Int J Clin Exp Pathol 2017;10(5):6054-6063
www.ijcep.com /ISSN:1936-2625/IJCEP0047970

Original Article

Panax notoginseng saponins exert osteogenic promotion effect on rabbit distraction osteogenesis model through TGF-β_1 signaling pathway

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Received January 3, 2017; Accepted March 29, 2017; Epub May 1, 2017; Published May 15, 2017

Abstract: The purpose of this study is to find an effective method to promote distraction osteogenesis and reduce complications. 32 adult New Zealand rabbits were randomly divided into 4 groups, which were control, low, middle and large dosage group. All of them were conducted operation for distraction osteogenesis (DO). In 3 treatment groups, PNS was given intramuscularly on the dose of 40 mg/80 mg/120 mg per day per kg since the next day of operation. General observation, X-ray observation, HE staining, SEM, ELISA and q-RCR were applied on the local tissue of DO zone, which were collected at the end of DO and 2 week fixation. At both time points, bone mineral density is higher in middle and high dose group; the percentage of trabecular bone area in 3 treatment groups was significantly higher than control group. At the end of DO, local tissue TFG-β_1 concentration in middle dose group and high dose group were higher than the control group. Local TGF-β_1 mRNA expression in low and middle dose group were significantly higher than control group ($P<0.05$). Automatic biochemical analyzer and ELISA were applied on serum isolated from the vein blood in all groups at three time points, which were the end of DO, 1 w fixation, 2 w fixation to determine the serum ALP and TGF-β_1 activity. Serum ALP activity in middle and high dose groups at the end of DO, high dose group at 1 w fixation were significantly higher than that in control group ($P<0.05$). Serum TGF-β_1 activity in middle and high dose groups at the end of DO and middle group at 1 w fixation were significantly higher than that in the control group ($P<0.05$). PNS can promote DO and the effect is partly mediated by TGF-β_1 signaling pathway.

Keywords: Panax notoginseng sopanins, distraction osteogenesis, TGF-β_1 signaling pathway, osteogenic promotion

Introduction

Mandibular distraction osteogenesis is currently an accepted method of treatment for patients requiring reconstruction of hypoplastic mandibles and large bone defects which were caused by tumor resection. To date some of the unsolved problems is how to promote the quantitative increase of mandible length and solve some disadvantages of distraction osteogenesis. The disadvantages of this technique include a prolonged treatment period, secondary surgical intervention and bone nonunion. Panax Notoginseng is a well-known traditional Chinese medicine which has been applied on promoting bone fracture healing since ancient time. Panax Notoginseng Saponins (PNS), believed as its active component has been developed as a medicine for the adjuvant treatment of thrombosis diseases in China. Recent studies reported that PNS could stimulate alkaline phosphatase (ALP) activities and increase the number of osteoblasts in vitro, promote the proliferation of bone marrow mesenchymal stromal cells of rats, and is effective in promoting local blood circulation after bone fracture on animal tests. These studies suggest that PNS may process therapeutic potency on treating bone nonunion in distraction osteogenesis, osteoporosis and osteonecrosis.

Materials and methods

Animal and reagents

This study was approved by the animal ethics committee of Guangxi Medical University. 32 healthy adult New Zealand rabbits (without gen-

der limitations) were supplied by the Experimental Animal Center of Guangxi Medical University in China. (License No.: SCXK GUI 2009-0002).

Reagents and equipment were showed as following, including PNS (Freeze-dried XueShuan Tong Injection, Wuzhou Pharmaceutical Group Co. Ltd, Guangxi, China, No. Z20025652, 150 mg/bottle); ELISA kit (Wuhan Huamei biotechnology company, China); total RNA-extracted kit (Corning Company, USA); reverse transcription kit (TAKARA Company, Japan); real-time PCR primer (Shanghai Bioengineering Co. Ltd, China); DAB kit (Beijing Zhongshan Biotechnological Co. Ltd, China); inverted aberration microscope (Zeiss Company, Germany); PCR instrument (Bio-Rad, USA; Scanning electron microscopy (Vega 3, Tescan, Czech Republic); Leica Q500IW Image analysis system (Leica, Germany). Automatic enzyme-linked immunosorbent assay systems (iMark, Bio-Rad, USA); Sliding Microtome (NVSLM1, WPI company, USA); rat anti-rabbit TGF beta 1 antibody (Novus Company, USA); goat anti-rabbit IgG (Biotech, USA).

