Propofol Promotes Ankle Fracture Healing in Children by Inhibiting Inflammatory Response

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Background: The present study aimed to investigate the potential effects of propofol on ankle fracture healing in children and the underlying molecular mechanisms.

Material/Methods: We first detected the levels of inflammatory cytokines from peripheral blood in children with or without ankle fracture using quantitative real-time polymerase chain reaction (qRT-PCR) and ELISA assay. Then, effects of propofol on inflammatory response in MG-63 cells were investigated. MG-63 cells were pre-treated with or without propofol and then stimulated with 1 μM bradykinin (BK). The productions of cytokines from MG-63 cells were determined by using qRT-PCR and Western blot assay. The expression levels of p-p38, NF-κB p-p65, NLRP3, ASC, caspase-1, and COX-2 were measured by Western blot and/or qRT-PCR.

Results: The results showed that, compared with the healthy children, the levels of tumor necrosis factor (TNF-α), interleukin (IL)-1β, and IL-6 were significantly up-regulated in children with fractured ankles. No cytotoxicity was observed in MG-63 cells after propofol treatment. BK treatment significantly enhanced TNF-α, IL-1β, and IL-6 expression levels, and these enhancements were reduced by propofol treatment in a dose-dependent manner. Moreover, BK-induced up-regulation of p-p38, NF-κB p-p65, NLRP3, ASC, caspase-1, and COX-2 was dose-dependently down-regulated by propofol treatment.

Conclusions: Propofol prevents inflammation in MG-63 cells by regulating p38MAPK-NF-κB pathway, NLRP3 inflammasome, and COX-2 expression. Our findings indicate the benefits of propofol in fracture healing, and provide a more theoretical basis for the clinical treatment of fractures.

MeSH Keywords: Ankle Fractures • Inflammation • NF-kappa B • p38 Mitogen-Activated Protein Kinases • Propofol

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Background

Ankle fracture is one of the most common bone injuries, with an incidence rate of 187 per 100,000 people, and it is especially common in children [1,2]. Due to the special characteristics of the bones of children, delayed diagnosis and treatment of ankle fractures in children may result in deformity and disability [3]. Therefore, timely diagnosis, treatment, and nursing care are very important in reducing pain and healing time of fractures. Pediatric care is especially important for children with ankle fracture due to their age, mobility, poor compliance, and unsafe conditions. The process of bone formation is a balance between osteoblast and osteoclast activity [4]. Fracture healing requires a large number of osteoblasts. After a fracture, the expression of inflammatory cytokines is positively correlated with the number of osteoclasts [5].

Bradykinin (BK), a well-known mediator of pain and inflammation, is also known to be involved in the process of bone resorption. Studies have indicated that BK decreases osteoblast differentiation, with a concomitant increase in osteoclast formation [6]. BK also stimulates bone resorption in vitro and synergistically potentiates interleukin-1 (IL-1)-induced bone resorption and prostaglandin (PG) production, indicating that kinins play an important role in inflammation-induced bone loss [7–9]. As a TNF-related cytokine, RANKL has been identified as an important factor in bone resorption [10,11]. RANKL can activate TNF receptor-associated factors (TRAFs) and the cognate receptor RANK on osteoclast progenitor cells, leading to osteoclast progenitor cell differentiation [10–12]. Moreover, BK has been reported to potentiate cytokine-induced prostaglandin production in osteoblasts by promoted cyclooxygenase 2 expression, resulting in the up-regulation of RANKL [13].

Propofol is an anesthetic, and many recent studies have found that it has a variety of effects other than anesthesia, such as anti-inflammatory effects [14–17]. To the best of our knowledge, there are no previous studies fully investigating the effects of propofol on ankle fracture healing and the potential underlying molecular mechanisms. Therefore, in the present study, we investigated the effects of propofol on the inflammatory process by using MG-63 cells stimulated with BK as an inflammatory process model, so as to provide new treatment strategies and theoretical bases for the clinical treatment of pediatric ankle fractures.

