European Review for Medical and Pharmacological Sciences 2018; 22: 2439-2446

Neurotrophin-3 improves fracture healing in rats

X. LI[1], D.-C. SUN[2], Y. LI[3], X.-Y. WU[4]

[1]Department of Hand surgery, Yantaishan Hospital, Yantai, China
[2]Department of Pharmacy, No. 107 Liberation Army Hospital, Yantai, China
[3]Department of Orthopedics, Qingdao Women and Children's Hospital, Qingdao, China
[4]The Third Department of Orthopedics, Lanling People's Hospital, Linyi, China

Xiao Li and Dacheng Sun contributed equally to the work

Abstract. – OBJECTIVE: This study aimed at investigating the role of Neurotrophin-3 (NT-3) in the bone fracture healing of rats and to provide a reference for clinical treatment.

MATERIALS AND METHODS: 40 Sprague-Dawley (SD) rats were randomly divided into control group and NT-3 group. The tibia fracture model was made in NT-3 group, and the tibia bone mineral density (BMD) was measured before and after the surgery. The biomechanics indexes were inspected after the surgery, including elasticity modulus, max load, and bending rigidity. The levels of bone morphogenetic protein (BMP)-2 and transforming growth factor (TGF)-β1 in serum were examined by enzyme-linked immunosorbent assay (ELISA). Reverse transcriptase-polymerase chain reaction (RT-PCR) was used to detect the levels of hypoxia-inducible factor (HIF)-1α and vascular endothelial growth factor (VEGF) mRNA expression in callus tissue. The pathological profile of tibia fracture healing was characterized after the surgery.

RESULTS: The levels of BMD in NT-3 group were significantly higher than that in control group after the surgery ($p < 0.05$). The levels of elasticity modulus, maximum load, stiffness of shinbone, BMP-2 and TGF-β1 were significantly higher in NT-3 group than those in control group after the surgery ($p < 0.05$). Compared with the control group, the expression of HIF-1α mRNA was significantly lower and the expression of VEGF mRNA was significantly higher in NT-3 group after the surgery ($p < 0.05$). Histological study showed that the periosteal reaction, capillary proliferation, cartilage cells production and ossification were happened after treating NT-3 for tibia fracture model rats.

CONCLUSIONS: NT-3 can significantly improve fracture healing in rats.

Key Words:
Neurotrophin-3, Fracture healing, Tibia fracture model.

Abbreviations

NT-3 = neurotrophin-3; BMD = bone mineral density; NFG = nerve growth factor; BDNF = brain-derived neurotrophic factor; BMP = bone morphogenetic protein; SD = Sprague-Dawley; TGF = transforming growth factor; ELISA = enzyme-linked immunosorbent assay; RT-PCR = reverse transcriptase-polymerase chain reaction; HIF = hypoxia-inducible factor; VEGF = vascular endothelial growth factor; SD = standard deviation; HE = hematoxylin-eosin staining.

Introduction

Bone fracture happens frequently in clinic. The incidence of long bone fractures in the west world is estimated to be 300 to 400 per 100,000 individuals each year[1]. About 5% to 10% of the majority heal satisfactory went on to delayed union or nonunion[2]. Besides, high-energy tibia fractures are serious injuries with longer healing period around 43 to 49 weeks[3]. Previous studies indicated that various populations were suffered from it[4-6]. It is important to develop novel therapies to accelerate the fracture healing process.

Fracture healing is a complex the organism itself repair process, needs a variety of growth factor involved[7]. Fracture healing is the recovering of the broken bones through a series of physiological and cellular pathways[8]. Coordinated bone resorption and formation were required for fracture healing process[9]. Osteoporosis, a common condition among elderly women or men, could increase the risk of hip and other fractures[10]. Osteogenesis happened during fracture healing through intramembranous and endochondral ossification[11]. Most fracture was related to low bone mineral density (BMD). The level of BMD has been a maker for predicting the risk of various fractures[12]. There are various reactions in fracture healing during the process, including inflammation, repair, and remodeling[13]. The repair and remodeling, as the mainly recapitulate the process of normal bone development, are happened at the same time. These reactions are overlapped in fracture healing

Corresponding Author: Xingyu Wu, MD; e-mail: wuxingyuyux@163.com

process. Meanwhile, the inflammation can induce gene expression and cell proliferation to promote healing[14].