Animal model preparation and drug administration

32 adult rabbits were randomly divided into 4 groups which are control group, low dose group, middle dose group, high dose group. All animals were conducted operation and installed the distractor on right mandible under the sterile procedure. The disconnected line was set on about 1 cm away from mandible angle. A 7-day distraction procedure began on the 8th day after operation, with a speed of 0.5 mm/time, twice a day. Animals in low/middle/high dose group were given PNS intramuscularly on dosage of 40 mg/80 mg/120 mg per day per kg of weight since the next day of operation. Those in control group were given equivalent NS. The injection went once a day and lasted till one day before execution.

Collection of tissue specimen: The animals were executed for mandible sample collection at two time points: the next day of the end of distraction (the end of DO) and after 2-week fixation (2 w fixation). At each time point, 4 animals in each group were executed. The samples of local tissue on the distraction zone were collected and properly preserved.

Collection of serum specimen: To isolate the serum, blood from the animal ear vein were collected at three time points: end of DO, after 1 w fixation, after 2 w fixation.

General observation and X-ray inspection of the distraction zone

Before removed the distractor, the isolated mandible sample were observed and taken X-ray photos.

Scanning electron microscopy observation on distraction zone osteogenesis

Fresh bone tissue in distraction gap was collected, and made about 3 mm×3 mm size tissue block, then put in 3% glutaraldehyde solution overnight. These tissue blocks were cleaned with PBS buffer for 15 minutes, totally 3 times, and then put in PBS buffer using ultrasonic cleaning within 10 min, and rinsed again and again with distilled water. In turn these tissue blocks were put into 50%, 70%, 50%, 70%, 100% (3 times), 100% ethanol and 100% isoamyl acetate (3 times), dehydration step by step, 10 minutes at a time. These tissue blocks were sprayed gold plating in vacuum. The specimens in different groups were observed under scanning electron microscope.

HE staining observation on distraction zone

The preserved tissue samples were processed and the HE staining slices were made for observing the structure of regenerated tissue in the distraction zone. Images were taken and analysis with Leica Q500IW. 4 field of vision were selected randomly for calculating the trabecular area percentage (Tb•Ar%). Data in this part were presented as mean + SD. Statistical significance was determined by two-tailed student's t-test. A *P*-value of 0.05 was considered statistically significant. A *P*-value was less than 0.01 and considered extremely significant. The data in different dose group were respectively compared with the control group on two time points, but the data were not compared in different time points.

Serum ALP activity examination

After collecting animal serum specimens, using automatic biochemical analyzer test ALP activity. After calculating the mean and standard

