The role of remifentanil in regulating mitochondrial autophagy in osteoclasts was investigated based on PINK1/Parkin pathway

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**Abstract**

This study aimed to explore the regulatory effect of remifentanil-mediated mitochondrial autophagy on osteoclast formation and further investigate its mechanism. Macrophage cell line RAW264.7 was taken and induced to differentiate into mature osteoclasts using nuclear factor kB receptor activating factor ligand (RANKL). The cell model was treated with different concentrations of remifentanil or down-regulated expression of mitochondrial autophagy-related gene PINK1. The survival, death and ROS production of osteoclasts were detected by CCK8 kit and flow cytometry, MMP level was detected by JC-1 method, mitochondrial morphology and autophagy were observed by transmission electron microscopy, and mitochondrial autophagy-related protein expression was detected by Western blot. The number of osteoclasts in the remifentanil-treated group was significantly reduced compared to the control group, accompanied by a reduction in reactive oxygen species (ROS) and mitochondrial membrane potential levels (MMP). Further results showed that remifentanil could significantly up-regulate the activity of PINK1/Parkin pathway, promote the occurrence of mitochondrial autophagy, and damaged mitochondria, and inhibit the formation of osteoclasts. Remifentanil successfully inhibited osteoclast formation by regulating mitochondrial autophagy mediated by PINK1/Parkin pathway. The results of this study revealed that remifentanil plays an important role in the physiology and pathology of osteoclasts, which may provide new ideas and strategies for the clinical treatment of remifentanil in tibial fractures.

**Keywords:** Remifentanil; PINK1/Parkin pathway; Osteoclast; Mitochondrial autophagy

1. Introduction

Tibial fractures, including tibial shaft fractures and tibial plateau fractures [1,2], often occur in middle-aged and elderly people over 50 years old [3]. The cause of the disease is often due to heavy blows, kicks, impact injuries or wheel running injuries, etc. The clinical manifestations are mainly leg swelling and pain, deformity and abnormal movement, knee swelling and pain, and activity disorders, which cause serious physical burdens to elderly patients [4]. At present, the treatment of tibial fractures generally relies on drugs and surgery. Among them, drugs are generally used to relieve pain, reduce swelling and prevent venous thrombosis [5], while surgery is divided into open reduction screw internal fixation and external fixation stent fixation [6-8]. Despite the continuous progress in the treatment of fractures in the medical field, there is still a significant proportion (up to 10%) of fracture cases with inadequate bone repair, which may lead to delayed recovery or incomplete healing of fractures [9,10]. Therefore, understanding the biological process of fracture healing is critical to the clinical development of new treatment and management strategies to help ensure optimal repair of damaged bones.

Fracture healing is an extremely complex process that involves bone reconstruction and formation at the end of the fracture. Among them, the most critical is the process of bone reconstruction, which requires the interaction between osteoblasts and osteoclasts to co-maintain [11]. During bone tissue renewal, the balance of bone/cartilage absorption by osteoclasts and bone formation by osteoblasts is disturbed, which adversely affects the speed and quality of fracture repair, thereby impairing bone microstructure and biomechanical properties. Previous studies have found that if osteoclast formation and/or activity is hindered, the absorption of cartilage callus and remodeling of hard bone callus will be hindered, thus improving the fracture healing process [12,13]. Autophagy is a non-selective process in which eukaryotic cells degrade cytoplasmic macromolecules and organelles in bulk. Recent studies have shown that abnormal autophagy levels are closely related to impaired osteoclast function and activity, and play a key role in bone metabolism disorders [14]. Osteoclasts have high energy requirements, and their functions and activities are regulated by mitochondria [15]. As a selective autophagy process, mitochondrial autophagy is closely associated with the pathogenesis of various metabolic bone diseases [16,17]. However, the role of mitochondrial autophagy in osteoclast formation remains to be further elucidated.
The mechanism of anesthesia drugs and organ protection has been a focus of research. At present, narcotic drugs with organ protection potential mainly include inhalation anesthetics such as isoflurane, sevoflurane, desflurane, emesil, antinociceptive, respiratory and sedative drugs such as ketamine and propofol, narcotic analogues such as fentanyl, remifentanil, morphine, and narcotic adjuvant drugs such as dexamethasone [18]. For example, it has been reported that propofol weakens osteoclast generation by inhibiting RANKL/OPG expression axis, thus producing beneficial effects on bone remodeling [19]. In addition, remifentanil can prevent oxidative damage of human fetal osteoblasts through mechanisms highly associated with autophagy [20]. Remifentanil belongs to a new class of opioid blocker drugs, which have rapid action, strong efficacy and a short half-life, and are widely used in anesthesia. Recent studies have shown that remifentanil can play a protective role in different types of cells by regulating the autophagy process [21,22]. In addition, we note that remifentanil has been shown to promote osteoblast differentiation and inhibit osteoclast differentiation and maturation in vitro, thereby reducing bone resorption [23]. But so far, the study of remifentanil-mediated mitochondrial autophagy in osteoclast formation has not been reported.

