded by microscope (Carl Zeiss, Göttingen, Germany), and the areas were picked out by randomly selecting six areas in three sections of the same specimen. All images were captured with a Cool CCD camera (SNAP-Pro c.f. Digital kit; Media Cybernetics, Silver Spring, MD, USA) and analyzed using Image-Pro Plus 6.0 image-analysis software (Cybernetics Media, Sliver Spring, MD, USA). The percentage of the positive staining cells were counted on all five frames, and we chose the averages as the results. Two independent evaluators who were blinded to the nature of the study performed the measurements on all specimens.

2.7 Cell preparation

After anaesthesia, 2-week-old Sprague-Dawley rats (Ke Yu animal breeding centre, Beijing) were sacrificed and

immersed in 75% alcohol for 5 minutes. BMMSCs were isolated from the bilateral femurs and tibias in a sterile environment, and then were inoculated in the culture flask (Corning incorporated, USA) at the condition of 37℃, 5% CO_2. The medium (α-MEM, Hyclone by Thermo Fisher Scientific, Waltham, MA, USA) was changed at the first 24 hours. Non-adherent cells were removed by replacing the medium after 3 days. The adherent cells were digested by 0.25% trypsin (Beyotime Biotechnology, China) and passaged when the area of culture bottle was covered approximately 80% to 90%. The second generation of BMMSCs was used in the experiment. The cells were seeded in 24-well culture plates at $2*10^4$ cells/$cm^{[2]}$. They were cultured separately and then divided into three groups including GM group with only growth medium (α-MEM including 10% fetal bovine serum (Gibco by GE Healthcare Life Sciences, Logan, UT, USA) and 1% penicillin and streptomycin (Hyclone)); OM group with osteogenic differentiation medium(Cyagen, Beijing, China), and ESW group with ESW [33] (Swiss DolorClast, Switzerland) added to the OM group. After grouping, the 24-well plates were treated with ESW (800 impulses, 0.6 bar, 4 Hz) through couplant and whereas the other wells were used as controls. Then all these cells were cultured at 37℃ in the presence of 5% CO_2. The culture medium was changed every 3 days during the experiments.

2.8 Mineralisation

The 24-well plates of every group were used to evaluate the calcium production by staining with 10 % Alizarin Red (Cyagen, Beijing, China) solution at 7^{th} day, 14^{th} day and 21^{st} day, respectively. Then, the red-positive regions of the plates were imaged and measured by using Olympus digital microscope (Olympus Corporation, Japan). In addition, to quantify the staining, 1 mL of 10 % Hexadecylpyridium chloride (Sinopharm, Beijing, China) was added to each well and incubated for 20 min to elute the stain. Finally, 400 μL of the eluted stain was added to cuvettes and read at 562 nm using a spectrophotometer (Purkinje general, Beijing, China).

2.9 Alkaline phosphatase (ALP)

The second generation of BMMSCs were added into 24-well culture plates ($2*10^4$ cells/cm^2), using the same protocol as above. The expressive level of ALP was examined in each group at 14^{th}, 21^{st} day. ALP staining was performed according to the manufacturer's instructions (Genmed, Scientifics, Inc.USA). Finally, the dyeing regions of the plates were imaged (SNAP-Pro c.f. Digital kit; Media Cybernetics, Silver Spring, MD, USA) and measured by using Olympus digital microscope (Olympus Corporation, Japan).

3. Statistical analysis

Statistical analysis was performed with the JMP 13 software program (SAS Institute, Cary, NC USA). Graphical results were displayed as the mean±standard error. Differences among groups were analyzed by Student's t-test (designated as $^{\#}P < 0.05$ and $^{\#\#}P < 0.001$; $^{*}P < 0.05$ and $^{**}P < 0.01$). In all cases, $P < 0.05$ was considered significant.