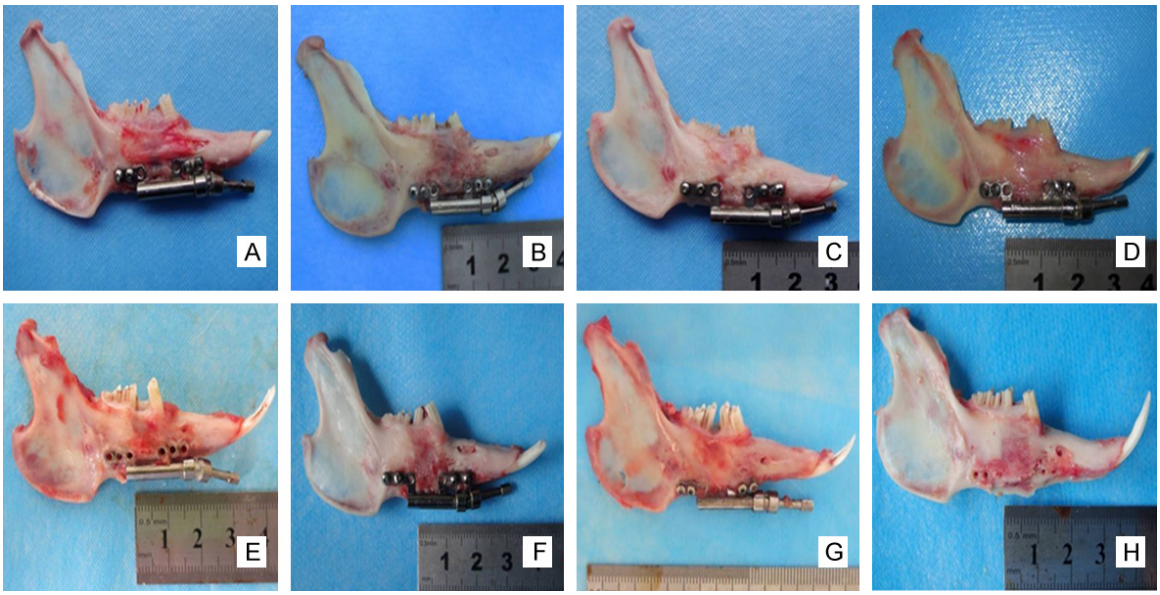

Figure 1. Mandibula of different groups at the end of distraction (A: Control; B: Low dose; C: Middle dose; D: High dose) and 2 w fixation (E: Control; F: Low dose; G: Middle dose; H: High dose).

deviation of results in all groups, the ALP activity were compared between PNS groups and the control group. Data in this part were presented as mean + SD. Statistical significance was determined by two-tailed student's t-test. A *P*-value of 0.05 was considered statistically significant. A *P*-value was less than 0.01 and considered extremely significant. The data in different dose group were respectively compared with the control group on three time points, but the data were not compared in different time points.

Serum TGF-β_1 activity and local TGF-β_1 concentration by ELISA

Experiments were done according to Wuhan Huamei biotechnology company kit (double antibody sandwich method) instructions. Data in the part of serum TGF-β_1 activity and local TGF-β_1 concentration by ELISA were presented as mean + SD. Statistical significance was determined by two-tailed student's t-test. A *P*-value of 0.05 was considered statistically significant. A *P*-value was less than 0.01 and considered extremely significant. The data in different dose group were respectively compared with the control group on different time points, but the data were not compared in different time points.

Impact of PNS on TGF-β_1 mRNA expression in distraction zone tissue (q-PCR)

Following the Instruction of AxyPrep® Total RNA Preparation Kit, the total RNA was extracted from 40 mg of tissue collected from the distraction zone. A small amount of liquid nitrogen was applied for grounding the sample. Extracted RNA (500 μg) was converted into cDNA via reverse transcriptase and was put into the real-time q-PCR: primer TGF-β_1-F: 5'-ACTGCTTCAGCTCCACAGAGA, TGF-β_1-R: 5'-GGACCTTGCTGTACTGGGTGT, amplified fragment 150 bp; GAPDH-75 bp, GAPDH-F: 5'-CCACTTTGTGAAGCTCATTTCCT, GAPDH: 5'-TCGTCCTCCTCTGGTGCTCT; the reaction condition of PCR was showed as following: pre-denaturation 95°C 30 s; PCR 95°C 5 s, 60°C 30 s, 40 circulations; 60°C-95°C melting curve analysis. The RQ value should be calculated via $2^{-\Delta\Delta}$Ct method and repeated three times for each sample. Data in this part were presented as mean + SD. Statistical significance was determined by two-tailed student's t-test. A *P*-value of 0.05 was considered statistically significant. A *P*-value was less than 0.01 and considered extremely significant. The data in different dose group were respectively compared with the control group on two time points, but the data were not compared in different time points.