Animal models have been used to investigate fracture healing from all perspectives. Many osteogenic molecules were proved to have the potential to improve the fracture healing[15]. Neurotrophic factors are involved in the regulation of the proliferation, survival, migration, and differentiation of cells in the nervous system. Neurotrophic factors such as nerve growth factor (NFG), brain-derived neurotrophic factor (BDNF), and neurotrophin-3 (NT-3), were key factors involved in nervous systems development16. NT-3 plays an important role in cell proliferation, differentiation, and neuroprotection[17]. NT-3 has been proved to be important in the development of nervous system[18,19]. Furthermore, NT-3 could promote oligodendrocytes proliferation, cuprizone-induced demyelination model in mice, combing with other factors[20]. Evidence implied that NT-3 also had a role in bone repair, which could induce bone marrow stromal cells into neuronal phenotype[21]. NT-3 was showed to improve vascularization and bone repair after bone injury[22]. However, there was little research about the functions of NT-3 in bone fracture healing. We aimed at investigating the functions of NT-3 in fracture healing of rats and to provide a reference for clinical treatment.

Materials and Methods

Experimental Animals

40 SPF level male Sprague-Dawley (SD) rats were provided by Shanghai Laboratory Animal Center of Chinese Academy of Sciences (Shanghai, China). The rats were 4 weeks old and the weight ranged from 100 to 130 g. The rats were reared in animal room (20-24°C, 12-h-light/12-h-dark cycle) with free access to food and water.

Animal Grouping and Fracture Model

All the experiments were approved by our institute's Subcommittee on Animal Studies. After feeding for 1 week, the 40 rats were randomly assigned to control group and NT-3 group, 20 in each group.

The rats received intraperitoneal injections of 10% chloral hydrate (North China Pharmaceutical Co., Ltd, Shijiazhuang, China) for general anesthesia before operation. The right legs of the rats were shaved and disinfected. Open fracture of the tibia was caused by carborundum disc. A steel K-wire was inserted from the border of the tibial plateau into the medullary canal for stabilization. The skin was stabbed by the wire and the tibia was immobilized by plaster. The rats were kept in special rearing cage with intramuscular of 10^5 U/d penicillin (North China Pharmaceutical Co., Ltd, Shijiazhuang, China) for 3 days to prevent infection. 0.3 μg NT-3 (Sinopharm Chemical Reagent Co., Ltd., Beijing, China) were injected to quadriceps of the lateral thigh instantly after the surgery for 1 week, while 0.5 ml saline were injected in the control group at the same time.

BMD Inspection

The tibia BMD was inspected before and after the surgery (2, 4, 6, 8, 10, 12 weeks) using Lunar-iDXA dual-energy X-ray absorptiometry (Sigma-Aldrich, St. Louis, MO, USA).

Biomechanics Testing

The rats were sacrificed at 12 weeks after operation. The tibia was dissected and frozen at -80°C with wrapping the gauze soaked in isotonic saline. After thawing at room temperature, samples were tested to failure in the three-point bending using AG-IS-20 universal testing machine (Shimadzu, Tokyo, Japan). The callus of the tibia served as the loading point during the testing with the lower supports placed 18 mm from the callus. The actuator was displaced at a rate of 1 mm/s until failure occurred. Load *vs.* displacement curve

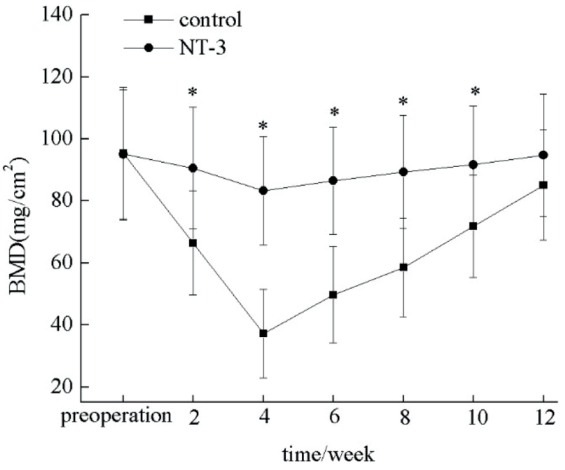

Figure 1. The levels of BMD in the rats of each group before surgery and after surgery (2 weeks, 4 weeks, 6 weeks, 8 weeks, 10 weeks, and 12 weeks) (N=10). * compared with control group, $p < 0.05$.

