Repetitive brief ischemia can promote bone healing in a rat tibia fracture model

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Abstract: Objective: To observe the effects of repetitive brief ischemia on promoting bone healing in a rat tibia fracture model. Methods: To achieve the aim of the study, rats with right tibia closed fractured were administered homemade tourniquet placed on right thigh 10 min inflated/10 min deflated 3 times ischemia treatment at three interval time (24 hours, 48 hours, and 72 hours) for 5 consecutive weeks after first ischemia treatment at 24 hours after bone fractured. Digital X-rays, computed tomography, serum insulin-like growth factors-2 (IGF-2), and Immunohistochemical Detection of IGF-2 in fractured bone area were detected. Results: The present data showed that repetitive brief ischemia treatment can increase the mean digital X-rays scores, BV, and TV and decrease the TMD and callus BMD. It can stimulate the secretion of serum IGF-2. Conclusion: Repetitive brief ischemia can promote bone healing by increasing the level of IGF-2 in a rat tibia fracture model.

Keywords: Repetitive brief ischemia, bone healing, promoting effects, IGF-2, tibia fracture, rat model

Introduction

Repetitive brief ischemia as a treatment is applied to reduce organ ischemia-reperfusion injury [1-3]. It can enhance the tolerance of the tissue in hypoxia environment [4, 5]. The bone architecture is distorted and blood vessels are ruptured when bone fractured. Blood in the fracture site rapidly coagulates and forms hematoma [6]. The destruction of vasculature causes the local tissue ischemia [7]. The cells cannot get adequate oxygen and nutrients. Part of cells is becoming necrosis or apoptosis. If the revascularization is impossible, fracture healing will be delayed or nonunion. Fracture itself cause substantial economic burden and delayed healing or nonunion add more [8-10]. Therefore, there is an incentive to develop a time and money saved therapy to enhance fracture healing to improve the life qualities of patients and reduce the costs. Some studies have shown that blood flow decreased triggers angiogenesis and can stimulate macrophage produces angiogenic factors [11-14]. Bone healing needs local angiogenesis. Tourniquet is common in clinical. It can make the limb repetitive brief ischemia. Since repetitive brief ischemia can reduce the necrosis or apoptosis of cell in hypoxia environment, we suppose that it can promote the bone healing [15-17]. To the best of our knowledge, the promoting effect of repetitive brief ischemia on bone healing has not been reported before. Therefore, the target of our study was to verify the promoting effect in a rat tibia closed fracture model and to explore the underlying mechanisms of this effect. Finally, we design different interval time of ischemia treatment to investigate the best treatment protocols of repetitive brief ischemia on bone healing and demonstrate our hypothesis.

Materials and methods

Animals

Female adult wistar rats (10 weeks-old; Charles River Laboratories, Beijing, China) were used in this study. All procedures were performed in accordance with the Guiding Principles in the Care and Use of Animals and approved by Capital Medical University Committee on the Use of Animals in Research and Education. The animals were maintained in a controlled
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condition of 12 h light/12 h dark at 23.6°C, humidified at 35%, and fed a standard chow diet with free water intake. At the end of the experiments, rats were anesthetized using pentobarbital sodium and euthanized.

Tibia fracture model

The right tibia of rat was fractured with a homemade fracture device after intraperitoneal anesthesia with pentobarbital sodium (40 mg/kg body weight). The right hind leg of rat was shaved and disinfected. A 1-mm steel K-wire was used to open the cortical bone and medullary canal by touching the border of the tibial plateau when the keen was bent 90°. The skin was stabbed directly by the wire and the wire was driven into the medullary canal up to the distal part of the tibia. After removing the wire, a weight (500 g) was dropped at a distance of 26 cm upon the middle of the tibia to make the closed fracture [18, 19]. After closed reduction, the tibia was subjected to intramedullary stabilization with 1-mm steel K-wire (Zimmer, USA). The implant was inserted from the border of the tibial plateau into the medullary canal (Figure 1). The protruding part of the wire was cut flush with the cortical level of the bone. After stabilization, X-ray examinations were performed to document the fracture reduction and the position of the implant (Figure 2). Animals excluded due to comminuted fractures.