Therefore, this study took the osteoclasts formed by the differentiation of mouse mononuclear macrophages as the research object. Through the intervention of remifentanil, molecular biology techniques were used to detect the mitochondrial morphology and function and the expression of mitochondrial autophagy-related proteins, and the mechanism of PINK1/Parvin pathway in the regulation of remifentanil on mitochondrial autophagy of osteoclasts was studied. It provides scientific theoretical basis for the clinical application of remifentanil in the treatment of fracture.

2. Materials and Methods

2.1. Induction and culture of osteoclasts

Mouse mononuclear macrophage cell line RAW264.7 was purchased from Wuhan Punose Life Technology Co., LTD. (Wuhan, China), and cultured in DMEM medium containing 10% FBS+1% penicillin-streptomycin. When the cells were fused to 80%–90%, the original medium was discarded and the cells were re-suspended after digestion with pancreatic enzymes. Subsequently, the supernatant was discarded, the cells were re-suspended with complete medium, and passed by 1:3 ~ 1:6, once a day.

RAW264.7 cells were prepared into cell suspension using α-MEM medium containing 10%FBS and inoculated into 24-well plates at a density of 3×10⁴ cells/ well. After 12h of cell incubation, RANKL (20-100 ng/ml) was added. The medium was replaced every 3 days and supplemented with RANKL (20-100 ng/ml). More obvious multinucleated osteoclasts will begin to appear after 4 days of differentiation and culture and gradually become abundant from day 5 to day 6. Finally, the osteoclast formation was identified by TRAP staining.

2.2. Experimental Grouping

Group 1: Osteoclasts at logarithmic growth stage were randomly divided into 7 groups: group 0, group 0.5, group 1, group 2, group 4, group 8, and group 16. For the treatment of remifentanil, different concentrations (0, 0.5, 1, 2, 4, 8 and 16 ng/ml) of remifentanil were incubated with osteoclasts. CCK-8 assay was used to analyze the effect of remifentanil on the survival rate of osteoclasts at different concentrations. The optimal dose of remifentanil was selected according to the cell activity and used as the experimental concentration for the following experiments.

Specific administration methods are shown in Table 1.

Group 2: Osteoclasts at logarithmic growth stage were randomly divided into 6 groups: Control group, Remi group, si-NC group, si-PINK1 group, Remi+si-NC group, and Remi+si-PINK1 group. The small interfering RNA plasmid targeting PINK1 (si-PINK1) and its negative control (si-NC) were synthesized by Suzhou Jima Gene Co., LTD. According to the supplier's instructions, the above plasmids were transfected into osteoclasts with Lipofectamine 3000 reagent, and the infection number was 50:1. The transfection efficiency was verified by the WB experiment. The specific administration methods are shown in Table 2.

2.3. CCK-8 method

The induced osteoclasts were inoculated into 96-well plates. After the cells were observed to be in good condition under an inverted phase contrast microscope, they were incubated at 37°C and 5% CO2 for 4-8 hours. After the cells were attached to the wall, they were treated in groups with 3 multiple pores in each group. 2 h before the end of incubation, 10 μL CCK8 reagent (Bioss, Wuhan, China) was added to each well. After incubation, OD values of each well were measured at 450 nm by RT-6000 enzyme-labeled analyzer (Rayto).