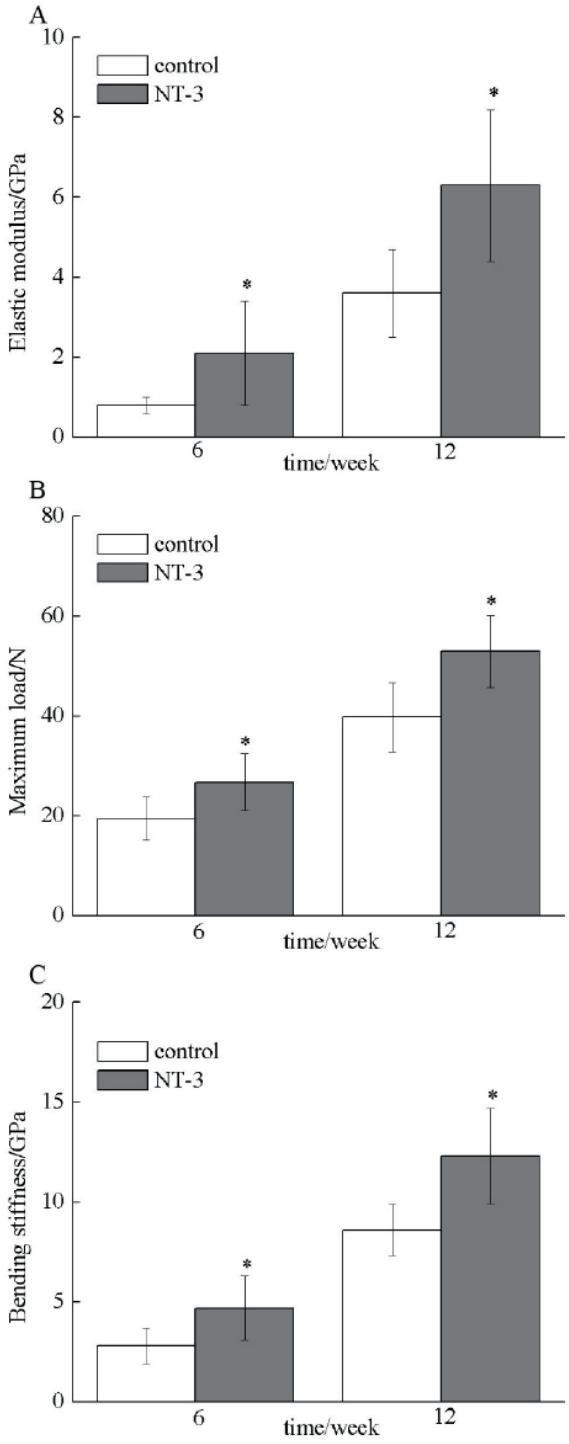

Figure 2. Biomechanical indexes of the tibia in the rats of each group before surgery and after surgery (6 weeks and 12 weeks). ***A,*** Elasticity modulus; ***B,*** Maximum load; ***C,*** Stiffness. *compared with control group, $p < 0.05$.

were recorded automatically. Elasticity modulus, maximum load, stiffness and other biomechanics indexes were calculated according to the curve.

Serum Cytokines Analysis

The levels of BMP-2 and TGF-β_1 in serum were measured by enzyme-linked immunosorbent assay (ELISA), according to the manufacturer's instructions (Aachen, Westphalia, Germany).

Reverse Transcriptase-polymerase Chain Reaction (RT-PCR)

Total RNA was extracted according to TRIzol kit (Invitrogen, Carlsbad, CA, USA), and RNA purity was determined by UV spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). The reverse transcription was performed using reverse transcription kit (TaKaRa, Tokyo, Japan). 1 μL of the cDNA configuration reaction system was prepared using the SYBR Green Master Mix (Promega, Madison, WI, USA) and iCycle iQ (Bio-Rad, Hercules, CA, USA). PCR primer sequences were listed following,

Vascular endothelial growth factor (VEGF): Forward: 5′- ATCTTCAAGCCGTCCTGTGTG-3′, Reverse: 5′- TGAGGTTTGATCCGCATGATC-3′; HIF-hypoxia-inducible factor (HIF-1α): Forward: 5′- CCCCTACTATGTCGCTTTCTTGG-3′, Reverse: 5′- GGTTTCTGCTGCCTTGTATGG-3′; β-actin: 5'-ACGGCCAGGT

CATCACTATTG-3', Reverse: 5'-CCTGCTTGCTGATCCACATCT-3'. β-actin was used as the endogenous control and $2^{-\Delta\Delta CT}$ method was used to analyze the data.

