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Original Article

Effect of Propofol Preconditioning on Hypoxic-Cultured Human Osteoblast

Ji Uk Yoon, Sang Wook Shin, Bong Soo Park*, Yong Ho Kim*, Mi Na Woo†, Ji Young Yoon‡, Cheul Hong Kim‡

Department of Anesthesia and Pain Medicine, School of Medicine, Pusan National University, Gyeongnam, Korea
*Department of Oral Anatomy, School of Dentistry, Pusan National University, Gyeongnam, Korea
†Department of Dental Anesthesia and Pain Medicine, Pusan National University Dental Hospital, Gyeongnam, Korea
‡Department of Dental Anesthesia and Pain Medicine, School of Dentistry, Pusan National University, Gyeongnam, Korea

Background: Angiogenesis has been recognized an essential precondition for osteogenesis. Because reduction and disruption of the blood supply to tissue cause tissue hypoxia, pathological bone loss affected by hypoxia often can occur in various clinical conditions. The effects of propofol on the process of osteogenesis have received little direct attention. Therefore, we investigated the effect of propofol on the growth and function of osteoblasts under hypoxic condition.
Methods: After propofol (3, 30, 300 μM) preconditioning for 2 hours, hFOB 1.19 human osteoblast cells were cultured under 1 % oxygen tension for 48 hours. Using real time PCR and western blot analysis, we analyzed the expression of, BMP-2, TGF-β1, type I collagen, osteocalcin, HIF-1s and Akt. Cell viability was also determined by MTT assay.
Results: Propofol preconditioning on hypoxic-cultured osteoblast promoted the expressions of BMP-2, TGF-β1, type I collagen and osteocalcin and induced hypoxia-mediated HIF-1 activation and the expression of Akt protein. Propofol with 300 μM significant decreased cell viability compared to control.
Conclusions: Clinically relevant concentrations of propofol are not cytotoxic to hypoxic osteoblasts in vitro. Propofol preconditioning on hypoxic-cultured osteoblast stimulates proliferation and differentiation of osteoblast through induced expression of BMP-2, TGF-β1, type I collagen and osteocalcin. Propofol might promote angiogenesis and bone regeneration under hypoxic condition.

Key Words: propofol; hypoxi; osteoblast

INTRODUCTION

Bone physiology is unusual in several respects. It appears an ability to regenerate and repair itself. A decrease of blood supply frequently occurs during impaired healing of fracture. This show that impaired angiogenic response is a major contributor to the pathology at the site of injury [1,2]. The processes of endochondral bone formation and fracture repair are related with the invasion of blood vessels [3].

PO_2 may decrease when the blood supply to tissues is reduced or disrupted. Reduction and disruption of the blood supply to tissue cause tissue hypoxia. Under clinical conditions such as orthopedic arthroplasty, orthognathic surgery, spine surgery and amputation, tissue hypoxia may occur due to reduction of blood supply. Hypoxia results in a failure to generate sufficient ATP to maintain essential cellular functions, whereas hyperoxia does in the production of damaging reactive oxygen intermediates. Thus, tight regulation of cellular oxygen concentrations within a narrow physiological range is very important to maintain tissue homeostasis.

Some studies have shown that hypoxia increases osteoblast vascular endothelial growth factor (VEGF) and insulin-like growth factor-2 (IGF-2) expression [4,5].

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Corresponding Author: Sang Wook Shin, Department of Dental Anesthesia and Pain Medicine, School of Dentistry, Pusan National University, Beomeo-ri, Mulgeum-eup, Yangsan-si, Gyeongsangnam-do 626-787, Korea
Tel: +82.55.360.5370 Fax: +82.55.360.5369 email: shinsw@pusan.ac.kr
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In addition, hypoxia enhances bone morphogenetic protein-2 (BMP-2) expression in osteoblasts by hypoxia inducible transcription factor-1α (HIF-1α) [6].

The hypoxia inducible transcription factor (HIF) is a heterodimer containing α and β subunits and it controls the oxygen-sensitive gene expression [7]. These target genes are related with various cellular processes including angiogenesis, energy metabolism, cell proliferation and survival, pH control, vasomotor control and matrix metabolism [8].

Recent reports have demonstrated hypoxia decreases osteogenic differentiation [9], cell proliferation [10,11] and osteocalcin [12] and it is necessary for growth arrest and survival of chondrocytes [13].