4. Results

4.1 Changes in biomechanical properties

The biomechanical properties of cartilage in medial tibia plateau of rats in different groups were evaluated by a micro-indentation test. Compared with sham group, OPOA significantly increased displacement of the stress-strain curve[Fig.1(c,d)]. After the treatment for four weeks, suitable strength (above 0.02N) of ESWT induced significantly decrease of the displacement compared to the OPOA rats without treated[Fig.1(e)]. Synchronously, the creep behaviors of these rats in different groups were evaluated[Fig.1(f)]. At fourthly weeks after surgery, the OPOA rats were with higher creep than the sham group's rats and the raised creep was with a significant difference compared with sham group's rats($P < 0.001$). After the ESWT, the creep of OPOA rats was significantly lower compared to the OPOA rats without treated($P<0.05$), although the creep was higher than the sham group($P<0.05$).

4.2 Micro-CT analysis

Micro-CT were execute to image subchondral bone cysts(SBCs) underneath region of degenerated cartilage in the tibia of rats in different groups[Fig.2(A)]. The analysis of micro-CT image shows the variation of microstructure in subchondral bone, including BV/TV, Tb.N, Tb.Th and Tb.Sp in rats of different groups, respectively. Compared with the sham group, BV/TV($P<0.001$), Tb.N($P<0.001$) and Tb.Th($P<0.001$) significantly were decreased, and Tb.Sp($P<0.05$) was increased in OPOA rats[Fig.2(B)]. After the treatment for four weeks, compared with the without treated OPOA rats, the BV/TV($P<0.05$), Tb.N($P<0.01$) and Tb.Th($P<0.01$) were significantly rised, and the Tb.Sp($P<0.01$) was significantly lowered.

4.3 Histopathology

Tissue sections of subchondral bone were dyed by hematoxylin-eosin(HE) and toluidine blue(Fig 3A). The micrograph of the sections was acquired, and then was used to score of histopathologic mankin (Fig 3B(a)) and toluidine blue(Fig 3B(b)). OPOA group appears to be significant raise of mankin score and toluidine blue score compared with sham group($P<0.05$), the increased trend of scores imply the degradation degree of cartilage. After ESWT, the mankin score($P<0.05$) and toluidine blue score($P<0.05$) were significantly decreased compared with OPOA group.

4.4 Immunohistochemical

BMP-2 and Osterix in these tissue sections were labeled by antibody(Fig 4A), respectively. The number of BMP-2 brown mark($P<0.001$) in representative images of subchondral bone section of OPOA group was lower than sham group. Same result also appear the section labeled by Osterix($P<0.001$). After the treatment, the number of labeled BMP-2($P<0.01$) and Osterix($P<0.01$) in sections was induced rise. Compared with OPOA group, ESWT significantly increase the number of BMP-2 and Osterix mark in subchondral bone.

4.5 Mineralisation and Alkaline phosphatase (ALP)

Induced osteoblast differentiation and proliferation by ESWT was detected for ascertaining of therapeutic mechanism to OPOA. After ESWT for 7^{th}, 14^{th}, 21^{st} day, the content of alizarin red S staining, that quantify calcium nodules of mineralizing in osteoblast was assied, respectively. Compared with the OM group absenting of treatment, ESWT gradually and significantly increase the number and intensity of the calcium nodules in treated cell for 14^{th} day($P<0.05$) and 21^{st} day($P<0.01$) (Fig.5A,5B). In addition, the ALP content in these cells treated for 14^{th} and 21^{st} day were significantly higher than without treated(Fig.5C).

5. Discussion

According to recent reviews, the complicated multiple-factor relate to result in the rise of morbidity of knee OA, including age, gender, obesity, trauma and abnormal bone metabolism[34]. All of these factors can be ultimately attributed to mechanical overload on articular cartilage. The early stage of osteoarthritis present deformed subchondral bone, and following severe degeneration of articular cartilage. Some researchers have shown that degenerated and softened cartilage might transmit excessive mechanical loading to the subchondral bone surface[35]. Over time, less denser and compliant bone can generate shear stress to induce the varying of physiological structure of subchondral bone, further to damage cartilage[36]. The mechanotransduction of excessive mechanical loading induced the hypothesized process: Impulse loading → Trabecular mirofracture → Bone remodelling → Resultant stiffening of bone → Increased stress on articular cartilage → Cartilage breakdown → Joint degeneration[37]. As OA, OPOA will also present deforming of subchondral bone and the deformation attenuate buffering capacity of subchondral bone to excessive mechanical loading. In addition, due to abnormal bone metabolism, OP may aggravate the decreasing of the biomechanical properties of subchondral bone[38]. Therefore, a suggested for OA clinic treatment was to target subchondral bone and improve the biomechanical properties of the tissue. Treatment of ESW can improve the metabolism of subchondral bone and promote osteogenesis[20]. It have been proved that intervening of mechanical stimulation of ESW to the subchondral bone in early stage of OA may reduce or retard the progress of articular cartilage degeneration and improve the biomechanical properties of articular cartilage[28]. For simplicity and handleability, indentation tests on cartilage had been widely used to detect and elevate mechanical properties of the

