Trehalose enhances bone fracture healing in a rat sleep deprivation model

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Background: The purpose of this study was to investigate whether sleep deprivation (SD) could delay bone fracture healing and evaluate the therapeutic effect of trehalose.

Methods: Eighteen 300–350 g female Sprague-Dawley rats were created a mid-femoral transverse osteotomy in the right thigh and divided into three groups (i.e., group 1: fracture; group 2: fracture + SD; and group 3: fracture + SD + trehalose). Seven days after surgery, the rats in group 2 and group 3 were started to get sleep-deprived for 18 h per day for 3 weeks. The rats in group 3 were injected with trehalose intraperitoneally at 1 g/kg/d for 3 weeks. Radiological and histological analyses were used to assess fracture healing quality. Circulating cytokines were detected by the end of the study. The expression of M1 and M2 macrophage markers were measured by quantitative real-time polymerase chain reaction (qPCR).

Results: X-rays showed group 2 experienced much poorer fracture healing. Micro CT demonstrated that the bone quality of the fracture callus site in group 2 was much worse than that in groups 1 and 3. Both haematoxylin eosin (H&E) and Masson staining revealed that the bone fracture of the group 2 healed worse. Elisa results demonstrated that the interleukin-1 beta (IL-1β) and tumor necrosis factor-alpha (TNF-α) of the rats in group 2 were significantly higher. *In vitro* study showed that 100 mM trehalose enhanced the expression of M2 macrophage markers (Arg-1 and IL-10), and decreased M1 macrophage polarization through the decreasing expression of IL-6.

Conclusions: The present study showed (SD) could delay bone fracture healing in a rat model. And, trehalose could promote the healing of delayed bone fracture union by down-regulating pro-inflammatory mediators and enhancing M2 polarization.

Keywords: Sleep deprivation (SD); bone fracture healing; trehalose; circulating cytokines; M2 polarization

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Introduction

Sleep deprivation (SD) is a common problem. An increase number of adults experience SD in modern society (1). It is reported that approximately one-third of adults get less than 6.5 h of sleep per night (2). While, the American National Sleep Foundation suggests that the healthy

grown-ups should sleep for 7–9 h/day. Similarly, many adolescents suffer from sleep problem because of stress or early school start time (3). SD may contribute to various kinds of disorders, such as diabetes (4), cardiovascular disease (5), circadian rhythm problems (6), and metabolic syndrome (7). Several studies have showed that SD may lead to decreased bone mineral density (BMD) (8-11). It has been reported that the individuals who sleep for 6 h or less have significantly lower BMD compared to the ones who sleep for 8 h (8). Similarly, other clinical surveys showed that patients with SD have lower BMD (9-11). An animal study showed that SD affects bone health by decreasing BMD and 25(OH)D (12). The study also demonstrated that SD affected BMD by decreasing osteogenesis and impairing mineralization of the newly formed bones.

A high number of people in the world are involved in fractures, which are the most frequently injuries of the musculoskeletal system (13). Up still now, a large proportion of all fractures still display delayed healing or nonunion (14). Delayed fracture healing is attributed to several risk factors, such as type of injury, fracture treatment, age, gender, comorbidities, alcohol consumption, smoking, and medications (15).

The switch from the initially pro-inflammatory M1 macrophage to the anti-inflammatory M2 phenotype is essential for bone fracture healing (16). We hypothesized that SD, a systemic inflammatory factor, may also affect bone fracture healing, and the medications that could enhance M2 polarization may help accelerate fracture healing.

Trehalose is a natural disaccharide with chemical chaperone activity. As it has the ability to maintain the stability of nucleic acid and protein, trehalose has been widely used as a stabilizer in drugs, foods, and transplantation surgery (17,18). Additionally, trehalose was reported to be bioactive in various kinds of disease (i.e., Alzheimer, osteoarthritis, and osteoporosis) (19-21). Recently, it has been suggested that trehalose has an anti-inflammatory effect and may be a new therapeutic approach for treatment of inflammation (22). However, whether trehalose could enhance the healing of pathological delayed bone fracture union and direct macrophage polarization requires further investigation.

In the present study, an experimental SD with femoral fracture model was used. The effect of SD and trehalose on healing quality of bone fracture, and anti-inflammatory activity of trehalose were evaluated.