Propofol is an intravenous anesthetic drug widely used for general anesthesia and immediate postoperative or prolonged sedation in ICU patients. Propofol has also been shown to have protective effects against hypoxia-induced apoptosis in alveolar epithelial cell and inhibit HIF-1α-hypoxia responsive element axis [14]. Propofol increases BMP and decreases oxidative stress in sepsis-induced acute kidney injury [15].

However, the effects of propofol on the function of osteoblasts, the bone forming cells, have received little direct attention. Active and pathological bone loss affected by hypoxia often occurs in various clinical conditions. The present study was designed to investigate the effect of propofol on the growth and function of osteoblasts under hypoxic condition.

MATERIALS AND METHODS

1. Cell Culture and Drug Treatment

A hFOB 1.19 human osteoblast cell line was purchased from the ATCC (Rockville, MD, USA). This cell line was maintained at 34℃ with 5% CO_2 in air atmosphere in D-MEM/F-12 medium with 4 mM L-glutamine, 1.5 μg/L sodium bicarbonate, 4.5 g/L glucose and 1.0 mM sodium pyruvate supplemented with 10% fetal bovine serum (FBS).

Propofol was from Astrazeneca Ltd., Italy as the commercially available solution Diprivan (1%). Osteoblasts were exposed to various concentrations of propofol (3, 30, 300 μM) for 2 hours.

2. Hypoxia of cultured osteoblasts

The cells were cultured under 1% oxygen tension. Cells were seeded on 96-well plate (1×10^4 cells) before exposure to hypoxia. Cells were gassed with 95% N2, and 5% CO_2 (Anaerobic System PROOX model 110; BioSpherix, USA) and incubated at 34℃ within the chamber for 48h.

3. Assay of cell viability

Cells were cultured in a 96-well plate and incubated for 48 h. 100 μl of a colorimetric 3-(4,5-dimethylthiazol-2-yl)-2,5- diphenyltetrazolium bromide (MTT) (0.5 mg/ml final concentration) was added and incubated in the dark for an additional 4 h to induce the production of formazan crystals at 37℃ and the supernatants were discarded. The medium was aspirated and formed formazan crystals were dissolved in DMSO. Cell viability was monitored on an ELISA reader (Sunrise Remote Control, Tecan, Austria) at 570 nm excitatory emission wave length.

4. Quantitative reverse transcriptional PCR

Total RNA was extracted from the hFOB cells using Trizol reagent (Invitrogen, Life technologies, Carlsbad, NM, USA) according to manufacturer's instructions. Total RNA (2 μg) was reverse transcribed using a RevertAid™ First Strand cDNA synthesis kit (Thermo, Fremont, CA, USA) according to the manufacturer's protocols. Real-time PCR was performed on ABI 7500 Fast Real-Time PCR

System (Applied Biosystems 7500 System Sequence Detection System (SDS) software version 2.0.1) using SYBR Green PCR Master Mi x (Applied Biosystems, Foster City, CA, USA). The primers for BMP-2 (forward: 5'-ACC AGG TTG GTG AAT CAG AA-3' and reverse: 5'-TTT GGC TTG ACG TTT TTC TC -3'), transforming growth factor-β1(TGF- β1) (forward: 5'-TCT TTT GAT GTC ACC GGA GT-3' and reverse: 5'-CGT GGA GCT GAA GCA ATA GT -3'), type I collagen (forward: 5'-CGT GGT GTA ACT GGT CCT TC-3' and reverse: 5'-ACC GGG CTC TCC CTT ATC -3') and osteocalcin (forward: 5'- ATG AGA GCC CTC ACA CTC CT-3' and reverse: 5'- GGA TTG AGC TCA CAC ACC TC-3') were used. GAPDH (forward: 5'-GGA AGG ACT CAT GAC CAC AG-3' and reverse: 5'-TTG GCA GGT TTT TCT AGA CG-3') was used as an internal control. The conditions for the PCR were as follows: 50℃ for 2 min, 95℃ for 10 min and 40 cycles of 95℃ for 15 sec, 60℃ for 1 min and 72℃ for 30 sec. Real-time PCR data were analyzed by the SDS 2.0.1 software package (Applied Biosystems, Foster, CA, USA).

5. Western blot assay

Cells were plated at a density of 1 x 105 cells in 6-well plates. Cells were washed twice with ice-cold PBS and centrifuged at 2,000 rpm for 10 min. Total cell proteins were lysed with a RIPA buffer [300 mM NaCl, 50 mM Tris-HCl (pH 7.6), 0.5% TritonX-100, 2 mM PMSF, 2 μg /ml aprotinin and 2 μg /ml leupeptin] and incubated at 4℃ for 1 h.