Histological Study

The specimens were retrieved after the surgery (2 weeks, 4 weeks, 6 weeks, 8 weeks). Tibia tissue slices were washed by dimethyl benzene for two times, 5 min each time, then immersed in absolute ethanol for 2 min, 95%, 85%, 75% ethanol successively for 1 min each. After that, the slices were washed with distilled water for 2 min and dyed by hematoxylin staining fluid for 5 min. The slices were checked and the pictures were got using Pathological microtome (Nikon, Chiyoda, Tokyo, Japan).

Statistical Analysis

SPSS19.0 (SPSS Inc., Chicago, IL, USA) was used to analyze the data. The data was showed with mean value ± standard deviation (SD) and comparisons between two groups were performed using *t*-test. $p < 0.05$ was considered to be statistically significant.

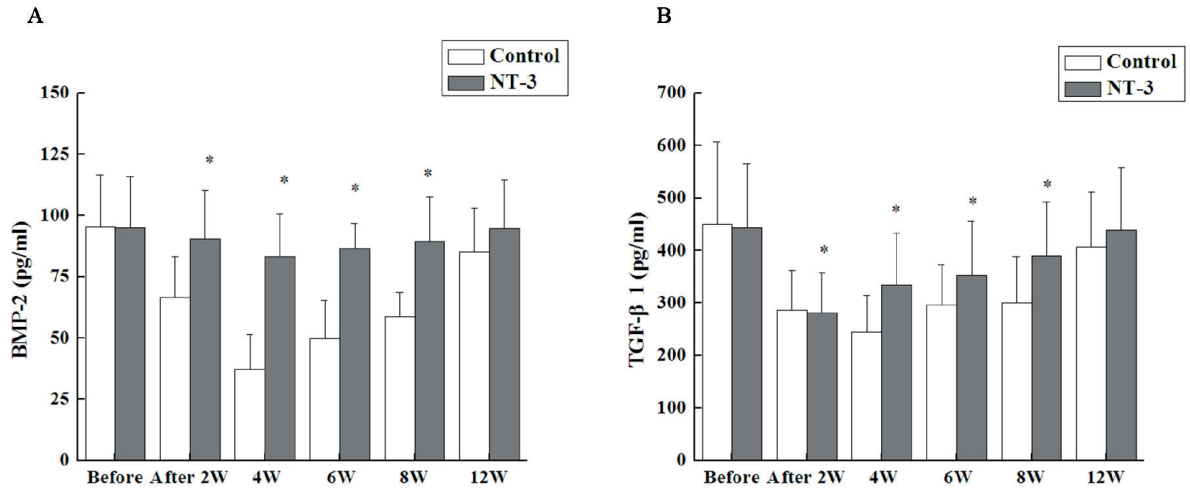

Figure 3. The levels of BMP-2 ***(A)*** and TGF-β1 ***(B)*** in serum of each group before surgery and after surgery (2 weeks, 4 weeks, 6 weeks, and 12 weeks). *compared with control group, $p < 0.05$.

Results

Effects of NT-3 on BMD Variation

BMD variation profile before and after fracture was showed in Figure 1. No significant difference ($p > 0.05$) was showed for the tibia BMD in the two groups before surgery. The BMD showed a decreasing tendency at 2-4 weeks, increasing tendency at 6 weeks after surgery in NT-3 group. However, the tibia BMD was higher in NT-3 group than that in control group at 2-10 weeks after the surgery ($p < 0.05$). At 12 week, the BMD level was still higher in NT-3 group than that in control group ($p > 0.05$).

Effects of NT-3 on Biomechanics Index

The elasticity modulus, maximum load, and stiffness were all significantly higher in NT-3 group than those in control group ($p < 0.05$) at 6 and 12 weeks after the surgery (Figure 2). The elasticity modulus of NT-3 group was almost 2.5 folds of control group at 6 weeks and less than 2 folds at 12 weeks. This means the elasticity modulus of NT-3 group increased faster than that control group. However, maximum load and stiffness showed almost the same fold of NT-3 group to control group.