Methods

Laboratory animals

All animal care and surgical procedures were carried out in accordance with the guidelines and approved by the Ethics Committee of Drum Tower Hospital, Medical School of Nanjing University, Nanjing, China. Eighteen 300–350 g female Sprague-Dawley rats obtained from the Laboratory Animal Center of Drum Tower Hospital were randomly divided into fracture group (group 1, n=6), fracture + SD group (group 2, n=6), and fracture + SD + Tre group (group 3, n=6). Before the experiments, all animals were housed under the same conditions (humidity, 45%±5%; temperature, 25±2 ℃; 12 h/12 h light/dark cycle) for a week. The rats were fed a regular diet twice a day and allowed free access to water during the whole study.

Bone fracture

A mid-femoral transverse osteotomy was created in each animal, as described previously (23,24). Briefly, the surgical procedure was performed under general anesthesia and sterile conditions. A lateral incision was made on the right thigh of the rats to expose the femur. A thin oscillating blade was used to create a mid-femoral transverse osteotomy. The periosteum of the fracture site was removed to avoid fast healing. Then, the articular surface of the distal femur was exposed by a middle incision on the right knee. A 1.1 mm Kirschner wire was inserted in the medullary canal to fix the fracture in a retrograde fashion. The wire was cut to the level of the articular surface of the knee. The incision was closed with absorbable suture.

Experimental SD and trehalose administrated

A modified multiple platform method that was illustrated previously was selected to make the SD model in the study (25,26). Six narrow (6.5 cm in diameter) platforms were placed in the water tanks (60×40×30 cm^3) that were made of organic glass. The rats were housed in the platforms. In the SD making group (groups 2 and 3), the water tank was filled with water to a level of 1 cm below the upper surface of the platforms. When the rats reached the paradoxical phase of sleep, which is characterized by muscle atonia, their face would touch the water, and they would wake up. The housing conditions in group 1 were similar, but there was no water in the water tank, and the rats could move freely and

fall into asleep. Seven days after bone fracture procedure, the rats were started to get sleep-deprived using the platform for 18 h (starting at 16:00 h) per day for 3 weeks. The rats were allowed to sleep in their normal cage for the remaining time (6 h, 10:00–16:00 h). All rats have free access to water and food. Other conditions were kept constant (humidity, 45%±5%; temperature, 25±2 ℃; 12 h/12 h light/dark cycle). The rats in group 3 were injected with trehalose intraperitoneally at 1 g/kg/d since SD procedure began.

Radiological analysis

The rats were sacrificed 4 weeks after surgery, and the lower limbs were harvested. After removing the soft tissue, the specimens were X-rayed (MX-20; Faxitron Biooptics, Tucson, USA) to evaluate bone union. The limbs were laterally placed and exposed to a voltage of 45 kV for 5 s. The callus area was measured by two independent observers.

The fracture callus were scanned with a micro CT system (uCT-80, Scanco Medical, Bassersdorf, Switzerland). The micro-CT scanner was set at a voltage of 70 kVp and a current of 114 μA to collect images at a resolution of 15.6 um per pixel. Serial cross-sectional images of the callus areas were collected to perform three-dimensional histomorphometric analysis. Several architectural and densitometry parameters were determined: BMD, mineralized volume fraction (BV/TV), trabecular thickness (Tb.Th), trabecular number (Tb.N), and trabecular separation (Tb.Sp) (27).

Histological analysis

After X-ray and micro CT scan, the specimens were fixed in 4% paraformaldehyde and then decalcified in a 5% EDTA solution. Afterward, the specimens were dehydrated with a series of ethanol rinses and embedded in paraffin. Then, longitudinal sections (5 μm thickness) were prepared using a microtome (Leica RM2165, Leica, Germany). Representative sections were stained with haematoxylin eosin (H&E) and Masson staining. The imagines were scanned using case viewer software 3.3 (JAVS, Inc., Louisville, USA).

Enzyme linked immunosorbent assay (ELISA)

Blood was collected from the rat hearts after anesthesia, and a total of 0.5–1.5 mL of blood was collected. Serum samples were obtained by centrifugation of blood at 3,000 rpm for 10 minutes and stored at –80 ℃ before analysis. The plasma concentrations of the biomarkers serum interleukin-1 beta (IL-1β) and tumor necrosis factor-alpha (TNF-α) were measured using an assay (Proteintech, Chicago, USA) according to the manufacturer's instructions.

Cell culture

RAW264.7 murine macrophage cells (Institute of Life Science Cell Culture Center, Shanghai, China) were cultured in α-minimal essential medium (α-MEM) supplemented with 10% fetal bovine serum and penicillin (100 U/mL) at 37 ℃ in a 5% CO_2 atmosphere.