The lysates were centrifuged at 14,000 revolutions per min for 15 min at 4℃, and sodium dodecyl sulfate (SDS) and sodium deoxycholic acid (0.2% final concentration) were added. Protein concentrations of cell lysates were determined with Bradford protein assay (Bio-Rad, Richmond, CA, USA) and BSA was used as a protein standard. A sample of 50 μg protein from each well was separated and loaded onto 7.5-10% SDS/PAGE. The gels were transferred to Nitrocellulose membrane (Amersham Pharmacia Biotech, Piscataway, UK) and reacted with each antibody. Immunostaining with antibodies was performed using SuperSignal West Pico enhanced chemiluminescence substrate and detected with Alpha Imager HP (Alpha Innotech, San Leandro, USA). Equivalent protein loading was confirmed by Ponceau S staining

6. Statistical analysis

Experiments were repeated five times. Multiple groups were compared using one-way analysis of variance (ANOVA) followed by a post hoc Tukey's test. The data were expressed as the mean ± standard deviation (SD). Values of $P < 0.05$ were considered significant (SPSS 13.0 Software, SPSS Inc., Chicago, IL, USA).

RESULT

1. Effect of propofol preconditioning on cell proliferation

The proliferation assay was performed at 48 h (Fig.1). Treatment with 3 and 30 μM for 2 h was not cytotoxic to osteoblasts. However, Treatment with 300 μM was significant decrease in cell viability compared to control ($P < 0.05$, respectively).

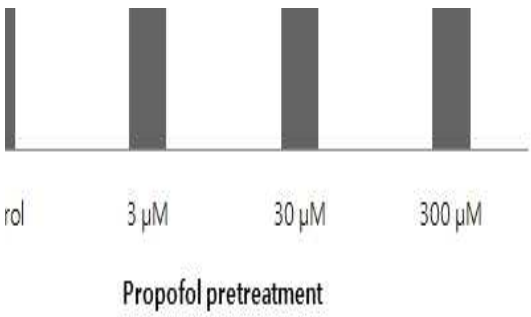

Fig. 1. Effect of propofol preconditioning on hFOB cell viability under hypoxia. Values are expressed as mean ± SD. *$P < 0.05$ as compared with control group.

2. Effect of propofol preconditioning on BMP-2, TGF-β1, type I collagen and osteocalcin

To examine the effect of propofol preconditioning on BMP-2, TGF-β1, type I collagen and osteocalcin in hFOB cells, cells were exposed to propofol at 3, 30 and 300 μM. Thereafter, the cells were cultured under 1% oxygen tension for 48 h.

The expression of BMP-2 mRNA was markedly increased throughout the experiment in all propofol treatment groups compared to the control group and peak the expression of BMP-2 mRNA was noted at treatment with 300 μM (Fig. 2A). The expression of TGF-β1 mRNA was slightly increased in 30 and 300 μM groups compared to the control group. However, Treatment with 3 μM did not affect the expression of TGF- β1 mRNA (Fig. 2B). The expression of Type I collagen was significantly increased in 30 and 300 μM groups compared to the control group. Unlike BMP-2 and TGF- β1, peak the expressions of type I collagen mRNA was noted at treatment with 30 μM (Fig. 2C). The expression level of osteocalcin mRNA was higher in 30 and 300 μM groups than in the control group (Fig. 2D). A similar effect of propofol was observed on the expression of TGF- β1 mRNA.

In Western blot analysis, similar to the results of PCR, propofol preconditioning is shown to increase the expressions of BMP-2, TGF-β1, type I collagen and osteocalcin protein (Fig. 3).

These results suggest that propofol on hypoxic osteoblasts induces the expressions of BMP-2, TGF-β1, type I collagen and osteocalcin.