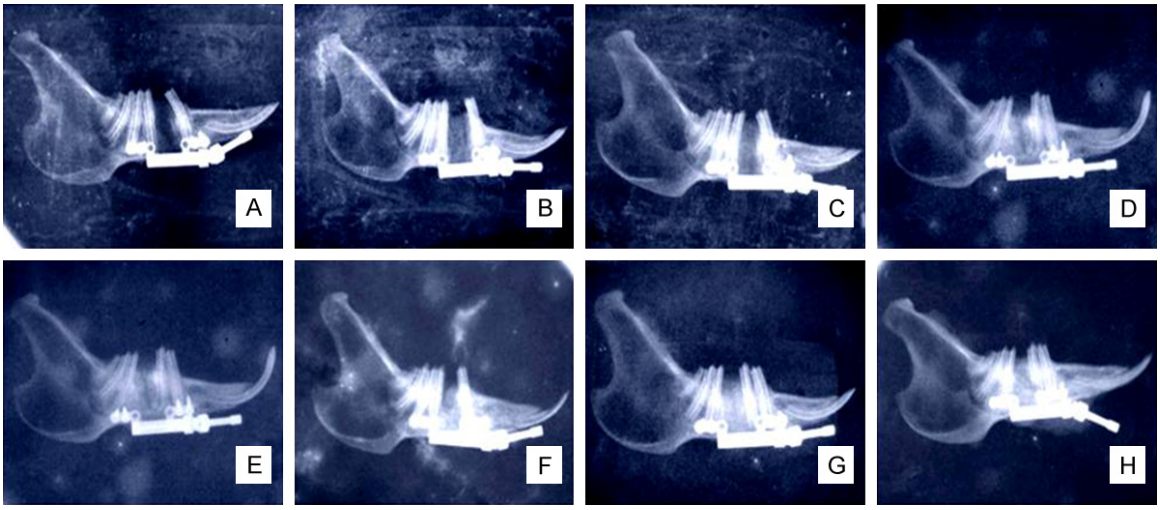

Figure 2. X-ray examination on mandibula of different groups at the end of DO (A: Control; B: Lowl dose; C: Middle dose; D: High dose) and 2-week fixation after DO (E: Control; F: Low dose; G: Middle dose; H: High dose).

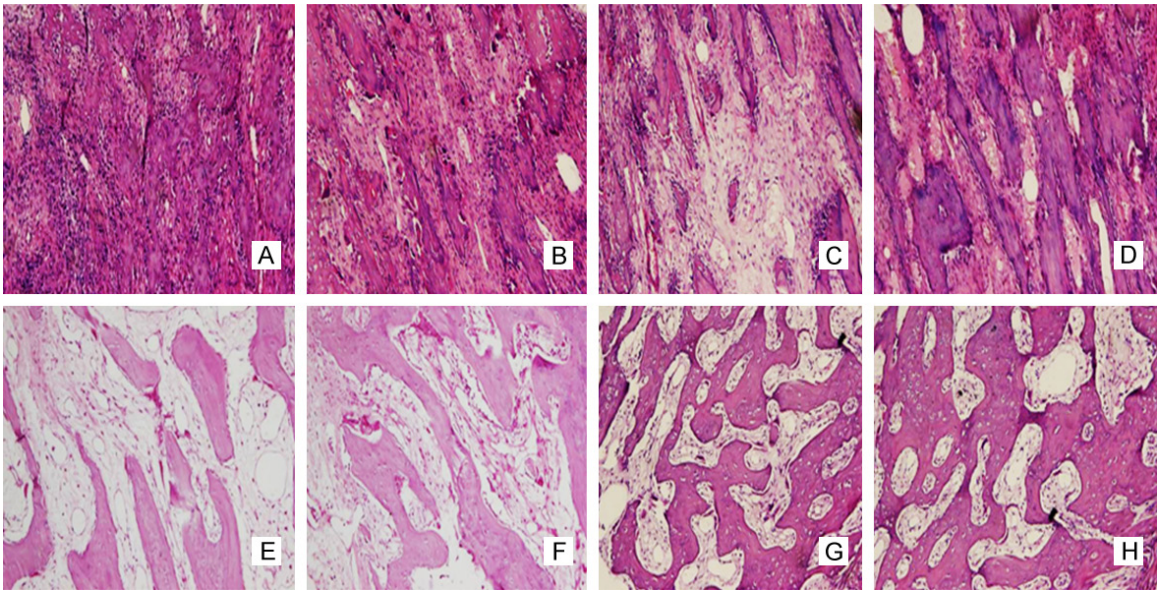

Figure 3. HE histology view of different groups at the end of DO 100× (A: Control; B: Low dose; C: Middle dose; D: High dose) and after 2-week fixation 100× (E: Control; F: Low dose; G: Middle dose; H: High dose).

Statistical analysis

Quantitative data were presented as mean + SD. Statistical significance was determined by two-tailed student's t-test. A *P*-value of 0.05 was considered statistically significant.

Results

General observation of the distraction zone

At the end of DO, distraction gaps in all groups were filled with regenerated callus tissue and covered with hard-to-remove connective tissue. Comparing with those in the control group, the boundary of distraction gap in PNS groups was not so clear. At the time point after 2 w fixation, distraction gaps in all groups were filled with regenerated bone tissue. The connective tissues were easy to remove so as to expose the newly generated bone cortex. Compared to the control group and low dose group, continuous smooth bone cortex was observed in middle and high dose group. In general, at both time points, regenerated tissue in PNS groups were

Table 1. Comparisons of the percentage of trabecula bone area (Tb•Ar%) among all groups ($\bar{x}$±s, *n*=4)

	Control	Low dose	Middle dose	High dose
At the end of DO	24.7±3.5	30.9±2.3*	34.0±4.7*	35.5±3.8*
2 w fixation	49.6±4.7	60.7±5.2*	64.7±6.3*	66.2±7.6*

*Significant differences (P<0.05) compared to the control group at the same time point (**Figure 3**).