2.4. Flow Cytometry (FCM)

Annexin v-FITC /PI kit (Beyotime, Shanghai, China) was used for staining and the apoptosis rate was evaluated by Attune Nxt flow cytometry (Thermo Fisher Scientific, Waltham, MA, USA). After incubation, 100 μL of untreated and treated cells were transferred into different tubes with 5 μL Annexin V-FITC and 5 μL PI added to

Table 1. Experimental group 1.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Model</th>
<th>Type of administration</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 group</td>
<td>osteoclast</td>
<td>No processing required</td>
</tr>
<tr>
<td>0.5 group</td>
<td>osteoclast</td>
<td>The osteoclasts were incubated with 0.5ng/ml remifentanil for 2h</td>
</tr>
<tr>
<td>1 group</td>
<td>osteoclast</td>
<td>The osteoclasts were incubated with 1ng/ml remifentanil for 2h</td>
</tr>
<tr>
<td>2 group</td>
<td>osteoclast</td>
<td>The osteoclasts were incubated with 2ng/ml remifentanil for 2h</td>
</tr>
<tr>
<td>4 group</td>
<td>osteoclast</td>
<td>The osteoclasts were incubated with 4ng/ml remifentanil for 2h</td>
</tr>
<tr>
<td>8 group</td>
<td>osteoclast</td>
<td>The osteoclasts were incubated with 8ng/ml remifentanil for 2h</td>
</tr>
<tr>
<td>16 group</td>
<td>osteoclast</td>
<td>The osteoclasts were incubated with 16ng/ml remifentanil for 2h</td>
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</tbody>
</table>
Each tube. The tubes were incubated in darkness at room temperature for 15 min and then analyzed within 1 hour by Attune NxT flow cytometry (Thermo Fisher Scientific, Waltham, MA, USA).

### 2.5. Determination of cell ROS production

After cell treatment, osteoclasts were collected and added with fluorescent probe DCFH-DA (1:1000) with a final concentration of 10 μM, and incubated in a constant temperature incubator for 30 min. Subsequently, the cells were washed with PBS 2 to 3 times and centrifuged for 5 minutes at a low temperature of 1000 rpm/min. The supernatant was discarded, cleaned with PBS, and the cells were re-suspended with 400 μL PBS and then analyzed sequentially with Attune NxT flow cytometry (Thermo Fisher Scientific, Waltham, MA, USA).

### 2.6. Determination of MMP level

The treated osteoclasts were collected and incubated with JC-1 probe (Beyotime, Shanghai, China) at 37°C in the dark for 30 min. Subsequently, the cells were washed three times with PBS to remove the free JC-1 probe. Finally, fluorescence microscopy was used to detect the fluorescence intensity of 525 nm emission wavelength and 488 nm excitation wavelength (green fluorescence) and 590 nm emission wavelength and 525 nm excitation wavelength (red fluorescence) respectively, and the fluorescence intensity ratio was calculated to reflect the MMP level.

### 2.7. Transmission electron microscopy (TEM)

The treated osteoclasts were collected and fixed in a dark environment at 4°C with 2.5% glutaraldehyde (Spicher, USA) for 2-4 h. The supernatant was abandoned, and the cells were rinsed with 0.1 M phosphoric acid bleach 3 times, and then fixed in 1% osmic acid (Ted Pella Inc., USA) for 2 h. The cells were dehydrated in cold-graded ethanol series (30, 50, 70, 80, 95 and 100% ethanol) and rinsed with 100% acetone three times. The cells were then impregnated and coated in salt. Finally, the blocks are cut and observed. Finally, the dye was double stained with 3% uranyl acetate and 2.7% lead citrate and observed under TEM (HITACHI, HT7700, Japan).