3. Effect of propofol preconditioning on HIF-1α, HIF-1β and Akt

In order to assess the effect of propofol precondi-

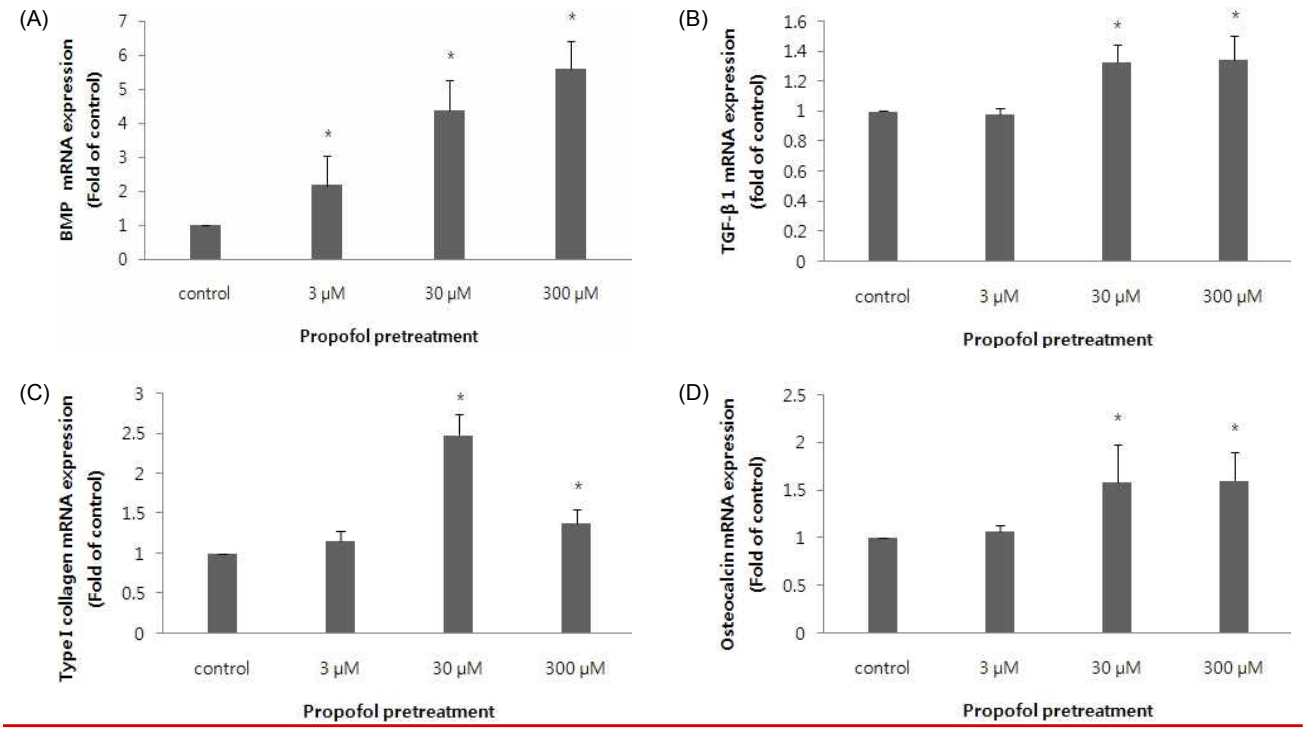

Fig. 2. Effect of propofol preconditioning on the expressions of BMP-2, TGF-β1, type I collagen and osteocalcin in hFOB cells under hypoxia. Values are expressed as mean ± SD.
*P < 0.05 as compared with control group.

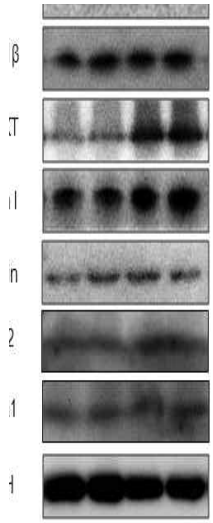

Fig. 3. In Western blot analysis, the Effect of propofol pre-conditioning on BMP-2, TGF-β1, type I collagen, osteocalcin, HIF-1α, HIF-1β and Akt. Propofol precon-ditioning is shown to increase the expressions of BMP-2, TGF-β1, type I collagen, osteocalcin, HIF-1 α and Akt protein. Unlike HIF-1α, the expression of HIF-1β were slightly increased by propofol.

tioning on the expressions of HIF-1α, HIF-1β and Akt, cells were exposed to propofol at 3, 30 and 300 μM and the cells were cultured under 1% oxygen tension for 48 h. In Western blot analysis, the expression of HIF-1α was significantly in all the propofol treatment groups com-pared to the control group. Unlike HIF-1α, the expression of HIF-1β were slightly increased by propofol. The expression of Akt was significantly increased in 30 and 300 μM groups compared to the control group (Fig. 3).

These results suggest that propofol on hypoxic osteo-blasts induces hypoxia-mediated HIF-1 activation and the expression of Akt protein.

DISCUSSION

The earlier studies have presented the effect of hypoxia on osteoblasts, but the effect of propofol on osteoblasts under hypoxia condition has not been documented until this study [4,6].