Effects of NT-3 on Serum Cytokines

The levels of NMP-2 and TGF-β1 in serum were 83.3 ± 17.5 pg/ml and 334.2 ± 99.6 pg/ml in NT-3 group at 4 weeks, respectively. Compared with the control group, the levels of NMP-2 and TGF-β1 were significantly increased ($p < 0.05$). As shown in Figure 3, the levels of NMP-2 and TGF-β1 increased in a time-dependent manner.

Effect of NT-3 on HIF-1α and VEGF mRNA Expression in Callus Tissue

Before surgery, the expression levels of HIF-1α and VEGF mRNA in the NT-3 group were 2.06 ± 0.51 eq and 0.85 ± 0.15 eq, respectively. The expression levels of HIF-1α and VEGF mRNA in the control group after surgery were 4.13 ± 0.83 eq and 0.61 ± 0.03 eq, respectively. The expression level of VEGF mRNA in the NT-3 group was significantly higher than that in control group, and the expression level of HIF-1α mRNA in the NT-3 group was significantly lower than that in control group ($p < 0.05$) (Figure 4).

NT-3 Ameliorated the Pathological Tissue

Hematoxylin-eosin staining (HE) staining analysis revealed that necrosis, periosteal reactive hyperplasia, inflammatory cell infiltration, and capillary proliferation happened in the tibia tissue of the control group at 2 weeks after surgery. A few fibroblast cells were also produced. While cell infiltration increased at 4 weeks. Fiber callus, cartilage, and a little ossification happened at 8 weeks in the control group (Figure 5). However, periosteal reaction, and capillary proliferation happened, and fibroblast cells were produced in the NT-3 group at 2 weeks after surgery. Cartilage cells and fiber osteogenesis were observed at 4 weeks. Cartilage cells proliferated, bone trabecu-

la formed and ossification happened were showed at 6 weeks. More ossification was observed at 8 weeks (Figure 5). The results mean that NT-3 accelerates the fracture healing in rats.

Discussion

The expression of NT-3 related genes has been reported previously[16]. We studied the effect of NT-3 treatment on fracture healing. This research points to a new possibility in the treatment of fracture.

Bone fragility can be defined by biomechanical parameters, and changed after fracture or restructuring of bone[23]. Fracture healing occurs with callus formation[24]. Measurements of bone mineral density can predict fracture risk. Osteoporosis was associated with a fracture rate approximately 4 times that of normal BMD[25]. BMD decreased after fracture and increased following the fracture healing process[26]. So BMD can be used as an index of fracture healing. BMD was measured before and after surgery in this study. BMD of tibia showed no significant difference in the two groups before surgery. However, the BMD of tibia increased significantly in the NT-3 group than that in control group in the following weeks after surgery (Figure 1), which indicated the fracture healing was much more improved in the NT-3 group.

The degree of fracture healing can be quantified by normalizing mechanical parameters of the bone[27], which means that the biomechanics indexes can be employed to evaluate the process of fracture healing. The significant difference of the Biomechanics indexes between the control and NT-3 group indicated the different bone healing after fracture. The elasticity modulus, maximum load and stiffness were all significantly higher in NT-3 group than those in control group ($p < 0.01$) at 6 and 12 weeks after the surgery. Therefore, it showed that the fracture healing in NT-3 group was much better than the control group.

The NT-3 expression was increased during the process of healing. The NT-3 and its receptors were expressed in the bone cells, indicated that NT-3 was involved in the formation of bone[28]. BMP-2 and TGF-β1 were required for the initiation of fracture healing. BMP-2 and neurotrophins cooperatively increase the number of striatal calbindin-positive neurons. Our study showed that the levels of NMP-2 and TGF-β1 in serum were significantly increased in NT-3 group. The expression level of VEGF mRNA in the NT-3 group was increased, and the expression level of HIF-1α mRNA in the NT-3 group was declined. This suggested that NT-3 may play an important role in the bone healing and bone formation.

The formation of a cartilaginous callus with later mineralization, resorption, and replacement with bone is the key feature of fracture healing. Our results showed that periosteal reaction, capillary proliferation, and fibroblast cells were produced in the NT-3 group at 2 weeks after surgery (Figure 5). Cartilaginous callus formation and new bones happened earlier in NT-3 group than that in control group. This indicated that fracture healing was accelerated in NT-3 group.