tissues. The test also applied in clinical diagnosis of related organizational lesion[39]. After the treatment of ESW, we test and ascertain the obviously improvement of the mechanical properties of cartilage in treated OPOA rats. The variety of cartilaginous mechanical properties among groups suggest that the changes of subchondral bone may correlate with strain-rate of the cartilage[40]. The micro-CT images showed the degradation of subchondral bone in OPOA rats and further assay proves the degree of the degradation through measuring of BV/TV, Tb.Th, Tb.N and Tb.Sp of subchondral bone. These datas also ascertain the more rapid losing of density in subchondral bone of OPOA rats compared with sham group. Through ESWT, OPOA rats was with significantly increased in BV/TV($P<0.05$), Tb.Th($P<0.01$), and Tb.N($P<0.01$) and with decreased in Tb.Sp($P<0.01$) compared with the rats absenting of treatment. The varied trend of growth factor of BMP-2($P<0.01$) and Osterix($P<0.01$) were also consistent with these changes from micro-CT image. The histological score of OPOA group was faster increase than sham group($P<0.001$). ESWT observably lower the score ($P<0.05$) of OPOA rats. These phenomenon and datas suggest that lesion of subchondral bone may precede the changes of the articular cartilage in this OPOA model. Therefore, it is a reasonable suggest that lesion of articular cartilage can be treated not only through the reducing of inflammation , but regulating of subchondral bone metabolism[41]. Moreover, osteoblasts play the important role in the regulation of osteogenesis. For the sensitive to mechanical stimulation, osteoblasts can be induced by ESW to improve mechanical properties of subchondral bone through increasing density in osteogenesis, and thus help cartilage to buffer the mechanical load[33][42]. In the experiment, we have confirmed that ESWT can up-regulate expression of alkaline phosphatase (ALP) and promote calcium deposit in osteoblasts, further promote the osteogenesis.

In recent researches, ESWT showed the potency to protect and prevent cartilage corroded[12][35]. Meanwhile, we detected the beneficial efficacy of ESWT to OPOA rats for four weeks treatment[43]. Compared with the temporary effects of suppressing inflammation treatments, ESWT had long lasting favorable effects on relieving of pain and improving of joint function[44] and the effects have been evidenced by pathomechanics detecting of OPOA rats with or without treatment. In other hand, there are several methodological challenges. There are several limitations in here study. Firstly, the effect of ESW to early stage of OPOA, a transient period, have been confirmed[12]. The treating effect to the middle and later periods of OPOA accompanied with damage of cartilage and degradation on subchondral bone should be researched further. Secondly, the treatment intensity was divided into three levels in clinic[45][46], but the method of determining optimal energy flow density of ESWT have not been fully elucidated. To individual treat, the method urgently need in clinic. These limitations of ESWT to OPOA may be solved through elucidating the biological mechanism of ESWT to bone tissues.

In conclusion, our research suggests that ESWT prevented cartilage damage progression in the development of OPOA, and the treat took effect through intervening to degradation of subchondral bone.