Experimental design

Rats were randomly distributed into four groups, twenty four per group. Rats of the control group received homemade tourniquet uninflated placed on right thigh 1 hour every 24 hours after tibia fractured. Rats of the other groups received homemade tourniquet placed on right thigh 10 min inflated/10 min deflated 3 times 24 hours after tibia fractured. Then, the rats were as this every 24 hours, every 48 hours, or every 72 hours. The pressure of the tourniquet was selected to block the blood flow and confirmed by the B-mode ultrasonic diagnostic equipment (Visual Sonics, Canada). Rats of the four groups received tourniquet treatment 5 weeks.

Radiographic evaluation: digital X-rays

Radiographs were taken throughout the observation period in posterior-anterior and lateral views at days 0 (days after fracture), 14, 28, 42, and 56. Digital X-rays were taken using an animal X-ray unit (Avchoice, Del, USA). The healing of the fracture was described by two independent observers using 7 scores method. Four cortices bridged is 7 scores, three cortices bridged is 6 scores, two cortices bridged showed by lateral radiograph is 5, two cortices bridged showed by anteroposterior radiograph is 4, one cortices bridged showed by anteroposterior radiograph and one cortices bridged showed by lateral radiograph is 3, onecortices bridged is 2, and no cortex bridged is 1 score.

Radiographic evaluation: computed tomography

Micro-CT images were acquired (Inveon, Siemens, Germany) of both the fractured and un-fractured tibia matching the regions of interest (ROI) of the middle of the tibia shaft (2 weeks after bone fracture). Sequential transaxial images through the tibia were obtained using an isotropic voxel size of 18 μm, an integration time of 250 ms, and peak tube voltage of 80 kV. The region of interest (ROI) then was 150 axial slices above and below the fracture line (Figure 3). A three-dimensional

Figure 1. K-wire was inserted from the border of the tibial plateau into the medullary canal.
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Gaussian filter (σ = 0.8) with a limited, finite filter support of 1 was used to suppress noise in the images, and mineralized tissue was segmented from air or soft tissue at threshold of 400 mg HA/cm. Total volume (TV), tissue mineral density (TMD), bone volume (BV), bone volume fraction (BV/TV), and callus mineral density (callus BMD) were determined on ROI that included all of the volume within the outer perimeter of the fracture callus or unfractured cortices.

Serum IGF-2 variation

IGF-2 was detected at days 1 (after tibia fractured), 3, 5, 7, 14, 28, 42, and 56. Detecting kits were purchased from TechLab, China. ELISA was used to test IGF-2.

Immunohistochemical detection of IGF-2 in fractured area

Bone samples were harvested at 2, 4, 6, and 8 weeks after the fracture and fixed. Bone specimens were decalcified with 5% EDTA, embedded in paraffin, sectioned at a thickness of 2 μm along the longitudinal axis, and stored at 80°C until staining. The samples stained with IGF-2 antibody purchased from EnoGene (China). The catalog number is E2A6810. The cells secreted IGF-2 stained brown and the other cells were not stained. Image-Pro Plus software (Version 9) was used to quantify the stained cells. The 3 mm above and below the fracture line was the observation area. Two blinded independent reviewers performed the analysis of all samples and the measurements were averaged.

Statistical analysis

All data are expressed as means ± SD and SPSS 13.0 was used for statistical analysis. Digital X-rays scores, serum IGF-2 variation, Immunohistochemical results of IGF-2 were...
subjected to statistical analysis using ANOVA for repeated measurement. Computed tomography results were subjected to statistical analysis using ANOVA for one-way ANOVA. Values of \( P < 0.05 \) were considered significant.