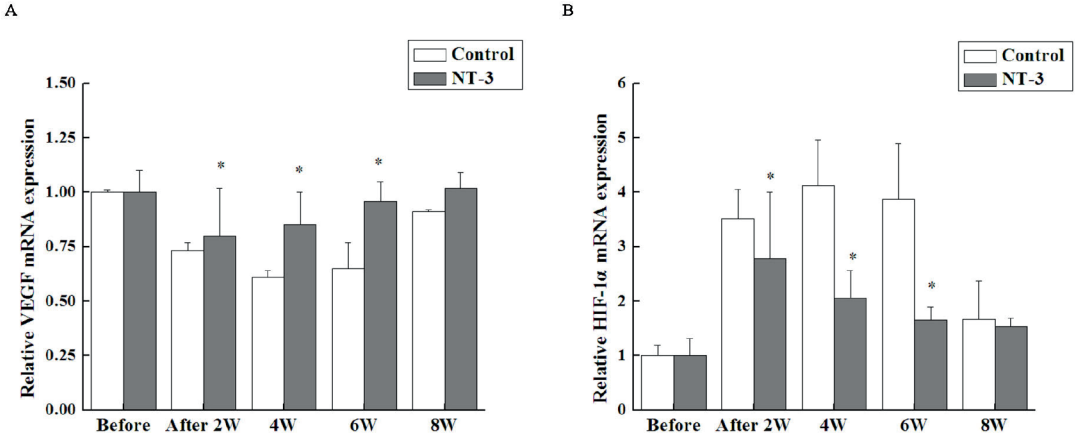

Figure 4. The expression of HIF-1α ***(A)*** and VEGF ***(B)*** mRNA in callus tissue of each group before surgery and after surgery (2 weeks, 4 weeks, 6 weeks, and 12 weeks). * compared with control group, $p < 0.05$