Cell viability assay

Cell viability assay of trehalose on RAW264.7 cells was assessed by CCK-8 assay according to the manufacturer's instructions. Cells were cultured in 96-well plates in α-MEM and incubated with different concentrations (0, 25, 50, 100 and 200 mM) of trehalose for 24 hours. CCK-8 reagents were added in each well for 3 hours at 37 ℃ and the absorbance was measured at 450 nm as an index of cell viability. For each of these experiments at least four parallel measurements were carried out.

Quantitative real-time polymerase chain reaction (qPCR)

Total RNA was extracted from RAW264.7 plated in 6-well plates using RNA-Quick Purification Kit (ES Science, Shanghai, China). HiScript II Q RT SuperMix for qPCR (Vazyme Biotech, Nanjing, China) was used to synthesize cDNA from total RNA. The PCR reactions were carried out on a light cycler instrument (LightCycler 480-II, Roche, Mannheim, Germany) using ChamQTM SYBR Colour qPCR Master Mix (Vazyme Biotech). Primers are described in *Table 1*. All data were normalized to β-actin. The relative quantification of RNA expression was performed using the $2^{-\Delta\Delta CT}$ method.

Statistical analysis

The data are expressed as mean ± standard deviation. One-way ANOVA (and nonparametric) test was used to assess the parameters among the three groups. P value ≤0.05 was considered a significant difference. The data were graphed

Table 1 List of primer sequences used for qPCR

Prime	Forward (5'-3')	Reverse (5'-3')
IL-6	GGCGGATCGGATGTTGTGAT	GGACCCCAGACAATCGGTTG
Arg-1	CTCCAAGCCAAAGTCCTTAGAG	AGGAGCTGTCATTAGGGACATC
IL-10	CGGGAAGACAATAACTGCACCC	CGGTTAGCAGTATGTTGTCCAGC
β-actin	CATGTACGTTGCTATCCAGGC	CTCCTTAATGTCACGCACGAT

qPCR, quantitative real-time polymerase chain reaction.

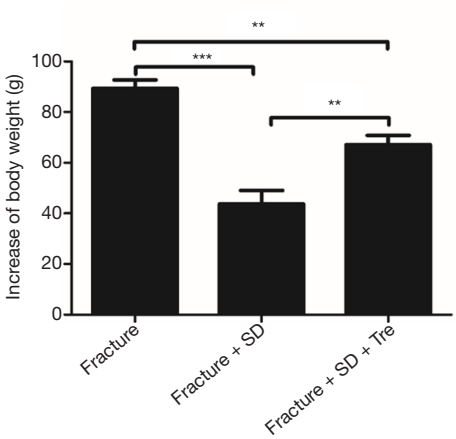

Figure 1 Increase of body weight in all the groups. The rats in fracture group (group 1) gained much more weight than that in fracture + SD group (group 2) and fracture + SD + Tre group (group 3). The body weight increase in group 2 was also less than that in group 3. **, P≤0.01 and ***, P≤0.001 indicate significance between two groups.

using GraphPad Prism software version 7 (GraphPad Software Inc., CA, USA).

Results

No rats dropped out before the end of the experiment. No adverse effect of trehalose was observed. The body weights of all the rats increased overtime. However, the rats in group 2 gained much less weight than the other two groups (*Figure 1*).

Radiological analysis

X-ray

X-rays taken at 4 weeks after surgery showed that the cortical gap was still apparent in group 2, indicating delayed bone union. By contrast, cortical gap disappeared at the fracture site in group 1 and group 3, and bridging callus formation was observed (*Figure 2*).

Micro CT

The micro CT images of the callus area in group 2 showed much worse bone reconstruction. While, the callus areas in groups 1 and 3 were reconstructed with intact cortical bone and nearly normal trabecular bone structure (*Figure 3A*,*B*,*C*). The BMD and BV/TV of the fracture callus site in group 2 were significantly less than the values in the other two groups, whereas the Tb.Sp was significantly higher (*Figure 3D*,*E*,*F*). The Tb.Th of group 2 was less than the other two groups, although no significant difference was detected (*Figure 3G*). The Tb.N in group 2 was significantly less than that of group 1; no significant difference was observed between group 2 and group 3 (*Figure 3H*).

Histological analysis

Groups 1 and 3 showed newly formed bone tissue at the fracture sites. Thick callus consisting of newly formed bone and chondrocytes could be observed in the two groups, resulting in bone union. In contrast, fibrotic tissue and chondrocytes could be observed in group 2, with no bridging bone formation. In group 2 inter-trabecula lacunas existed with mild inflammatory cell infiltration. However, no obvious inflammatory cell infiltration was observed in groups 1 and 3 (*Figure 4*).