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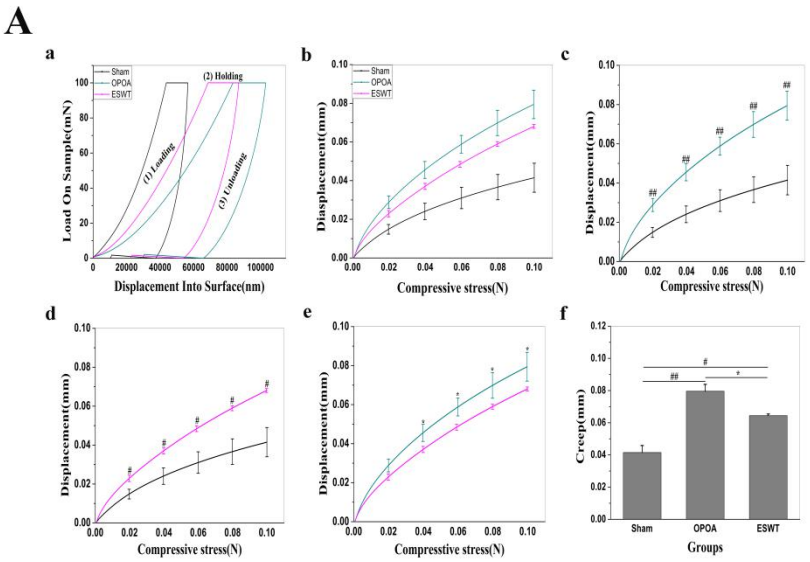

Fig.1. Effects of ESWT on the biomechanical of cartilage in the medial tibia of each group. (a)Representative nano-indentation load-displacement curve from the cartilage.(b) Stress–displacement curve at 4 weeks after ESWT. (c, d)OPOA rats with or without were respectively compared with Sham group($^{\#}$P<0.05, $^{\#\#}$P<0.001). (e) ESWT group compared with OPOA group (*P<0.05, **P<0.01). (f)Creep behaviors response of cartilage in the medial tibia ($^{\#}$P<0.05 and $^{\#\#}$P<0.001 represents the comparison among the groups of OPOA and ESWT with Sham group; *P<0.05 and **P<0.01 represents the comparison between ESWT group and OPOA group)..

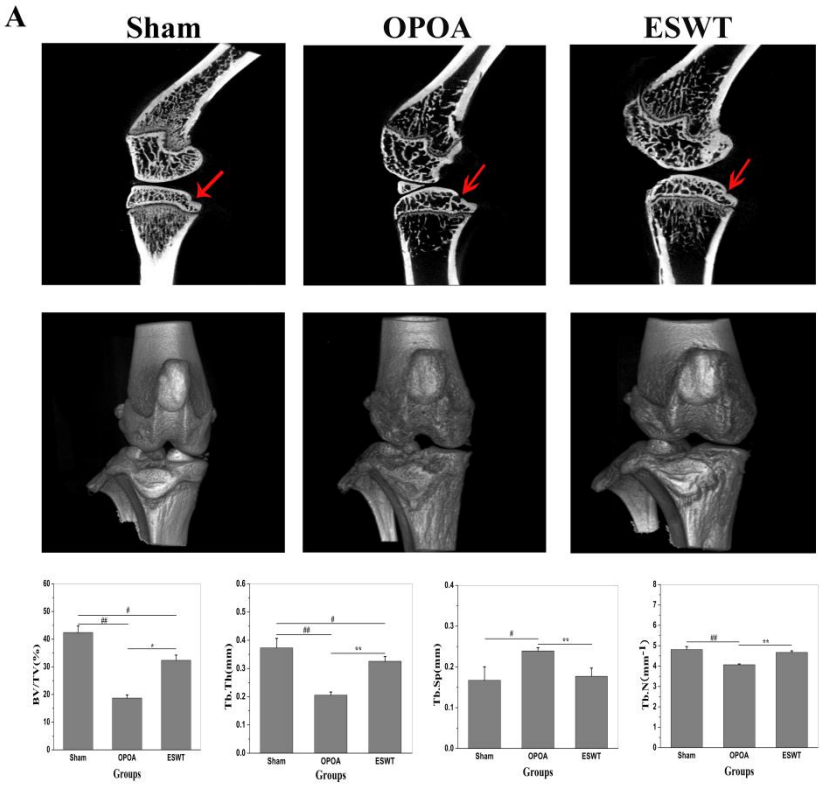

Fig.2. Characterization of subchondral bone changes in rats. (A) showed Axial and Coronal of the subchondral bone of all groups. The two-dimensional images of the knee joints show the presence of SBCs (red arrow) in the medial tibial plateau. (B) Micro-CT analysis of subchondral bone after ESW treatment from BV/TV, Tb.N, Tb.Sp and Tb.Th datas for all groups($^{\#}$P<0.05 and $^{\#\#}$P<0.001 represents the comparison among the groups of OPOA and ESWT with Sham group; *P<0.05 and **P<0.01 represents the comparison between ESWT group and OPOA group).