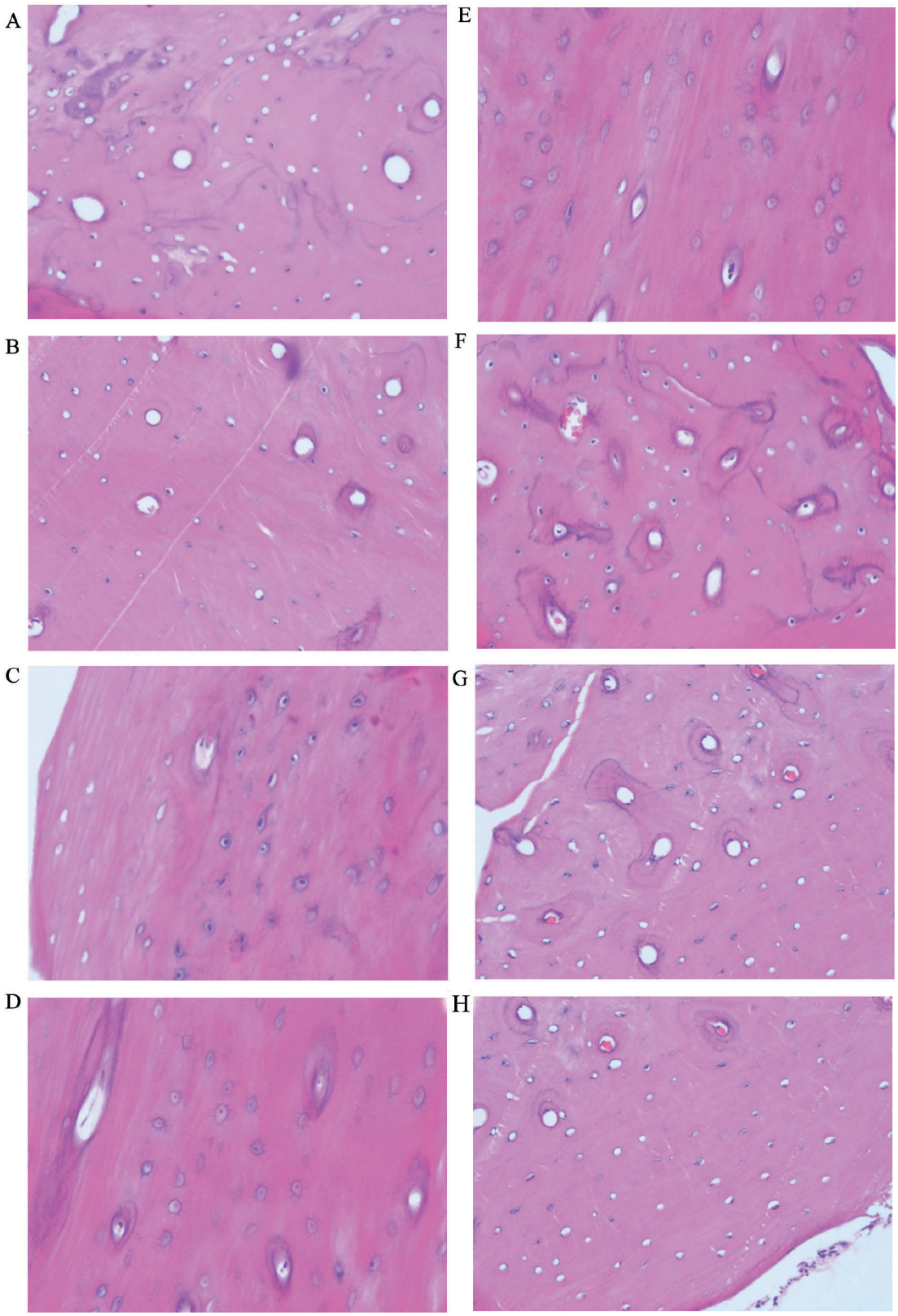

Figure 5. The changes of pathological tissue in tibia of the rats in each group (×100, N=10). ***A,*** Control group, 2 weeks after surgery; ***B,*** Control group, 4 weeks after surgery; **C,** Control group, 6 weeks after surgery; ***D,*** Control group, 8 weeks after surgery; ***E,*** NT-3 group, 2 weeks after surgery; ***F,*** NT-3 group, 4 weeks after surgery; ***G,*** NT-3 group, 6 weeks after surgery; ***H,*** NT-3 group, 8 weeks after surgery.

Conclusions

NT-3 can improve fracture healing in rats by regulating the tibia BMD, biomechanical indexes, and the bone formation.

Conflict of Interest

The Authors declare that they have no conflict of interest.