Serum IL-1β and TNF-α

The concentration of serum TNF-α and IL-1β was significantly higher in group 2 compared with the other two groups (*Figure 5*). No significant difference was observed between groups 1 and 3 (*Figure 5*).

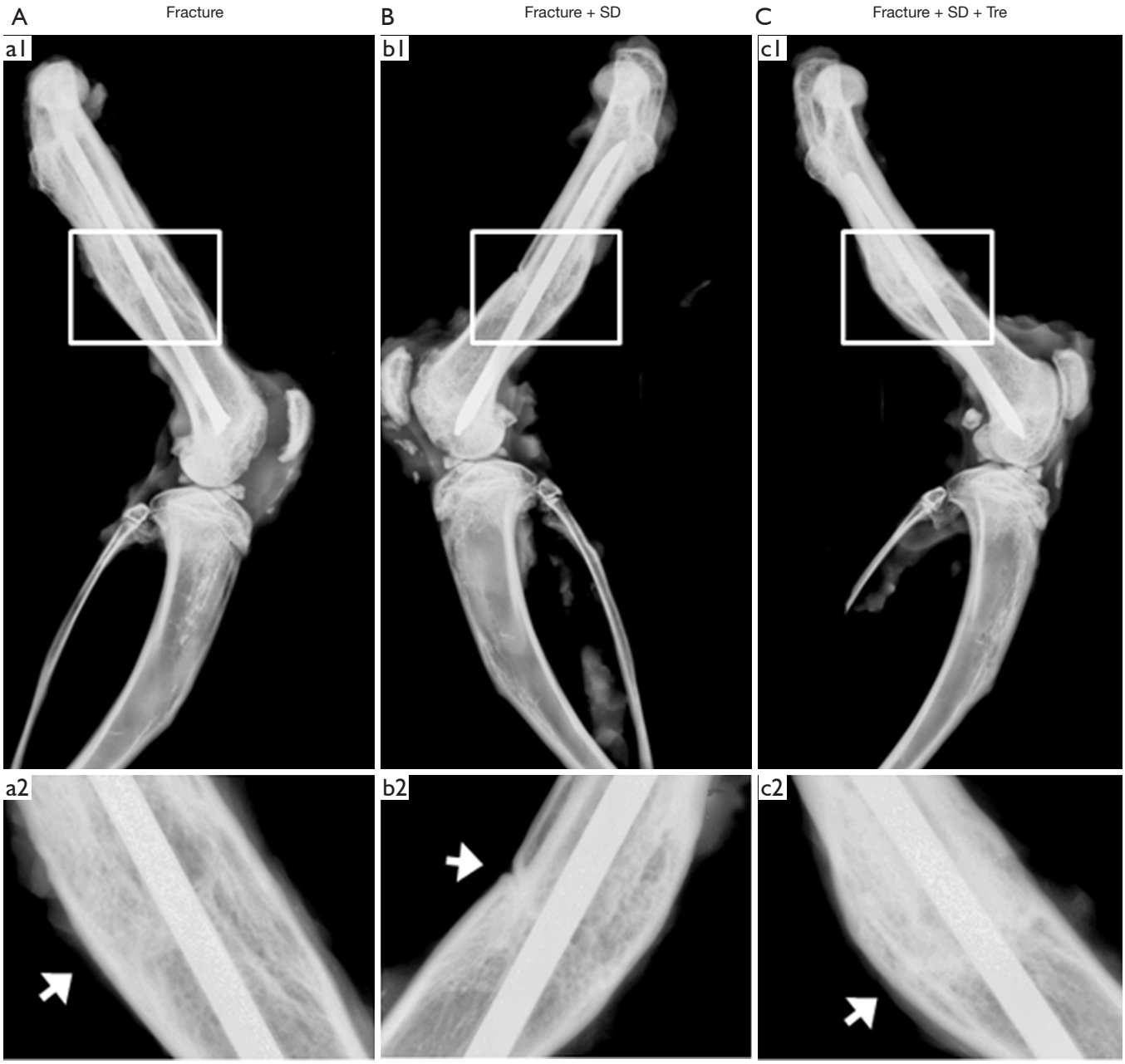

Figure 2 The X-ray analysis of the fracture healing in three groups (A,B,C). The X-rays (a1, b1, and c1) of the lower extremity showed that fracture + SD group (group 2) (b1) had delayed bone union, while the fracture (group 1) (a1) and fracture + SD + Tre group (group 3) (c1) had established fracture healing. The magnified images (a2, b2, and c2) showed the cortical gap was still apparent in group 2 (b2), while not in group 1 (a2) and group 3 (c2). Arrows show fracture sites.