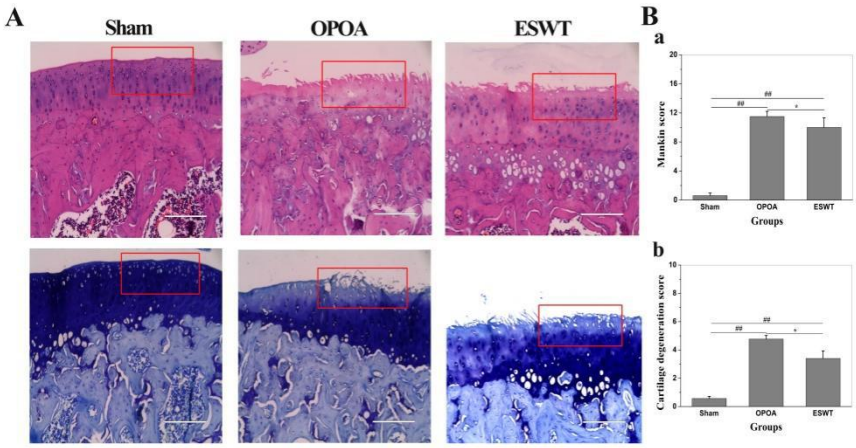

Fig.3.Histopathologic examination showed the cartilage damages (red frame) in osteoarthritis of the knee (A). Mankin score (a) and toluidine blue (b) score increased in OPOA group, as compared with sham group (indicated as $^{\#}P<0.05$ and $^{\#\#}P<0.001$). Meanwhile, ESWT significantly decreased Mankin score and toluidine blue score compared with the OPOA group ($^{*}P<0.05$ and $^{**}P<0.01$), and has significantly increased compared with sham group ($^{\#}P<0.05$ and $^{\#\#}P<0.001$). The scare bar represents 200 μm.

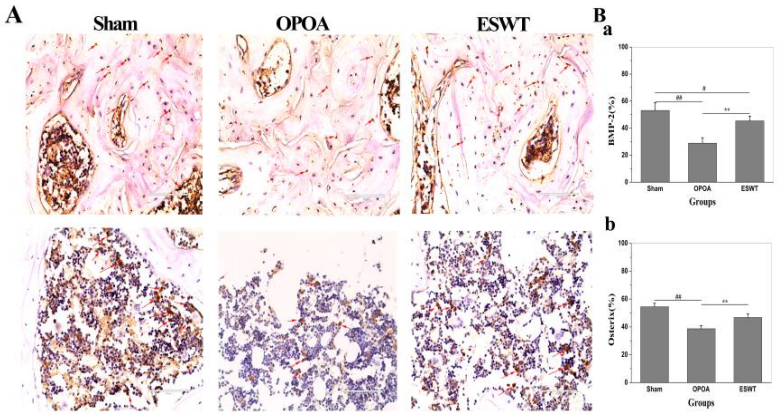

Fig.4. Immunohistochemical analysis for molecular changes (red arrow) in subchondral bone of the knee in representative images of microscopic features(A), and the results in graphs(B). The expressions of BMP-2 and Osterix (B) were significantly decreased in the OPOA group, as compared with the Sham group (indicated as $^{\#}P < 0.05$ and $^{\#\#}P < 0.001$). ESWT significantly increased the expressions of BMP-2 and Osterix, as compared with OPOA group (indicated as $^{*}P < 0.05$ and $^{**}P<0.01$). and has significantly different compared with sham group ($^{\#}P<0.05$ and $^{\#\#}P<0.001$). The scale bar represents 100 μm.