References

1) Meling T, Harboe K, Soreide K. Incidence of traumatic long-bone fractures requiring in-hospital management: a prospective age- and gender-specific analysis of 4890 fractures. Injury2009; 17: 1212-1219.
2) Kawaguchi H, Nakamura K, Tabata Y, Ikada Y, Aoyama I, Anzai J, Nakamura T, Hiyama Y, Tamura M. Acceleration of fracture healing in nonhuman primates by fibroblast growth factor-2. J Clin Endocrinol Metab 2001; 86: 875-880.
3) Keating JF, Blachut PA, O'Brien PJ, Court-Brown CM. Reamed nailing of Gustilo grade-IIIB tibial fractures. J Bone Joint Surg Br 2000; 82: 1113-1116.
4) Liang Y, Chu P, Wang X. Health-related quality of life of Chinese earthquake survivors: a case study of five hard-hit disaster counties in Sichuan. Soc Indic Res 2014; 119: 943-966.
5) Liang Y, Cao R. Employment assistance policies of Chinese government play positive roles! The impact of post-earthquake employment assistance policies on the health-related quality of life of Chinese earthquake populations. Soc Indic Res 2015; 120: 835-857.
6) Di S G, Del P A, Covelli B, Abete E, Varriale E, Salzano S, Postiglione L. Vitamin D receptor polymorphisms as tool for early screening of severe bone loss in women patients with rheumatoid arthritis. Eur Rev Med Pharmacol Sci 2016; 20: 4664-4669.
7) Garcia-Alias G, Petrosya HA, Schnell L, Horner PJ, Bowers WJ, Mendell LM, Fawcett JW, Arvanian VL. Chondroitinase ABC combined with neurotrophin NT-3 secretion and NR2D expression promotes axonal plasticity and functional recovery in rats with lateral hemisection of the spinal cord. J Neurosci 2011; 31: 17788-17799.
8) Marsell R, Einhorn TA. The biology of fracture healing. Injury 2011; 42: 551-555.
9) Munns CF, Rauch F, Zeitlin L, Fassier F, Glorieux FH. Delayed osteotomy but not fracture healing in pediatric osteogenesis imperfecta patients receiving pamidronate. J Bone Miner Res 2004; 19: 1779-1786.
10) Zhang Y, Wang N, Ma J, Chen XC, Li Z, Zhao W. Expression profile analysis of new candidate genes for the therapy of primary osteoporosis. Eur Rev Med Pharmacol Sci 2016; 20: 433-440.
11) Cho TJ, Gerstenfeld LC, Einhorn TA. Differential temporal expression of members of the transforming growth factor beta superfamily during murine fracture healing. J Bone Miner Res 2002; 17: 513-520.
12) Chen Z, Zhao GH, Zhang YK, Shen GS, Xu YJ, Xu NW. Research on the correlation of diabetes mellitus complicated with osteoporosis with lipid metabolism, adipokines and inflammatory factors and its regression analysis. Eur Rev Med Pharmacol Sci 2017; 21: 3900-3905.
13) Curtis KM, Gomez LA, Schiller PC. Rac1b regulates NT3-stimulated Mek-Erk signaling, directing marrow-isolated adult multilineage inducible (MIAMI) cells toward an early neuronal phenotype. Mol Cell Neurosci 2012; 49: 138-148.
14) Claes L, Recknagel S, Ignatius A. Fracture healing under healthy and inflammatory conditions. Nat Rev Rheumatol 2012; 8: 133-143.
15) Tsuji K, Bandyopadhyay A, Harfe BD, Cox K, Kakar S, Gerstenfeld L, Einhorn T, Tabin CJ, Rosen V. BMP2 activity, although dispensable for bone formation, is required for the initiation of fracture healing. Nat Genet 2006; 38: 1424-1429.
16) Asaumi K, Nakanishi T, Asahara H, Inoue H, Takigawa M. Expression of neurotrophins and their receptors (TRK) during fracture healing. Bone 2000; 26: 625-633.
17) Chen HH, Frank E. Development and specification of muscle sensory neurons. Curr Opin Neurobiol 1999; 9: 405-409.
18) Bernd P. The role of neurotrophins during early development. Gene Expr 2008; 14: 241-250.
19) Zhou XF, Li WP, Zhou HH, Zhong JH, Mi JX, Wu LL, Xian CJ. Differential effects of endogenous brain-derived neurotrophic factor on the survival of axotomized sensory neurons in dorsal root ganglia: a possible role for the p75 neurotrophin receptor. Neuroscience 2005; 132: 591-603.
20) Kumar S, Biancotti JC, Yamaguchi M, Vellis J. Combination of growth factors enhances remyelination in a cuprizone-induced demyelination mouse model. Neurochem Res 2007; 32: 783-797.
21) Xian CJ, Zhou XF. Treating skeletal pain: limitations of conventional anti-inflammatory drugs, and anti-neurotrophic factor as a possible alternative. Nat Clin Pract Rheumatol 2009; 5: 92-98.
22) Su YW, Chung R, Ruan CS, Chim SM, Kuek V, Dwivedi PP, Hassanshahi M, Chen KM, Xie Y, Chen L, Foster BK, Rosen V, Zhou XF, Xu J, Xian CJ. Neurotrophin-3 induces BMP-2 and VEGF activities and promotes the bony repair of injured growth plate cartilage and bone in rats. J Bone Miner Res 2016; 31: 1258-1274.
23) Turner CH. Biomechanics of bone: determinants of skeletal fragility and bone quality. Osteoporos Int 2002; 13: 97-104.
24) Hak DJ, Toker S, Yi C, Toreson J. The influence of fracture fixation biomechanics on fracture healing. Orthopedics 2010; 33: 752-755.

25) Yin LL, Jing N, Zhao ZY, Liu XH, Du J, Qin LL, Song JG, Xu Y. Relationship between the gene polymorphism of osteoprotegerin and bone mineral density in hemodialysis patients. Eur Rev Med Pharmacol Sci 2017; 21: 37-41.

26) Namkung-Matthai H, Appleyard R, Jansen J, Hao LJ, Maastricht S, Swain M, Mason RS, Murrell GA, Diwan AD, Diamond T. Osteoporosis influences the early period of fracture healing in a rat osteoporotic model. Bone 2001; 28: 80-86.

27) Funk JR, Hale JE, Carmines D, Gooch HL, Hurwitz SR. Biomechanical evaluation of early fracture healing in normal and diabetic rats. J Orthop Res 2000; 18: 126-132.

28) Aiga A, Asaumi K, Lee YJ, Kadota H, Mitani S, Ozaki T, Takigawa M. Expression of neurotrophins and their receptors tropomyosin-related kinases (Trk) under tension-stress during distraction osteogenesis. Acta Med Okayama 2006; 60: 267-277.