Original

Investigation on the Action and Effect of Culture Supernatant of Human Dental Pulp Stem Cells Using Rats with Medication-Related Osteonecrosis of the Jaw

Fumihiko Abe¹, Haruka Takahashi¹,² and Akira Tanaka¹,²

¹Department of Oral and Maxillofacial Surgery, The Nippon Dental University School of Life Dentistry at Niigata, Niigata, Japan
²Division of Cell Regeneration and Transplantation, Advanced Research Center School of Life Dentistry at Niigata, The Nippon Dental University
Niigata, Japan
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Abstract: The incidence of osteonecrosis of the jaw (ONJ) in association with bisphosphonate (BP) preparations (medication-related ONJ, MRONJ) has reportedly increased rapidly in the recent times. Despite the increase, specific cure remains to be established. In this study, MRONJ model rats were constructed, the action and effect of dental pulp stem cell-conditioned medium (DPSC-CM) on MRONJ were evaluated. Following BP preparation (sodium zoledronate, ZOL) administration to rats via the jugular vein, the first molar of the right maxilla was extracted to obtain MRONJ model rats, which served as the control group. Model rats were further administered with either Dulbecco Modified Eagle Medium (DMEM) or DPSC-CM via the jugular vein. These groups were comparatively analyzed, and the jawbone at the site of dental extraction was used as the test sample. Compared with control group and DMEM group, the DPSC-CM group exhibited unique macroscopic findings characterized by the narrowed width of bone exposure and increased mucous coating. Histological analysis revealed a significant decrease in the number of hollow bone lacunae, implying improved osteonecrosis. In immunohistochemical studies, it was discerned that ZOL administration promoted osteoprotegerin (OPG) production in the osteoblasts and suppressed the synthesis of receptor activator of nuclear factor kappa-B ligand (RANKL). Conversely, the DPSC-CM group displayed suppressed OPG and accelerated RANKL expressions, with an increase in the number of vascular endothelial growth factor-positive cells. Furthermore, Tartrate-resistant acid phosphatase/alkaline phosphatase (TRAP/ALP) staining suggested improved bone remodeling capability in this group. Thus, DPSC-CM administration improves ischemic osteonecrosis and may, therefore, be an effective therapeutic option.

Key words: MRONJ, Dental pulp stem cells-conditioned medium (DPSC-CM), Vascular endothelial growth factor (VEGF), Osteoprotegerin (OPG), Receptor activator of nuclear factor kappa-B ligand (RANKL)

Introduction

Bisphosphonate (BP) preparations are useful in treating osteoporosis, Paget disease of bone, bone metastasis of malignant tumors, multiple myeloma, and hypercalcemia, as well as in preventing and improving a range of bone-related conditions¹,². However, BP preparations tend to induce osteoclast (OC) apoptosis and suppress angiogenesis³. Although medication-related osteonecrosis of the jaw (MRONJ) has been reported as a symptom in association with BP preparations, a specific cure is yet to be established. Various therapeutic methods have been proposed until date to emphasize conservative and non-invasive modalities, but these procedures merely delay symptom progression and fail to achieve complete remission. Consequently, surgical incision is ultimately the only option for several patients. Thus, developing an effective cure for MRONJ remains a challenge. Past reports have indicated suggested improved bone remodeling capability in this group. Thus, DPSC-CM administration improves ischemic osteonecrosis and may, therefore, be an effective therapeutic option.

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The gizone (Life Technologies; Thermo Fisher Scientific, Inc.) and O.1% Fun. The process of MRONJ model rats used in the experiment. The condition of the mucous coating and the extent of bone exposure in the extracted tooth sockets were observed. The proximal, distal, and buccolingual diameters of the exposed bone were measured using a caliper. For statistical analysis, Kruskal–Wallis and Steel–Dwass tests (Excel Statistics) were conducted, with $P < 0.05$ set as the level of significance.

**Histological evaluation**

After immersion and fixation with 4% paraformaldehyde, the maxillary bone was decalcified in 10% EDTA (pH 7.4, 4°C) for 4 weeks. As per the conventional protocol, the sample was dehydrated and embedded in paraffin. Segments of 6-μm thickness were subjected to hematoxylin–eosin (HE) staining, and the number of empty bone lacunae was counted. For measurement, four segments were randomly extracted from each animal. The bone part corresponding to the first molar of the right maxilla was analyzed. Fig. 1 shows a schematic diagram of the experimental plan.

This experiment was approved by the Animal Experiment Ethics Committee of the Nippon Dental University School of Life Dentistry at Niigata (Approval No: EC-NG-H-121).