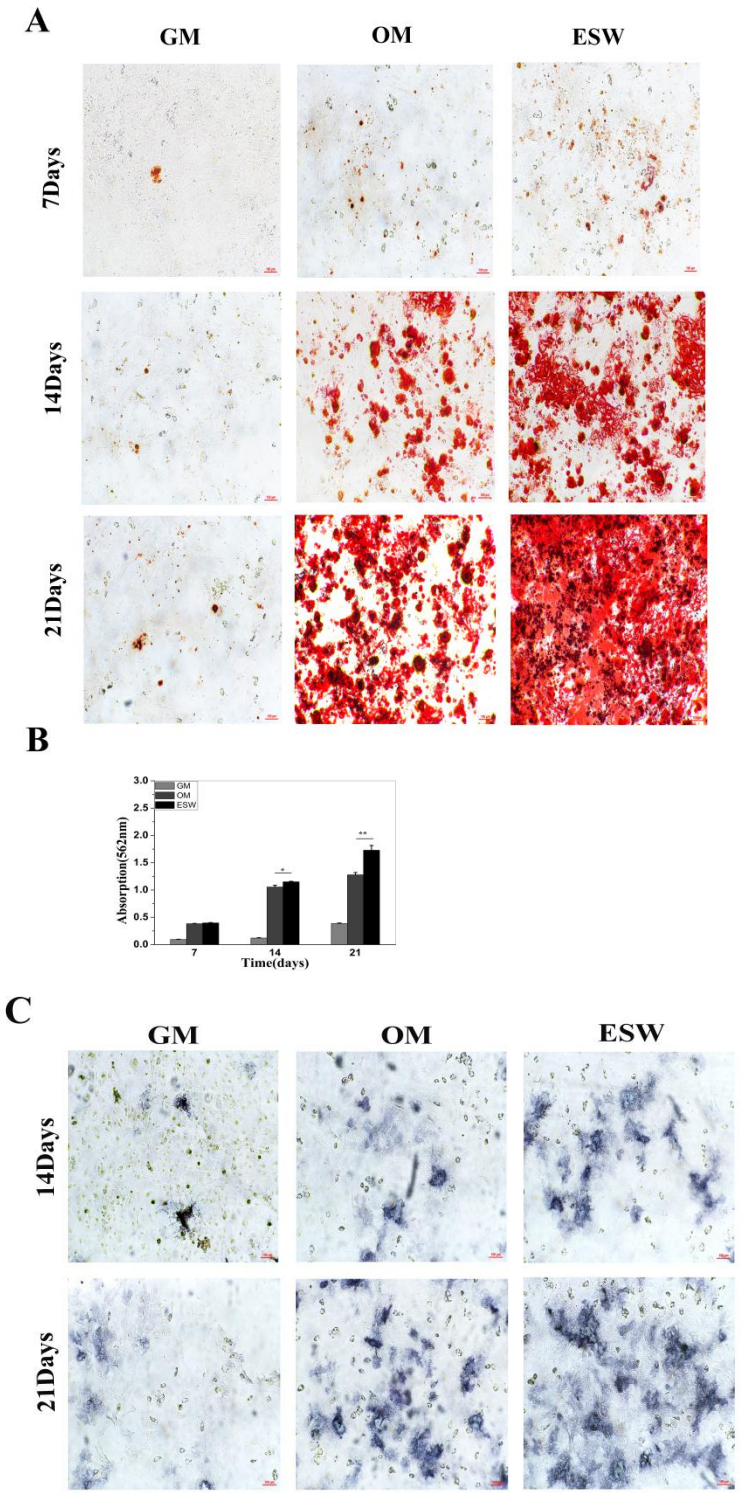

Fig.5. Alizarin red S for calcium was shown at 7th, 14th, 21st day and the expression of ALP was shown at 14th and 21st day(A and C). The number and intensity of the calcium nodules was shown(B).The groups of OM and ESW significantly increased at 14th and 21st day, as compared with Sham group. Meanwhile, the group of ESW significantly increased at 14th and 21st day, as compared with OM group(*P<0.05 and **P<0.01). The expression of ALP of OM and ESW groups had an increasing tendency at 14th and 21st day, as compared with Sham group. Meanwhile, compared with OM group, the group of ESW had an increasing tendency(C). Scale bar = 100 µm. GM = general medium; OM = osteogenic differentiation medium.