Results

Effect of repetitive brief ischemia treatment on digital X-rays scores

Repetitive brief ischemia increased the mean digital X-rays scores and there were no changes in different intermittence ischemia at the observation time points (Figure 4). These results reproduce our previous findings and suggest that repetitive brief ischemia contributes to bone formation.

Effect of repetitive brief ischemia treatment on computed tomography

Repetitive brief ischemia at every 24 hours after bone fracture can increase the TV and BV and decrease the TMD and callus BMD compared to the other ischemia treatment groups and control group. There is no difference between the groups at the level of BV/TV (Figure 5). The results suggest that the promoting effect of frequent ischemia on bone healing is better than ischemia which Intermittent time is longer at 2 weeks after bone fracture. Frequent ischemia accelerates the callus formation and ossification.

Discussion

In this study, we show repetitive brief ischemia with different time intervals of the injured right limb. The tibia of the right limb was made closed fracture by homemade device. Every time repetitive brief ischemia was homemade tourniquet placed on right thigh 10 min inflated/10 min deflated 3 times. Treatment groups were received repetitive brief ischemia 24 hours after tibia fractured and distributed into groups of ischemia with different time intervals. Three time intervals used in our study, 24 hours, 48 hours, or 72 hours. Uninflated tourniquet placed on right thigh 1 hour every 24 hours after tibia fractured as the control group. Our aim in this work was to identify the repetitive brief ischemia promoted the bone healing. An expression of serum metabolite and the Immunohistochemical detection in fractured area

Effect of repetitive brief ischemia treatment on serum IGF-2 variation

Repetitive brief ischemia at every 24 hours after bone fracture can increase the mean levels of serum IGF-2 compared to the other groups at days (after bone fracture) 1, 3, 5, and 7 (Figure 6). The levels of serum IGF-2 in frequent ischemia group are lower than the other ischemia treatment groups at weeks 2, 4, and 6 after bone fracture. There are no differences between the four groups on serum IGF-2 at 8 weeks.

Effect of repetitive brief ischemia treatment on immunohistochemical detection of IGF-2 in fractured area

Repetitive brief ischemia at every 48 hours or 72 hours after the first ischemia treatment can increase the levels of IGF-2 positive cells in the fracture area compared to the other groups at weeks 2, 4, and 6 (Figure 7). Repetitive brief ischemia at every 24 hours does not increase the levels of IGF-2 positive cells in the fracture area. There are no differences between the four groups at 8 weeks.
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Figure 5. Effect of repetitive brief ischemia treatment on computed tomography at 2 weeks after bone fracture. Repetitive brief ischemia at every 24 hours after bone fracture can increase the TV and BV and decrease the TMD and callus BMD compared the other ischemia treatment groups and control group. There is no difference between the groups at the level of BV/TV. Values are means ± SD, n = 12. Statistical test: one-way ANOVA. ***significantly different from control group at P < 0.001, **significantly different from control group at P < 0.01, *significantly different from control group at P < 0.05.

Figure 6. Effect of brief ischemia treatment on serum IGF-2. Repetitive brief ischemia at every 24 hours after bone fracture can increase the mean levels of serum IGF-2 compared the other groups at days (after bone fracture) 1, 3, 5, and 7. The mean levels of serum IGF-2 of frequent ischemia are lower than the other ischemia treatment groups at weeks 2, 4, and 6 after bone fracture. There are no differences between the four groups on serum IGF-2 at 8 weeks. Values are means ± SD, n = 12. Statistical test: ANOVA for repeated measurement data.

bone represent the potential mechanism by which the ischemia treatment increases the IGF-2.