**Materials and Methods**

**Construction of MRONJ model rats**

We used 8-week-old male Wistar rats (CLEA Japan, Inc., Tokyo, Japan), which were maintained in a breeding room set at a 12-h light–dark cycle. A mixed anesthetic agent comprising medetomidine hydrochloride (Kyoritsu, Tokyo, Japan) (0.15 mg/kg), midazolam (Astellas Pharma, Tokyo, Japan) (2 mg/kg), and butorphanol tartrate (Falma, Tokyo, Japan) (2.5 mg/kg) was used. As an anesthetic antagonist, atipamezole hydrochloride (Kyoritsu, Tokyo, Japan) (1.5 mg/kg) was intraperitoneally injected. Under controlled anesthesia, ZOL (Zometa®, Novartis Pharma K. K., Tokyo, Japan) was administered at a dose of 160 μg/kg via the jugular vein at 1 ml/body. The CM group was administered DPSC-CM via the jugular vein at 1 ml/body. These additional administrations were performed on the same day as that of the final ZOL. The rats were killed after 2 weeks. The maxillary bone extracted from each animal was immersed and fixed in 4% paraformaldehyde (Wako, Osaka, Japan). The bone part corresponding to the first molar of the right maxilla was analyzed. Fig. 1 shows a schematic diagram of the experimental plan.

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**Macroscopic evaluation of tooth sockets**

The condition of the mucous coating and the extent of bone exposure in the extracted tooth sockets were observed. The proximal, distal, and buccolingual diameters of the exposed bone were measured using a caliper. For statistical analysis, Kruskal–Wallis and Steel–Dwass tests (Excel Statistics) were conducted, with $P < 0.05$ set as the level of significance.

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In accordance with the DAB method, OPG, RANKL, and vascular endothelial growth factor (VEGF) were stained. Endogenous peroxidase

![Figure 1. Preparation of the MRONJ model rats](image-url)
was inactivated using the peroxidase-blocking solution Dako REAL™ (Dako, Tokyo, Japan). Anti-sRANKL (rabbit polyclonal-antibody, dilution 1:1000; ab62516, Abcam) and anti-OPG (rabbit polyclonal-antibody, dilution 1:1000; ab73400, Abcam) antibodies were allowed to react with the segments overnight at 4°C. Only the anti-OPG antibody was subjected to antigen activation with citric acid (pH 6.0) before the primary antibody was added. Reaction with the secondary antibody (EnVision™ + Dual link System-HRP, Dako, Tokyo, Japan) followed; the samples were colored, nuclear stained, penetrated, and embedded.

TRAP/ALP staining was performed using the TRAP/ALP Staining Kit® (Wako, Osaka, Japan) as per the manufacturer’s instructions. OCs and OBs were observed. Specifically, detached OCs (DAOCs) and OCs attached to the bone surface (OCABS) were noticed. OBs adjacent to OCABS were also noted. The cells were counted, and statistical analysis was conducted in the same way as HE staining, with \( P < 0.05 \) set as the level of significance.

**Results**

**Macroscopic findings in the extracted tooth sockets**

The exposed alveolar bone width in the extracted socket was 3.73 (±1.69) mm for the proximal/distal diameter and 1.66 (±0.64) mm for the buccolingual diameter in the control group, 3.25 (±1.64) mm for the proximal/distal diameter and 1.84 (±0.77) mm for the buccolingual diameter in the DMEM group, and 0.48 (±0.76) mm for the proximal/distal diameter and 0.30 (±0.25) mm for the buccolingual diameter in the CM group. Thus, the exposed bone width in the CM group was significantly reduced compared with that in the other groups (Fig. 2). Moreover, 2 samples in this group achieved complete closure (Fig. 3). Compared with the control and DMEM groups, the CM group exhibited a
Figure 4. Histological analysis by HE staining. A: Control group, B: DMEM group, C: CM group. Black arrowheads: empty bone lacunae; white arrow heads: epithelial tissue. HE staining revealed numerous empty bone lacunae in the jawbone around the extracted tooth socket in the control and DMEM groups. In contrast, the CM group exhibited a significant decrease in the number of empty bone lacunae, and the epithelial tissue was observed to have formed.

Figure 5. Comparison of the number of empty bone lacunae. Significant difference between a and b ($P < 0.05$), Steel-Dwass test.

Figure 6. Immunological findings (OPG-positive cells). A: Control group, B: DMEM group, C: CM group. Black arrowheads: OPG-positive cells. Numerous OPG-positive cells in the control and DMEM groups, whereas the CM group showed a significant decrease in the number of such cells.
Significant decrease in bone exposure and mucous-coating formation.