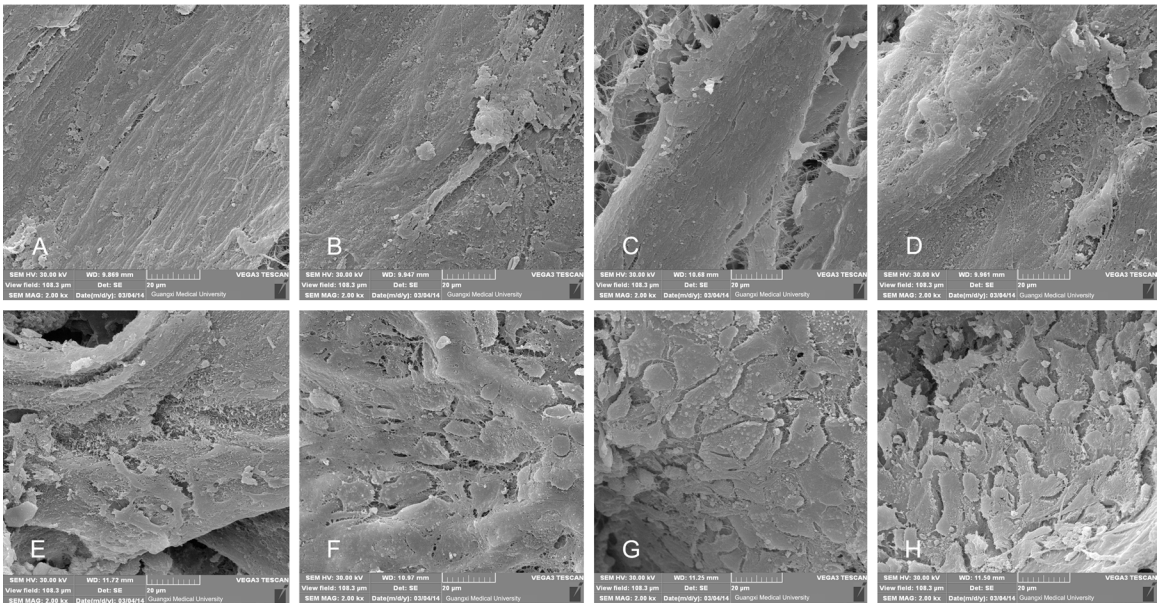

Figure 4. SEM view of different groups at the end of DO 2000× (A: Control; B: Lowl dose; C: Middle dose; D: High dose) and after 2-week fixation 2000× (E: Control; F: Low dose; G: Middle dose; H: High dose).

harder than those in the control group. Unilateral mandible in all animals were successfully lengthened about 7 mm. No significant difference on distraction length was found among these 4 groups (**Figure 1**).

X-ray inspection of the distraction zone

The shadow in middle and high dose group was deeper than control and low dose group. It shows bone mineral density is higher in middle and high dose group.

After 2 w fixation, there was a small amount of uneven X-ray transmission belt in control group. The shadow in each PNS group was slightly shallow than normal bone in distraction gap, which shows bone density increased significantly and formed continuous bone cortex. Bone quality is higher in PNS group (**Figure 2**).

HE staining observation on distraction zone

As seen on the slices of the sample obtained after 2 w fixation, more thick trabecula were observed in the distraction gap, while less fiber tissue were seen. Many osteoblasts were found laid around trabecula. Compared to the control group, more trabecula with less space was seen in the 3 PNS groups. Thickness of trabecula increased with the dosage, which indicated the maturity of new generated bone tissue (**Figure 3**; **Table 1**).

Scanning electron microscopy observation on distraction zone osteogenesis

At the end of DO, there existed a large amount of collagen fibers in distraction zone in different groups, whose arrangement is the same with and distraction direction. Fibrous tissue was seen in control group and low dose group. A

Table 2. Serum ALP activity in PNS groups and the control ($\bar{x}\pm s$) (U/L)

Group	Control	Low dose	Middle dose	High dose
At the end of DO	97.94±15.63	113.56±25.29	121.18±22.78*	150.56±17.03**
1 w fixation	86.88±22.15	81.25±17.69	83.63±15.65	126.88±9.44**
2 w fixation	81.13±13.93	82.63±8.28	80.00±13.57	88.75±9.25

Extremely significant differences (P<0.01) and *significant differences (P<0.05) compared to the control group at the same time point (Figure 5**).