### 2.8. Western blot (WB)

Proteins in cells and mitochondria were extracted using RIPA (Beyotime, Shanghai, China) lysis buffer. The protein concentration was set by BCA protein assay kit (NCM Biotech, China). All cell lysates containing 40 μg protein were subjected to SDS-PAGE gel electrophoresis and electrophoretically imprinted onto PVDF membranes. Subsequently, the membrane was sealed with TTBS containing 5% skim milk at room temperature for 2 hours and incubated with the following primary antibody: GAPDH Monoclonal antibody (60004-1-Ig, Proteintech, Rosemont, IL, USA) and PINK1 Polyclonal antibody (23274-1-AP, Proteintech, Rosemont, IL, USA), Anti-COX IV antibody - Mitochondrial Loading Control (ab16056, Abcam, Cambridge, MA, USA), Anti-Parkin antibody (ab77924, Abcam, Cambridge, MA, USA), Anti-LC3B antibody (ab186735, Abcam, Cambridge, MA, USA), Anti-TOMM20 antibody (ab188935, Abcam, Cambridge, MA, USA), Anti-SQSTM1/p62 antibody (ab51520, Abcam, Cambridge, MA, USA), Anti-Cox IV antibody (ab109012, Abcam, Cambridge, MA, USA), Prestained Protein Marker II (G2058-250UL, Servicebio, Beijing, China). After that, the membrane containing protein bands was incubated with HRP polymerized secondary antibody at room temperature for 2 hours. Finally, the imprinting was visualized with the detection system of JP-K6000 chemiluminescence instrument (Jiapeng, Shanghai, China).

### 2.9. Statistical Analysis

Data were statistically analyzed and mapped by GraphPad Prism 9 (Version 9.5.1, La Jolla, CA, USA) and Image J software, and typesetting by Photoshop software. All the data were expressed as Means±SD. T-Test was used for pound-wise comparison, and One-way test was used for multiple comparisons. A P-value less than 0.05 was considered a significant difference.

### 3. Results

#### 3.1. Effects of remifentanil on survival rate and apoptosis rate of osteoclasts

To investigate the effect of remifentanil on the survival rate of osteoclasts, we treated osteoclasts with different concentrations of remifentanil (0, 0.5, 1, 2, 4, 8 and 16 ng/ml) for 2 hours, and then measured the survival rate by CCK-8 method. As demonstrated in Figure 1A, the survival rate of osteoclasts treated with remifentanil at different concentrations decreased vs. the control group (P<0.001), in which 8 ng/mL of remifentanil had more significant suppression on the survival rate of osteoclasts, so 8 ng/mL of remifentanil was selected for follow-up experiments. Next, we evaluated the apopto-

### Table 2. Experimental group 2.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Model</th>
<th>Type of administration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control group</td>
<td>osteoclast</td>
<td>No processing required</td>
</tr>
<tr>
<td>Remi group</td>
<td>osteoclast</td>
<td>The osteoclasts were incubated with 8ng/ml remifentanil for 2h</td>
</tr>
<tr>
<td>si-NC group</td>
<td>osteoclast</td>
<td>si-NC plasmid was constructed and transfected into osteoclasts with Lipofectamine 3000 transfection reagent</td>
</tr>
<tr>
<td>si-PINK1 group</td>
<td>osteoclast</td>
<td>The carrier plasmid with low expression of PINK1 (si-PINK1) was constructed and transfected into osteoclasts with Lipofectamine 3000 transfection reagent</td>
</tr>
<tr>
<td>Remi+si-NC group</td>
<td>osteoclast</td>
<td>8ng/ml remifentanil was incubated with osteoclasts for 2 hours and si-NC was transfected into osteoclasts with Lipofectamine 3000 transfection reagent</td>
</tr>
<tr>
<td>Remi+si-Parkin group</td>
<td>osteoclast</td>
<td>8ng/ml remifentanil was incubated with osteoclasts for 2 hours and si-Parkin was transfected into osteoclasts with Lipofectamine 3000 transfection reagent</td>
</tr>
</tbody>
</table>
sis of osteoclasts, and the results revealed that the apoptosis rate of osteoclasts in the Remi group was higher than that in the Control group, as exhibited in Figure 1B (P<0.05). These results manifested that remifentanil treated osteoclasts for a short time could decrease the survival rate of osteoclasts, but promote their apoptosis.