Material and Methods

Clinical specimens

Peripheral blood samples (2 ml per individual) were obtained from 30 children with ankle fracture (average age: 7±1.1 years; sex ration: 1:1) and 30 healthy children (average age: 6.8±1.2 years; sex ration: 1:1) at Children’s Hospital Affiliated to Nanjing Medical University between April 2015 to April 2016. The present study was approved by the Ethics Committee of Children’s Hospital Affiliated to Nanjing Medical University, and each patient provided informed consent.

Cell culture

MG-63 cells, a type of human osteoblastic osteosarcoma cell line which expresses osteoblastic phenotypes, were obtained from the American Type Culture Collection (cat. no. CRL-1427; Manassas, VA, USA). MG-63 cells were seeded into culture plates and grown in α-Minimum Essential medium (MEM) containing 10% fetal calf serum (FCS) (Gibco; Thermo Fisher Scientific, Inc.). When cells were cultured to 80–90% confluence, they were then washed twice with PBS and once with serum-free α-MEM. Subsequently, the cells were cultured in 1, 5, and 10 μg/ml of propofol [18] containing α-MEM/1% FCS for 24 h, then we added BK (1 μM) and cultured them for another 24 h.

Cell viability assay

MG-63 cell viability was detected by use of the MTT Assay Kit (Beyotime, Shanghai, China) 48 h after treatment, according to the manufacture’s protocol. A Bio-Rad iMark plate reader (Bio-Rad Laboratories) was used to measure the absorbance at a wavelength of 540 nm.

ELISA assay

The serum levels of TNF-α, IL-1β, and IL-6 in children were determined by using ELISA assay according to the manufacturer’s instructions for each kit (Elabscience Biotechnology Co., Ltd., Wuhan, Hubei, China).

QRT-PCR

Total RNA was isolated from MG-63 cells and peripheral blood samples using Trizol (Invitrogen), following the manufacturer’s instruction. cDNAs were synthesized by performing reverse transcription assay using the ThermoScript RT-PCR system (Invitrogen, Grand Island, NY, USA). Then, the synthesized cDNAs were analyzed using the TaqMan Universal PCR Master Mix kit under the ABI PRISM 7900 HT sequence-detection system. Amplification conditions were as follows: 95°C for 10 min, followed by 40 cycles of 95°C for 10 s and 60°C for 60 s. GAPDH was used as the endogenous control. The primer sequences used were as follows:

- TNF-α-forward, 5'-GAAGTCACAGAGGACGACT-3’ reverse, 5'-CTCTCCTCTGGAGGGCTTGA-3’
- IL-1β-forward, 5'-AGCAGAATCGCGACCTCCT-3’ reverse, 5'-GAAGGCAGGCTGCGATC-3’
- IL-6-forward, 5'-CCCGAGAGAGACCTCACAG-3’
reverse, 5'-CAGAATTGCCATTGCACA-3';
COX-2-forward, 5'-TCCATTGACCAGAGCAGAGA-3'
reverse, 5'-TCTGGACGAGGTTTTTCCAC-3';
NLRP3-forward, 5'-GATCTTCGCTGCGATCAACAG-3'
reverse, 5'-CGTGCATTATCTGAACCCCAC-3';
ASC-forward, 5'-GCAATGTGCTGACTGAAGGA-3'
reverse, 5'-TGTTCCAGGTCTGTCACCAA-3';
caspase-1-forward, 5'-GCACAAGACCTCTGACAGCA-3'
reverse, 5'-TTGGGCAGTTCTTGGTATTC-3';
GAPDH-forward, 5'-AAAATCAAGTGGGGCGATGC-3'
reverse, 5'-AGGAGGCATTGCTGATGATCT-3'.

The 2–ΔΔCq method was used to analyze the relative gene expressions [19].