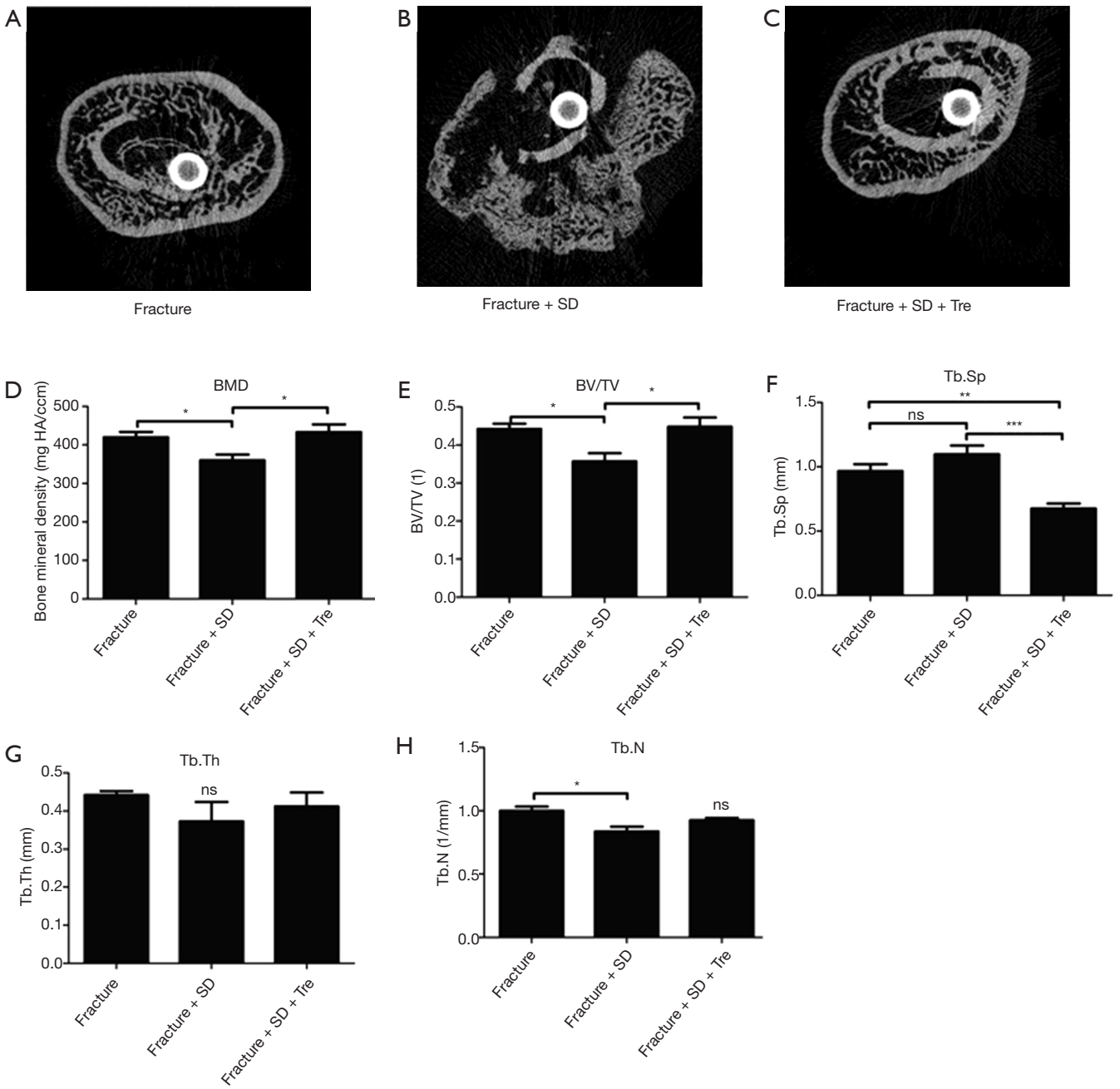

Figure 3 The micro CT images (A,B,C) showed that the fracture group (group 1) (A) and fracture + SD + Tre group (group 3) (C) had much better bone reconstruction in the callus area than that of the fracture + SD group (group 2) (B). The group 2 had significantly less values of BMD (D) and BV/TV (E), and significantly higher value of Tb.Sp (F) in the fracture callus site. The Tb.Th (G) of the fracture callus site in group 2 was less than the other two groups, although no significant difference was detected. The Tb.N (H) in group 2 was significantly less than that of group 1, while had no difference with that of group 3. *, P≤0.05; **, P≤0.01; and ***, P≤0.001 indicate significance between two groups; ns, indicates no significant difference was detected.