3.2. Effects of remifentanil on mitochondrial morphology and function of osteoclasts

Studies have revealed that osteoclasts are closely related to autophagy [14]. Due to the rich mitochondria in osteoclasts, mitochondrial autophagy is a selective autophagy process, and its relationship with osteoclast activation has not been clarified. Therefore, FCM and JC-1 probes were applied to detect ROS and MMP content respectively, and transmission electron microscopy was applied to detect mitochondrial ultrastructure and autophagy of osteoclasts. The results demonstrated in Figure 2A-C that remifentanil could depress ROS and MMP levels of osteoclasts but accelerated mitochondrial damage and mitochondrial autophagy. Furthermore, PINK1/Parkin pathway is a classic ubiquitin-dependent pathway mediating mitochondrial autophagy. WB experiment results showed that compared with the Control group, The protein expressions of mito-PINK1, mito-Parkin, cyto-Parkin and TOMM20 and the levels of mito-LC3B/mito-LC3I in Remi group were increased, while mito-p62 protein expression decreased (P<0.01) (Figure 2D), suggesting that remifentanil may promote mitochondrial autophagy by regulating PINK1/Parkin pathway.

The above data illustrate that the ROS and MMP levels of osteoclasts can be reduced by the short-term treatment of remifentanil, damage mitochondria, and promote the formation of mitochondrial autophagosomes.

3.3. Effects of blocking PINK1/Parkin pathway activation on osteoclast survival and mitochondrial autophagy

PINK1/Parkin pathway is a ubiquitin-dependent pathway mediating mitochondrial autophagy, but the role of PINK1/Parkin pathway in mitochondrial autophagy of osteoclasts has not been clarified. Therefore, we specifically knocked down the expression of PINK1 in osteoclasts using RNA interference technology, and the transfection efficiency was confirmed by WB assay. As exhibited in Figure 3A, vs. the si-NC group, the expression level of PINK1 protein in osteoclasts in the si-PINK1 group was significantly decreased (P<0.01). Subsequently, we further evaluated the effects of PINK1 knockdown on osteoclast survival and apoptosis. As shown in Figure 3B/C, vs. the si-NC
of WB experiment showed that the expression of mito-PINK1, mito-Parkin, cyto-Parkin, TOMM20 protein and the levels of mito-LC3I/mito-LC3II were decreased, but mito-p62 protein expression increased (P<0.05) (Figure 4G). This suggests that down-regulating PINK1/Parkin pathway can reverse the promoting effect of remifentanil on mitochondrial autophagy.

These data suggest that down-regulation of PINK1/Parkin pathway can reverse the effects of remifentanil on osteoclast survival and mitochondrial autophagy.

4. Discussion

Osteoclasts are the main cell types of bone resorption, and they participate in bone metabolism by absorbing and degrading bone tissue [24]. Moderate enhancement of osteoclast activity helps to maintain bone health and stability of bone mass, but excessive bone resorption may lead to instability of bone and delayed healing [25,26]. Autophagy is an intracellular degradation pathway that improves cell survival by recycling damaged organelles and proteins. The enhancement of osteoclast activity requires organelles and intracellular protein conversion, and autophagy is involved in this process [27]. However, as a selective autophagy process, the relationship between mitochondrial autophagy and remifentanil-mediated osteoclast formation has been rarely reported. Previous studies have shown that remifentanil can play aprotective role in different types of cells by regulating the autophagy process [21,22]. Meanwhile, in this study, we found that remifentanil affects osteoclast survival in a dose-dependent manner. However, it is not clear whether remifentanil mediates mitochondrial autophagy to play an active role in osteoclasts. Therefore, in this study, remifentanil was applied to osteoclasts for a short period of time, and combined with the classical PINK1/Parkin pathway of mitochondrial autophagy, to explore the changes in mitochondrial function and morphology of osteoclasts.