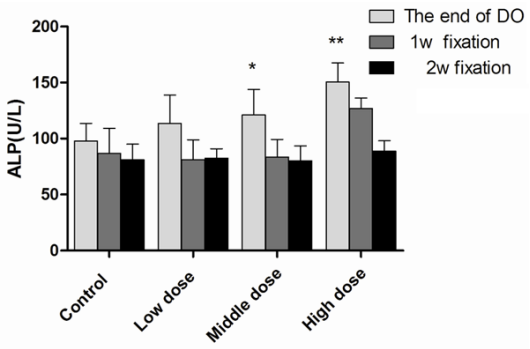

Figure 5. Comparison of serum ALP concentration among groups with different fixation time ($\bar{x}\pm s$).

large number of flat epithelioid cells were found in middle and high dose group, these cells may be osteoblast or undifferentiated stem cells.

After 2 w fixation, there existed a little fibrous tissue into distraction zone in different groups. Tissue in distraction zone also had no obvious arrangement orientation. There was concave and convex porous structure formation into partial area, and a layer of epithelioid cells (osteogenesis or osteoclast) were above them. It shows that tissue is in the process of strong bone repair. Each PNS group has a lot of flat epithelioid cells, and cell number is higher than the control group, which prompts PNS groups have more exuberant bone formation effects and higher bone healing (**Figure 4**).

Serum ALP activity examination (**Table 2**; **Figure 5**).

Serum TGF-β_1 activity and local TGF-β_1 concentration by ELISA (**Tables 3**, **4**; **Figure 6A**, **6B**).

Impact of PNS on TGF-β_1 mRNA expression in distraction zone tissue (q-PCR)

As shown in **Figure 6C**, at the end of DO, the TGF-β_1 mRNA relative expression in low and middle dose group were significantly higher than that in control group (P<0.05), but the expression in high dose group was not (P>0.05). As shown in **Figure 6C**, at the time point of 2 w fixation, the expression in 3 PNS groups was increased but the differences were not significant (P>0.05).

Discussion

Distraction Osteogenesis (DO) is an endogenous tissue engineering technology for new bone formation by taking advantage of the callus healing mechanism. In DO, bone is firstly sectioned into two or several segments and a special designed distractor is fixed on bone segments; distraction in certain speed to opposite direction activates the endogenous potency of tissue regeneration and new bone is continuously formed in the distraction gap so as to lengthen the bone [1]. Due to its broad application prospect in cranial and maxillofacial plastic, tumor surgery reconstruction, implant reparation of alveolar bone, DO has become a research hotspot on oral and maxillofacial surgery. However, the main disadvantage of DO is its prolonged course of treatment. Traditional Chinese medicine (TCM) enjoys a long history in orthopedics and traumatology. Therefore, from the perspective of TCM, seeking approaches for promoting osteogenesis will increasingly become the new path for bone healing study in DO.

Panax Notoginseng Saponins (PNS) is the main active component of Sanqi, a Chinese herb which is with the function of promoting blood circulation to remove stasis and broadly used for trauma and bone fracture in the clinical practice of Chinese Medicine [2-4]. PNS is the combination of a variety of saponins, including Ginsenoside Rb1, Rb2, Rc, Rd, Re, Rf, Rg1, Rg2, Rh1 and notoginsenoside R1, R2, R3, R4, R6 and other saponins. Due to the similarity among these compounds, most pharmacologi-

Table 3. Serum TGF-β_1 activity in PNS groups and the control ($\bar{x}$±s, ng/ml, n=4)

Group	Control	Low dose	Middle dose	High dose
At the end of DO	9.63±2.64	9.55±2.33	13.82±3.98*	15.95±4.54**
1 w fixation	7.75±1.58	7.94±3.15	14.39±4.75*	12.98±5.75
2 w fixation	6.33±0.90	5.97±1.44	7.02±2.23	6.91±2.16

Extremely significant differences (P<0.01) and *significant differences (P<0.05) compared to the control group at the same time point (Figure 6A**).