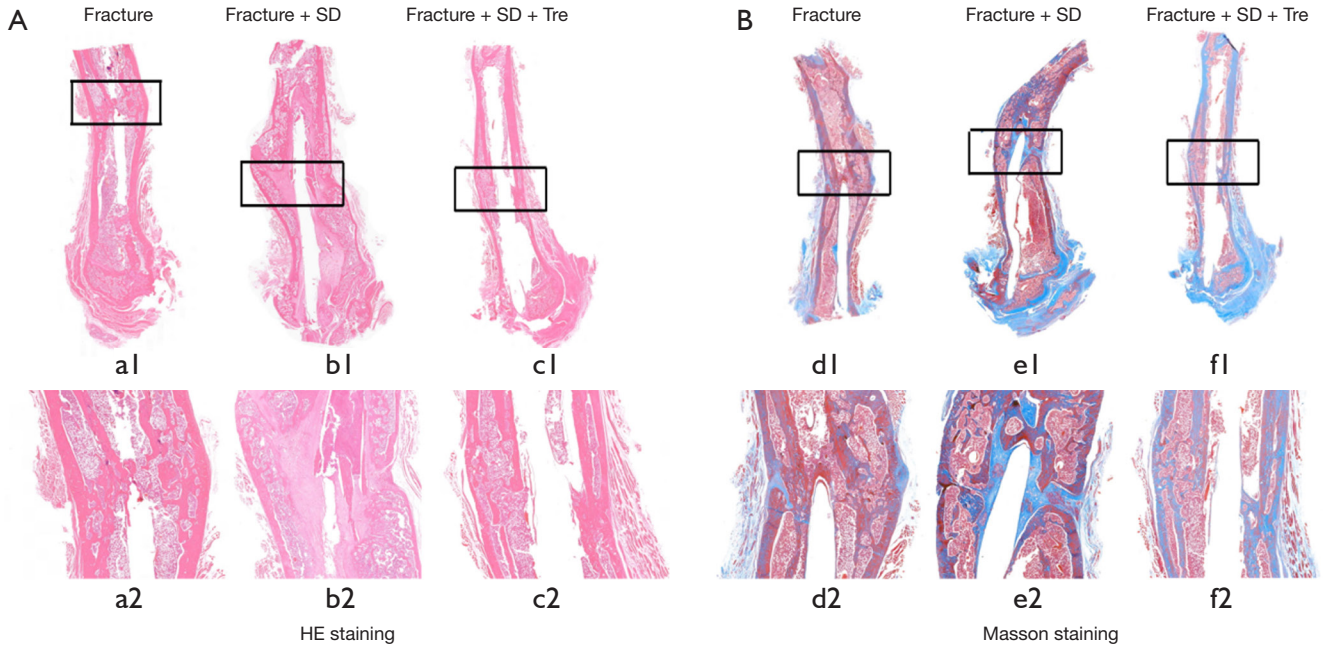

Figure 4 The histological analysis of the fracture healing in three groups (A,B). HE staining of the femurs (a1, b1, and c1) and the magnified images of the callus areas (a2, b2, c2) showed that the fracture + SD group (group 2) (b1, b2) had only fibrotic tissue and chondrocytes in the callus area, while the fracture group (group 1) (a1, a2) and fracture + SD + Tre group (group 3) (c1, c2) could observe newborn bones. In group 2, inter-trabecula lacunas existed with mild inflammatory cell infiltration.no obvious inflammatory cell infiltration was observed in groups 1 and 3. Similarly, Masson staining of the femurs (d1, e1, and f1) and the magnified images of the callus areas (d2, e2, and f2) demonstrated that group 2 (e1, e2) had less newborn born compared to the other two groups. The magnification in the upper row was 0.2× and lower row was 1×.

Trehalose affects cell viability of RAW264.7 cells *in vitro*

Cytotoxicity of trehalose on RAW264.7 cells was assessed by CCK-8 assay. RAW264.7 cell proliferation was significantly decreased with the high concentration of 100 mM trehalose, while the cell viability was not affected by trehalose at low concentrations (*Figure 6A*).

Trehalose regulates M1 and M2 inflammatory macrophage polarization in LPS-stimulated RAW264.7 cells

It is noteworthy that the level of M1 phenotype marker IL-6 increased significantly when the RAW264.7 cells were cultured in LPS medium compared with the control. However, trehalose effectively inhibited LPS-induced IL-6 expression (*Figure 6B*). By contrast, pro-inflammatory cytokines Arg-1 and IL-10 in M2 macrophages were markedly increased induced by LPS, trehalose still increased these levels when the cells were treated with a combination of LPS and trehalose (*Figure 6C*).