There are some studies on the contradictory effects of hypoxia on osteoblasts and osteoblastic cells. Park et al.[12] showed that hypoxia reduced the expression of Type I collagen and osteocalcin in human osteoblastic cells. In addition, hypoxia reduced the expression of osteocalcin, TGF-β, collagen in rat calvarial osteoblasts in a time-dependent manner [10]. Tseng et al. [6] have investigated the relationship between hypoxia and BMP-2 expression. In contrast to other cytokines and proteins, they demonstrated that hypoxia induced BMP-2 ex-pression via ILK/Akt/mTOR and HIF-1α pathways in osteoblasts time-dependently.

TGF- β1 is an important autocrine regulator of bone formation and a powerful bone growth stimulant at the level of pre-osteoblasts [16,17]. It promotes the synthesis of collagen, osteocalcin, and other extracellular matrix proteins [18]. Type I collagen is the major component of bone extra-cellular matrix and plays an important role in cell adhesion, proliferation and differentiation of the osteoblast [19,20]. Osteocalcin is a important marker of mature osteoblasts. The protein is shown to play a role in the differentiation of osteoblast progenitor cells, with significant up-regulation observed in both matrix syn-thesis and mineralization [20,21]. BMP-2 like other bone morphogenetic proteins has been demonstrated to play a crucial role in inducing osteoblast differentiation and bone formation during embryonic skeletal development and postnatal bone remodeling [22,23]. It is involved in the hedgehog pathway, TGF beta signaling pathway, and in cytokine-cytokine receptor interaction. In addition, several studies have demonstrated that Akt-related signaling pathways are involved osteoblast differentia-tion. Furthermore, Akt suppresses osteoblast apoptosis [24-26].

The hypoxia-inducible factor-1 (HIF-1) pathway is the central regulator of adaptive responses to low oxygen availability and is required for normal skeletal develop-ment [27]. Under hypoxia, HIF-1α protein is markedly

stabilized, translocates to the nucleus. The HIF-1 α and HIF-1β complex can then bind to hypoxia response elements (HREs) located in gene promoters to regulate transcription of vascular endothelial growth factor, erythropoietin, iNOS, and glycolytic enzymes that induce cellular adaptation to hypoxia [28,29]. The expression of VEGF via the activation of the PI-3 kinase pathway has also been observed to be mediated by HIF-1 α [30,31].

Propofol is extensively used for the induction and maintenance of anesthesia during surgery and for ICU sedation [32-34]. It has recently focused greater attention due to its anti-apoptosis capability and antioxidant property [35-38]. However, the direct effect of propofol on hypoxic osteoblasts has not been reported so far. Our preliminary experiment had showed that propofol pretreatment with 3 and 30 μM exhibits no cytotoxicity to hypoxic osteoblasts. But there were significant difference in cell viability between the control group and 300 μM group (Fig. 1). Several previous studies presented similar results of propofol at therapeutic concentrations having no effect on macrophage and osteoblast viability. However, propofol at a high concentration (300 μM) increased lactate dehydrogenase release and led to an arrest of the cell cycle in the G1/S phase [39]. The clinically relevant concentration of propofol was 3-11 μg/ml (approximately 17-62 μM /l) [40,41]. Therefore, clinically relevant concentrations of propofol are not harmful to osteoblasts.

In this study, we showed that propofol preconditioning on hypoxic osteoblasts induced the expressions of BMP-2, TGF-β1, type I collagen and osteocalcin. Chief of all, the expression of BMP-2 was markedly increased. These results indicate that propofol preconditioning promotes osteoblast proliferation and differentiation under hypoxic condition. In Western blot analysis, results of the present study demonstrated that propofol preconditioning on hypoxic osteoblasts increased HIF-1 activation and the expression of Akt protein. These finding are different from those of He, X.Y et al. [14], who studied effect of propofol on hypoxia-induced apoptosis in alveolar epithelial type II cells.

Because HIF-1 activation induce angiogenesis in hypoxic osteoblasts, this study suggests that propofol preconditioning accelerates bone regeneration. But, further studies about VEGF and several other genes will be required to ascertain effects of propofol preconditioning on angiogenesis and bone regeneration.

In conclusion, this study shows that clinically relevant concentrations of propofol are not cytotoxic to hypoxic osteoblasts in vitro and demonstrates that propofol preconditioning on hypoxic-cultured osteoblast stimulates proliferation and differentiation of osteoblast through induced expression of BMP-2, TGF-β1, type I collagen and osteocalcin. We assume propofol preconditioning promote angiogenesis and bone regeneration through HIF-1 activation.

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