Frequent ischemia treatment (24 hours interval) increases the X-rays scores, BV, and TV and decreases the TMD and callus BMD. Ischemia
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Figure 7. Effect of repetitive brief ischemia treatment on Immunohistochemical Detection of IGF-2 in Fractured Bone. Values are means ± SD, n = 12. Statistical test: ANOVA for repeated measurement data.

treatment may be an important role to activate of the cartilage formation and ossification. Many studies have clearly shown that ischemia can stimulate tissue to produce hypoxia inducible factor (HIF) [12, 20, 21]. HIF can make erythropoiesis and vascularization by regulating the expression of target genes. HIF promotes energy metabolism and decrease the cell apoptosis to make tissue adapt the hypoxia environment [12, 22]. It also plays an important role on stimulating cell differentiation [23, 24]. The bone architecture distorted and blood vessels ruptured when bone fractured. The vasculature fails to provide oxygen and nutrients in the fracture site. Ischemia may accelerate revascularization and make tissue adapt the bad environment to promote bone healing.

Radiographic results demonstrate that frequent ischemia treatment promotes bone healing. We detected the serum metabolite to explore the exact mechanism of repetitive brief ischemia on promoting bone healing. The results show that serum IGF-2 increase faster during tibia fractured 1 week in frequent ischemia treatment group compared the other groups. Frequent ischemia can stimulate the IGF-2 secretion in the initial stage of bone healing. It indicates that frequent ischemia makes multiple genes expression in multiple cells in the fracture site to produce multiple bioactivators to promote tissue reconstruction. The mechanism is complicated and comprehensive. We suspect that the effect of frequent ischemia treatment is not limited in the injured limb and is in many organs. We sustain the ischemia treatment 5 weeks and the serum IGF-2 in frequent ischemia group is not statistically significant differences with the control group after bone fractured 4 weeks. We surmise that initiation factors of ischemia treatment have feedback regulation. The initiation factors exceed threshold values by frequent ischemia and may be inhibited by the downstream biological materials or the initiation factors themselves. The next hypothesis is that the body has tolerated the ischemia treatment after a long-term frequent ischemia. Frequent ischemia treatment makes the cells in the fracture site adapt the repetitive brief hypoxia environment and the ischemia stress becomes a pathological normalcy for these cells. Although the serum IGF-2 is no difference with the control group, the cells in the fracture site is not the normal cells. However, this hypothesis needs cell analysis to prove.

Frequent ischemia treatment can promote bone healing but the promoting effect is not lasting according to the serum metabolite detection. The transition is at 2 weeks after bone fractured. To make the duration of promotion longer, we change the interval time of every time repetitive brief ischemia treatment from 24 hours to 48 hours or 72 hours and this is base on our hypothesis. The results of experiments show that the interval time of ischemia treatment prolonged can extend the duration of the promoting effect of repetitive brief ischemia treatment on bone healing. Serum IGF-2 in groups of Interval time used 48 hours or 72 hours are higher compared the other groups at weeks 4 and 6 after bone fractured. It indicates that the promoting effect is lasting until 6 weeks after tibia fractured. The transition is at 8 weeks and is 6 weeks later than frequent ischemia. We surmise that this new ischemia treatment delays the initiation factors grow and make the cells in the fracture site not completely adapt the hypoxia environment. The unadapted cells can continue to secrete the metabolite to promote tissue reconstruction.

Then we made the Immunohistochemical detection in fractured bone. The results indicate that brief ischemia can stimulate the cells in the fracture area to secrete IGF-2. Repetitive
brief ischemia at every 48 hours or 72 hours after the first ischemia treatment has the more promotion effects. With the extension of time, the promoting effects weakened gradually. The Immunohistochemical detection is conform to the serum metabolite results. The reason that increases the levels of serum IGF-2 is the cells in the fracture area.

Conclusion

In conclusion, we have revealed that repetitive brief ischemia promote the bone healing by increasing the serum IGF-2. However, the promoting effect of frequent ischemia is not lasting and we prolong the interval time of every time ischemia treatment. The duration of promoting effect of the new ischemia treatment is longer than frequent ischemia.

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Disclosure of conflict of interest

None.

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