Table 4. Local TGF-β_1 concentration of PNS groups and the control ($\bar{x}$±s, *n*=4, ng/g)

Group	Control	Low dose	Middle dose	High dose
At the end of DO	6.86±1.65	6.57±0.73	9.86±1.51*	9.69±2.55*
2 w fixation	5.26±1.05	5.72±0.61	6.63±1.42	6.70±2.36

*Significant differences (P<0.05) compared to the control group at the same time point (**Figure 6B**).

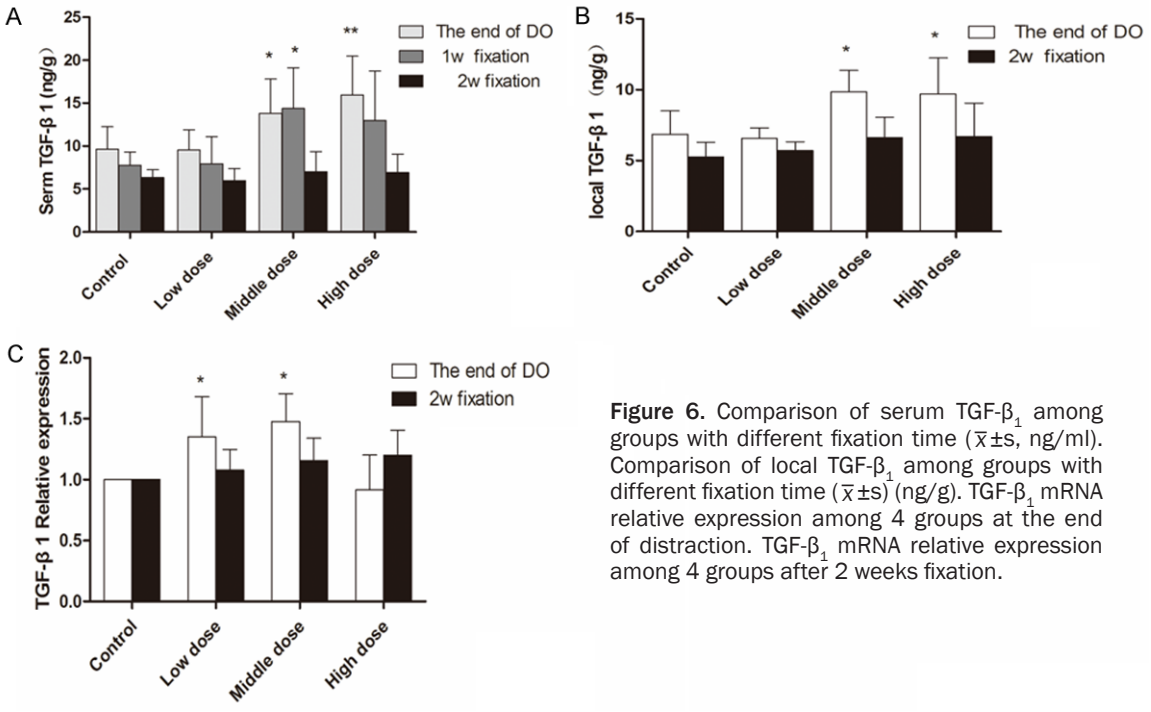

Figure 6. Comparison of serum TGF-β_1 among groups with different fixation time ($\bar{x}$±s, ng/ml). Comparison of local TGF-β_1 among groups with different fixation time ($\bar{x}$±s) (ng/g). TGF-β_1 mRNA relative expression among 4 groups at the end of distraction. TGF-β_1 mRNA relative expression among 4 groups after 2 weeks fixation.

cal researches on Sanqi were conducted with its main extract, PNS.

Some studies on the in vivo and in vitro osteogenesis effect of PNS had been reported, but most of them focused on the blood supply improving effect of it. It is reported that intravenous infusion of PNS could significantly release the swelling and pain and improve the concrescence in Colles facture and tibiofibular fractures patients. The possible mechanism is adjusting the hemodynamics and hemorheology to improve the local blood supply. Yang Jun reported that PNS can shorten the facture concrescence time and improve the quality of callus in rats and believed that is related to lowing the whole blood viscosity and plasma viscosity, so as to improve the local blood supply in facture [5]. It is reported that PNS can promote the mature of cells of tendon-bone interface, promote the formation of collagen growth on tendon-bone interface, so as to improve the osteogenesis and improve the process and quality of tendon-bone healing. Fierro [6] found that intra-

peritonealinjection of PNS increased the expression of HIF-1α gene in callus in left radius fracture model of rats, indicated the osteogenesis mechanism of it may related to angiogenesis inducing so as to increase the blood supply at facture zone.