Discussion

Several studies have reported the effect of SD on bone, mainly focused on the association between SD and BMD (8-12). Both clinical surveys (8-11) and animal laboratory studies (12) showed that short sleep duration resulted in lower BMD and worse bone mass accrual. Several mechanisms have been proposed as possible explanation for the decreased BMD. Higher cortisol levels observed during SD may lead to decreased bone formation and BMD (28). SD also alters the levels of several hormones, proteins, and minerals that may play a pivotal role in bone metabolism, such as osteocalcin, growth hormone, insulin-like-growth factor, parathyroid hormone, and so on (29,30). Bone metabolism also deeply influenced the process of fracture healing. Maybe, SD had adverse effect on fracture healing through the similar mechanisms.

We speculated that the reasons lie in the effect of SD on fracture healing are multifactorial. First, circadian rhythm disruption, which is a well-accepted consequence of SD, would be expected to affect bone formation and

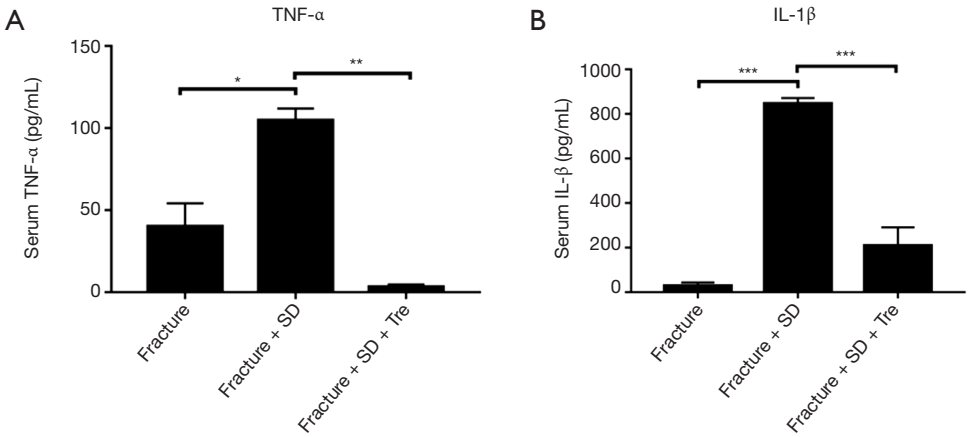

Figure 5 Elisa analysis of serum TNF-α and IL-1β in the three groups. The concentration of serum TNF-α (A) and IL-1β (B) was significantly higher in fracture + SD group (group 2) compared with the other two groups. No significant difference was observed between fracture (group 1) and fracture + SD + Tre group (group 3). *, P≤0.05, **, P≤0.01, and ***, P≤0.001 indicate significance between two groups.