ROS produced by mitochondria is a derivative of O2, and the production of ROS can act as a REDOX signal to regulate autophagy [28,29]. ROS can be used as a signaling molecule for many life activities, activating autophagy to clear mitochondria [30]. This study found that remifentanil could reduce ROS levels in osteoclasts. In addition, as a sensitive indicator of mitochondrial function, MMP reduces membrane potential when mitochondria are depolarized [31]. In this process, the mitochondria will be damaged and edema and disintegration will occur. This study found that remifentanil treatment could reduce MMP levels in osteoclasts. Mitochondrial morphology is the basis of function, and the change in mitochondrial function is closely related to the change of its morphology. In autophagy studies, autophagosomes or autophagolysosomes can be directly observed through transmission electron microscopy. Therefore, the ultrastructure of mitochondria and mitochondrial autophagosomes can also be observed by transmission electron microscopy [32]. In this study, transmission electron microscopy showed that after remifentanil treatment, mitochondrial morphology of osteoclasts was changed to different degrees, mitochondrial swelling, ridge blur and even dissolution, and mitochondrial autophagosomes were formed. We hypothesize that these results may be due to the compensatory mechanism of the cell. In a short period of time, remifentanil treatment of cells causes minor damage to the mitochondria, and the
cells are stimulated to activate autophagy and clear the damaged mitochondria to maintain the intracellular balance.

PINK1/Parkin pathway is a ubiquitin-dependent pathway that mediates mitochondrial autophagy [33]. When mitochondria are damaged, PINK1 accumulates stably on the outer membrane of mitochondria as a molecular receptor and activates Parkin translocation in the cytoplasm to the surface of the damaged mitochondria [34]. Activated Parkin can ubiquitinate and degrade proteins on the mitochondrial surface, while adaptor protein P62 accumulates around damaged mitochondria and can specifically bind to LC3 to mediate mitochondrial autophagy and degrade damaged mitochondria [35-37]. We have confirmed that remifentanil is related to mitochondrial autophagy in osteoclasts, and it causes mitochondrial function and morphological changes, but the study on the mechanism of remifentanil on mitochondrial autophagy in osteoclasts has not been reported. Therefore, we treated cells with remifentanil to investigate the role of PINK1/Parkin pathway in the regulation of mitochondrial autophagy in osteoclasts by remifentanil. Here, we treated osteoclasts with remifentanil and found that PINK1 expression was increased, Parkin was transferred from cytoplasm to mitochondria and protein expression was increased, suggesting that remifentanil was likely to enhance mitochondrial autophagy in osteoclasts by promoting PINK1/Parkin pathway. Next, we knocked down the expression of PINK1 in remifentanil-treated osteoclasts and clarified this experimental conclusion through a replacement experiment. We first found that down-regulating PINK1/Parkin pathways reversed the effects of remifentanil on osteoclast survival and mitochondrial autophagy.

In summary, remifentanil treatment significantly up-regulated the expression of mitochondrial PINK1/Parkin pathway proteins in osteoclasts, reduced the levels of ROS and MMP, and led to mitochondrial damage and activation of mitochondrial autophagy. This suggests that remifentanil can significantly enhance mitochondrial autophagy in osteoclasts through the PINK1/Parkin pathway. However, there are some limitations to this study. First, our findings are based on cell model studies and need to be further verified in animal models. In addition, the mechanism of whether remifentanil affects osteoclast differentiation through mitochondrial autophagy remains to be further explored. Therefore, more investigations are needed in the future to further clarify.

5. Conclusion
In this study, we highlight the key role of remifentanil in promoting mitochondrial autophagy of osteoclasts through upregulation of PINK1/Parkin pathway, which may provide theoretical basis for clinical use of remifentanil in the treatment of fracture healing or other diseases related to bone metabolism. Finally, our study does not imply remifentanil is used as a therapeutic agent but rather suggests the use of this anesthetic in surgery, especially for fracture healing or other bone metabolism-related diseases.

Conflict of Interests
The author has no conflicts with any step of the article preparation.

Consent for publications
The author read and approved the final manuscript for publication.

Ethics approval and consent to participate
No human or animals were used in the present research.

Informed Consent
The authors declare that no patients were used in this study.

Availability of data and material
The data that support the findings of this study are available from the corresponding author upon reasonable request.

Authors’ contributions
Pufeng Ye, Guifeng Pan and Zhenjiang Mai designed the study and performed the experiments, Yuanfeng Li and Aobo Li collected the data, Jianbin Zhang and Mingxiu Xin analyzed the data, Pufeng Ye, Guifeng Pan and Zhenjiang Mai prepared the manuscript. All authors read and approved the final manuscript.

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Remifentanil's impact on osteoclast mitochondrial autophagy

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