In this study, commonly used experiments for observed the healing of fracture and bone regeneration were conducted to evaluate the impact of PNS on rabbit DO model. In the X-ray observation on distraction zone, it was observed that the longer the fixation, the closer the shade of distraction zone is to normal bone tissue on the same mandible sample. It indicated the bone density increased with fixation time. The low-density gap in the middle of distraction zone indicated the healing process were started from bone stump of both sides, which were the general pattern of bone healing and agreed with literatures. At both time points, the bone density was higher in the medium dosage group and large dosage group, comparing to the control group. In the HE staining slice observation, at the end of distraction, the direction of bone trabecula were in accord with the direction of distraction. At the point of 2 W fixation, the bone trabecula were bigger than those at the end of distraction. This is agreed with the general development rules in DO. Generally speaking, the bone trabecula in the PNS groups were bigger and more mature than those in the control group which is agreed with literatures. At both time points, the trabecular bone area in PNS groups was significantly higher than that in control group. It indicated that the bone quality is higher in PNS groups than that in the control group. Results of X-ray inspection, SEM inspection and HE staining essay indicated PNS is with the potency of promoting bone healing.

As a member of the family of TGF-β super family, TGF-β_1 is an important cytokine regulating the osteogenesis and inserts great influence to the balance between resorption and formation in the process of facture healing, bone regeneration and bone reconstitution. Some study [7-9] showed that the osteogenesis regulating functions includes: inducing the transformation of mesenchymal cells, regulating the differentiation of osteoblasts and chondroblasts, promoting the synthesis and excretion of extracellular matrix, regulating the reconstitution and remodeling of bone tissue via regulating the function of osteoclasts, regulating other growth factors and hormones. Krafft [10] found that the stimulation of the distraction force can continuously increase the expression of TGF-β_1 in the distraction zone in the dog mandible DO model. Other report [7] also indicated that TGF-β_1 was involved in the process of promoting osteogenesis in rabbit mandible DO model.

Observing on the bone healing related cytokines is the key to the study on the healing process and its molecular mechanism of bone trauma such as DO operation, facture, bone defect and so on. Benisch [11] found that the strongest expression of TGF-β_1 gene was spotted right at the end of distraction and decreased as time goes on. Kim [12] reported that the TGF-β_1 protein content in the distraction zone reached to the highest point after 2-week fixation (4 weeks after operation) in rabbit mandible DO model. In this study, the q-PCR results showed the TGF-β_1 mRNA expression was higher at the time point of the end of distraction than that of 2 w fixation, which indicated the expression decreased along with the process of healing. And the expression in high dose group was significantly higher than the control at the end of DO but no significant difference had been observed after 2 w fixation. The reason could be: the bone maturity at the time point of 2 w fixation was higher than that at the end of DO and the more mature the bone tissue, the less TGF-β_1 will be expressed. The phenomenon can also be observed in the dog mandible DO model in the previous study of our team.

As a cytokine for regulating bone reconstruction, TGF-β_1 plays an important role in fracture healing, new bone regeneration as well as the balance between resorption and formation during bone reconstruction. It was reported [8, 9] that TGF-β_1 exerts five positive effects on regulating osteogenesis, including the transformation of mesenchymal cells, the differentiation of osteoblasts and chondroblasts, the formation and excretion of extracellular matrix, the repairing and reconstruction of bone tissues via the regulation of osteoclasts, as well as the regulation of other hormones and growth factors. TGF-β_1 was likely to be the target of osteogenesis-promoting effect for most drugs.

However, the mechanism of osteogenesis might involve in several cell signal pathways, including TGF-β pathway, Notch pathway [3, 13-15], Wnt pathway [16], Hedgehog pathway [17] and MAPK pathway [18]. Further research will be launched to identify which pathway and target plays a dominant role in promoting osteogenesis.

In summary, PNS has an osteogenic promoting effect on rabbit DO model by up-regulating the gene expression of TGF-β_1. PNS can promote new bone formation and may solve prolonged treatment period, secondary surgical intervention and bone nonunion. Panax Notoginseng as a well-known traditional Chinese medicine may be an effective medicine to reduce complications of distraction osteogenesis in the future.

Acknowledgements

This work was funded by grants from the National Natural Science Foundation of China (81160132).

Disclosure of conflict of interest

None.

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