Western blot

After specific treatment, MG-63 cells were washed with PBS 3 times, then total cell proteins were extracted using lysis buffer (Cell Signaling Technology, Danvers, MA, USA). The BCA protein assay kit (Thermo Fisher Scientific, Inc.) was used to detect the protein concentration, following the manufacturer’s instructions. Equal amounts of proteins (25 µg) were resolved by 10% SDS-PAGE, transferred onto polyvinylidene fluoride membranes (EMD Millipore, Billerica, MA, USA), and then blocked with 5% skim milk at room temperature for 2 h. After incubation with a primary antibody against TNF-α, IL-1β, IL-6, p-p38, p-p65, NLRP3, ASC, caspase-1, COX-2 (all Cell Signaling Technology Inc., Danvers, MA, USA; dilution ratio: 1: 1000), and β-actin (Cell Signaling Technology Inc., Danvers, MA, USA; dilution ratio: 1: 5000) overnight at 4°C, the membrane were then incubated with a horseradish peroxidase-conjugated secondary antibody, Anti-rabbit IgG, and HRP-linked antibody (dilution ratio: 1: 5000; Cell Signaling Technology Inc., Danvers, MA, USA) at room temperature for 4 h. Bands were visualized by using the enhanced chemiluminescence detection system (Cell Signaling Technology).

Statistical analysis

All experiments were repeated at least 3 times. Data are expressed as the mean ± SD. Statistical comparisons between 2 groups were analyzed with the Student’s t test and between multiple groups with ANOVA followed by Bonferroni post hoc test. p<0.05 was considered significantly significant.

Results

Inflammatory cytokines are increased in children with fractured ankle

The mRNA and protein levels of the inflammatory cytokines (TNF-α, IL-1β, and IL-6) in the serum of children with or without fractured ankle were detected by qRT-PCR and ELISA, respectively. Controls: healthy children; Patients: children with fractured ankle. **, p<0.01 vs. Control.

Figure 1. The levels of TNF-α, IL-1β, and IL-6 significantly enhanced in children with fractured ankle. The mRNA and protein levels of TNF-α, IL-1β and IL-6 in children with or without fractured ankle were detected by qRT-PCR and ELISA, respectively. Controls: healthy children; Patients: children with fractured ankle. **, p<0.01 vs. Control.
fractured ankle were detected by using qRT-PCR and ELISA assay, respectively. We found that compared with the healthy children, the levels of TNF-α, IL-1β, and IL-6 were significantly up-regulated in children with fractured ankle (Figure 1). The data indicated that inflammation is involved in the progression of fractured ankle.

Propofol shows no cytotoxicity in MG-63 cells

MG-63 cells were treated with 1, 5, and 10 μg/ml propofol for 48 h, then cell viability was analyzed. Our results demonstrate that no significant changes were observed in different groups, indicating propofol has no cytotoxicity in MG-63 cells (Figure 2).

Propofol significantly inhibits BK-induced increase of TNF-α, IL-1β, and IL-6

Compared with the control group, the mRNA and protein levels of TNF-α, IL-1β, and IL-6 were significantly increased in the BK group, and these increases were eliminated by propofol...
Figure 4. Propofol significantly inhibits BK-induced activation of p38MAPK-NF-κB pathway. After treatment with 1, 5, and 10 μg/ml propofol, protein levels of p-p38, p-p65, and p65 were detected by Western blot analysis. Con – control group; BK – 1 μM BK treatment group; BK + P1 – 1 μM BK + 1 μg/ml propofol treatment group; BK + P5 – 1 μM BK + 5 μg/ml propofol treatment group; BK + P10 – 1 μM BK + 10 μg/ml propofol treatment group. ** p<0.01 vs. Con; * p<0.05 vs. BK; ## p<0.01 vs. BK.

Figure 5. Propofol significantly inhibits BK-induced increase of NLRP3 inflammasome. After treatment with 1, 5, and 10 μg/ml propofol, protein and mRNA levels of NLRP3, ASC, and caspase-1 were detected by Western blot analysis. Con – control group; BK – 1 μM BK treatment group; BK + P1 – 1 μM BK + 1 μg/ml propofol treatment group; BK + P5 – 1 μM BK + 5 μg/ml propofol treatment group; BK + P10 – 1 μM BK + 10 μg/ml propofol treatment group. ** p<0.01 vs. Con; * p<0.05 vs. BK; ## p<0.01 vs. BK.
Propofol significantly inhibits BK-induced activation of p38MAPK-NF-κB pathway

To explore the molecular mechanism of the anti-inflammatory effects of propofol in a BK-induced model of fractured ankle in MG-63 cells, the p38MAPK-NF-κB pathway was analyzed. As shown in Figure 4, compared with the control group, the protein levels of p-p38 and p-p65 in BK-treated MG-63 cells significantly enhanced, indicating activation of the p38MAPK-NF-κB pathway. This activation was dose-dependently inhibited by propofol (1, 5, and 10 μg/ml) treatment.