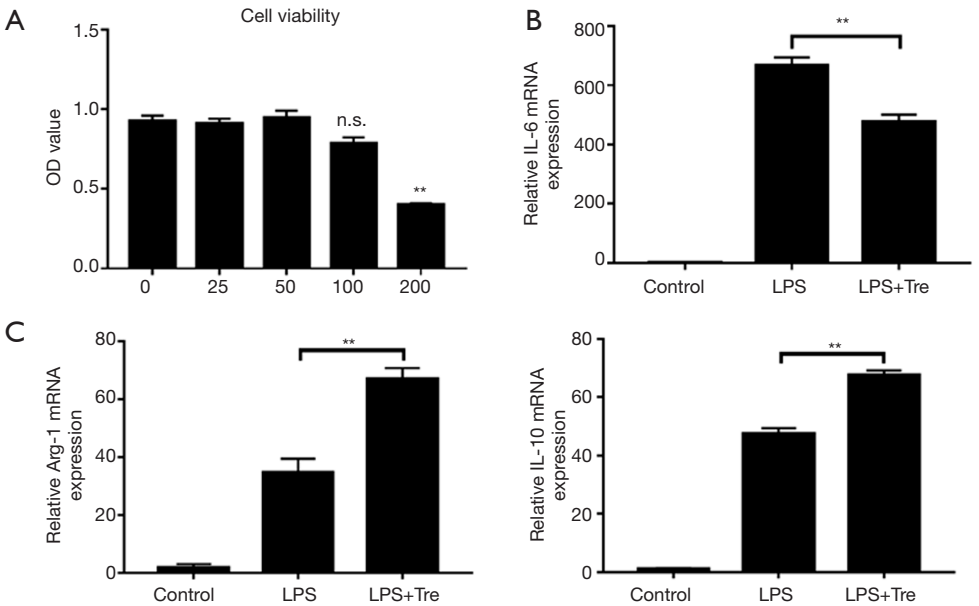

Figure 6 Trehalose regulates cell viability and LPS-induced M1 and M2 inflammatory macrophage polarization. (A) RAW264.7 cells were cultured with the indicated concentrations (0, 25, 50, 100 and 200 mM) of trehalose in the growth medium for 1 days. Cell proliferation was determined using CCK-8 assay. **, P<0.01 compared to that in the control group. (B,C) Total RNAs were isolated from RAW264.7 cells cultured with trehalose (100 mM) for 1 days and the expression levels of M1, M2 macrophage markers were evaluated by real-time PCR using IL-6, Arg-1, IL-10 and β-actin gene-specific primers. **, P<0.01 compared to that in LPS group.

bone metabolism by influencing the secretion of several hormones, proteins, and minerals (31). It was described that SD may result in growth hormones deficit (32). Growth hormone is an important regulator of postnatal growth and bone formation (33,34). Vitamin D, an important hormone for bone health has been reported to be associated with SD. Clinical research showed that low serum 25(OH)D level in older men is related with short sleep duration and low sleep efficiency (35). An animal study reported that rats in the SD group had

significantly decreased levels of serum 25(OH)D (25). IGF-1, which is important for periosteal expansion was also found a lower concentration with SD patients (36). Whereas, SD may promote the release of cortisol, a catabolic hormone that is associated with decreased BMD and increased fracture risk (37,38). On the other hand, it was reported that the skeleton may be a ductless gland that could produce a hormone, which influences bone mass and bone metabolism (39,40). And, the endocrine function of the bone may be disturbed by SD. Second, SD may lead to negative energy balance. We observed that the body weight gains of rats in fracture + SD group were attenuated. Similar with our results, previous study showed that SD rats lost 15% of their body weight, whereas, the food intake increased 2 to 3 folds (41). The negative energy balance may have adverse effect on bone formation. It is reported that negative energy may have inverse effect on osteocalcin concentrations (42). Another study showed that the rats with negative energy balance in the SD group had decreased bone mass (25). Other examples of hypermetabolism, such as induced by thyroid hormone administration also contributed to osteopenia (43). Third, SD may result in systemic inflammation (44). This inflammation may be related to decreased bone formation and osteoporosis. An animal study using a rat model of bone loss induced by SD observed upregulation of proinflammatory mediators such as serum IL-1β, cyclooxygenase (COX)-2, and TNF-α (45). Other studies have demonstrated high levels of circulating cytokines and cortisol were associated with increased fracture risk, altered bone metabolism, and low BMD (46,47). The present study also showed SD significantly upregulated serum IL-1β and TNF-α, which is consistent with previous studies.

Trehalose may promote bone fracture healing by suppressing systemic inflammation in the present study. In fact, previous studies demonstrated that trehalose may inhibit the inflammatory cascade (48). The present study also demonstrated that trehalose treatment suppresses pro-inflammatory cytokines IL-6 release by enhancing M2 macrophage polarization (mediated by increases in Arg-1 and IL-10 expression) in RAW264.7 cells following LPS stimulation. Although a relative predominance of M1:M2 cells in callus is still not understood (49), induction of M2 macrophages was found to significantly enhance bone formation, which appears to be good to fracture healing and successful regeneration (50). Shifting the inflammatory reaction towards M2 upon treatment of trehalose could improve the healing outcome and prevent delayed bone regeneration resulted from SD.

There are several limitations to the present study. First, no biomechanical study was performed to assess the fracture healing quality in the present study. Second, the period of 3 weeks SD was relatively short, and the extent of restrictive sleep duration was not considered. Third, the effect of oral administration trehalose was not investigated in the present study, although a recent study showed that trehalose would be digested into glucose molecules in mammalian digestive tract, and would lose effectiveness when taken orally. Different groups of varied sleep duration should be established in the future studies. And, the exact mechanism of trehalose needs more investigation.

In conclusion, this study revealed that SD had adverse effect on bone fracture healing. Sleep quality and duration should be improved to accelerate recovery of the patients with bone fracture. Trehalose may be good to the bone fracture ones with sleep problem by suppressing inflammation and enhancing M2 polarization.

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Footnote

Conflicts of interest: The authors have no conflicts of interest to declare.

Ethical Statement: All animals procedures were accordance with the guidelines and approved by the Ethics Committee of Drum Tower Hospital, Medical School of Nanjing University, Nanjing, China (Ethical approval number: 20171002).

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