Propofol significantly inhibits BK-induced increase of NLRP3 inflammasome

In addition, the NLRP3 inflammasome was assessed in the present study. As shown in Figure 5, compared with the control group, the protein and mRNA levels of NLRP3, ASC, and caspase-1 in BK-treated MG-63 cells were significantly up-regulated, and these up-regulations were eliminated by propofol (1, 5, and 10 μg/ml) treatment in a dose-dependent manner.

Propofol significantly inhibits BK-induced increase of COX-2

Finally, the protein and mRNA levels of COX-2 in MG-63 cells was analyzed in our study. The results indicated that compared with the control group, mRNA and protein levels of COX-2 were significantly increased in the BK group, and they were inhibited by propofol (1, 5, and 10 μg/ml) treatment in a dose-dependent manner (Figure 6).

Discussion

Fractures in children are very common, especially ankle fractures in children. Childhood is the most vigorous growth stage. The growth and development of children’s skeletal system from the anatomy, mechanical properties, injuries, and healing characteristics are significantly different from adults, so pediatric care is especially important for children with ankle fracture. In order to prevent various complications, promote fracture healing, and improve the quality of care, the nursing of children with fractures should take appropriate nursing measures that take into account their characteristics that are different from those of adults. Childhood ankle fracture may result in serious consequences due to the absence of timely diagnosis and treatment and reasonable care. Therefore, it is urgent to seek effective treatments for fractured ankle.

In trauma-induced fracture, persistent inflammation directly affects osteagenesis. However, the underlying molecular mechanisms remain largely unknown, and studies on the relationship between inflammation and osteoblast/osteoclast have attracted increasing attention [20]. The normal physiological inflammatory response is a form of body defense response that favors fracture healing; however, long-term chronic inflammatory reactions induced by infections or other factors are detrimental to fracture healing [21]. Studies have suggested that the level of TNF-α was significantly higher in the blood of rheumatoid arthritis patients [22]. In addition, TNF-α, IL-1β, IL-6, and other inflammatory factors can stimulate the differentiation and formation of osteoclasts [14–17]. The present study showed that compared with healthy children, the expression levels of TNF-α, IL-1β, and IL-6 were significantly up-regulated in children with fractured ankle. We were eager to know if there are effective treatments of fractured ankle via regulating inflammation.
Recently, propofol has been found to have various effects other than anesthesia, such as anti-inflammatory effects. Propofol has been reported to relieve inflammatory response in LPS-activated microglia [14]. Zhang et al. reported that propofol could inhibit NF-κB activation in ovalbumin induced allergic asthma in mice [15]. Liu et al. suggested that propofol-attenuated intermittent hypoxia induced up-regulation of pro-inflammatory cytokines in microglia through inhibiting NF-κB/p38MAPK pathway activation [16]. Ma et al. indicated that propofol has anti-inflammatory effects via inhibiting the NLRP3 inflammasome [17]. Another study indicated that propofol reduced the expression of COX-2 without affecting amniotic membrane cell viability [23]. Based on previous studies, we investigated the potential effects of propofol on ankle fracture healing and the underlying molecular mechanisms. MG-63 cells were treated with 1 μM of BK to construct the inflammatory cell model. Our results suggest that BK-treatment significantly enhanced TNF-α, IL-1β, and IL-6 expression levels, and these enhancements were reduced by propofol treatment in a dose-dependent manner. In addition, BK-induced up-regulations of p-p38, p-p65, NLRP3, ASC, caspase-1, and COX-2 were dose-dependently down-regulated by propofol treatment.

Taken together, the data of the present study indicate that propofol exerts an anti-inflammatory effect on BK-treated MG-63 cells though regulating the p38MAPK-NF-κB pathway, NLRP3 inflammasome, and COX-2 expression.

Conclusions

Our data indicate the benefit of propofol for fracture healing, and provide a more theoretical basis for the clinical treatment of fractures.

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