**Histological changes in the extracted tooth sockets**

HE staining revealed numerous empty bone lacunae in the jawbone around the extracted tooth socket in the control and DMEM groups, indicating the onset of MRONJ (Fig. 4A, B black arrowheads). In contrast, the CM group exhibited a significant decrease in the number of empty bone lacunae (Fig. 5), and the epithelial tissue was observed to have formed (Fig. 4C white arrowheads). MRONJ symptoms improved in this category.

Immunological staining revealed numerous OPG-positive cells in the control and DMEM groups (Fig. 6A, B), whereas the CM group showed a significant decrease in the number of such cells (Figs. 6C and 7A). RANKL-positive cells were scarcely detected in the control and DMEM groups (Fig. 8A, B), whereas they were significantly increased in the CM group (Figs. 8C and 7B). Similarly, the number of...
VEGF-positive cells was less in the control and DMEM groups (Fig. 9A, B), whereas they were significantly increased in the CM group (Figs. 9C and 7C). These results suggest that DPSC-CM administration promotes OB and OC activities in the jawbone around the extracted tooth sockets of MRONJ model rats.

**Condition of jawbone remodeling**

TRAP/ALP staining revealed only a few OCs in the control and DMEM groups, but these were significantly increased in the CM group (Fig. 10A). The number of DAOCs was high in the control and DMEM groups, whereas it was significantly decreased in the CM group (Figs. 10B and 11). The numbers of OCABS and OBs adjacent to OCABS were significantly elevated in the CM group than in the control and DMEM groups (Figs. 10C, D and 11C). These results indicate an improved jawbone remodeling ability in MRONJ model rats.

**Growth factors in DPSC-CM**

ELISA results reported 123 (±9.09) pg/ml of the osteogenesis-relat-
ed factor MCP-1 and 1499 (±126.5) pg/ml of VEGF in DPSC-CM.

Discussion

In this study, MRONJ model rats were constructed by administering ZOL via the jugular vein. Dental extraction was performed, and the action and effect of DPSC-CM were evaluated. Macroscopic findings of the extracted tooth sockets revealed reduced bone exposure. Healthy rats form an epithelial coating 14 days after dental extraction. In the present study, MRONJ model rats encountered a delay in this process because ZOL inhibited the angiogenesis and chemotaxis of oral epithelial cells. DPSC-CM contains angiogenic growth factors, such as VEGF and interleukin-8 (IL-8). The detection of VEGF was also expected from the DPSC-CM used in this experiment, and the immunostaining performed in the present study signified an increase in VEGF-positive cells. These factors may have induced epithelial tissue formation by increasing the number of vascular endothelial cells and promoting chemotaxis, thereby improving the bone exposure.

HE staining revealed an elevation in the number of empty bone lacunae in the jawbone tissues of the model rats constructed by ZOL administration, indicating the onset of MRONJ. DPSC-CM administration decreased the number of empty bone lacunae. According to Williams et al., samples with ≥5 empty bone lacunae can be considered to have osteonecrosis due to MRONJ. In the present study, all samples in the control and DMEM groups had ≥5 empty bone lacunae, whereas none in the CM group had ≥2 empty bone lacunae, suggesting that the DPSC-CM administration to MRONJ model rats improved osteonecrosis.

OPG and RANKL are proteins secreted by OBs and function as remodeling regulatory factors. When the balance between the proteins is maintained, they regulate differentiation and activation of OCs, thereby promoting normal bone remodeling. OPG and RANKL are also used as bone repair markers.

Several reports have indicated that ZOL is absorbed into OCs and suppresses bone absorption by inducing apoptosis. Meanwhile, ZOL acts on OBs to enhance OPG production while suppressing cell proliferation and RANKL expression, thereby regulating the differentiation and activation of OCs. Moreover, ZOL inhibits the migration and differentiation of OC precursors by suppressing IL-6, macrophage colony-stimulating factor (M-CSF), and MCP-1 expressions in OBs. As reported by these studies, ZOL influences OBs in diverse ways. In the present study, immunostaining revealed a significant increase in RANKL-positive cells and a significant decrease in OPG-positive cells in MRONJ model rats exposed to DPSC-CM. While inhibiting the apoptotic action of osteoclasts by ZOL, DPSC-CM may induce the differentiation and activation of OCs by suppressing OPG production and promoting RANKL expression in OBs. MRONJ model rats had fewer VEGF-positive cells and experienced a delay in mucous-coating formation and ischemic osteonecrosis because of the angiogenesis inhibitory action of ZOL. The results imply that DPSC-CM administration increases VEGF-positive cells and enhances angiogenesis. The DPSC-CM used in this study expressed VEGF.

TRAP/ALP staining demonstrated a significant increase in OCs, which could be attributed to DPSC-CM administration. An increase in DAOCs and decrease in OCABS are findings unique to apoptosis in ZOL-exposed OCs. Furthermore, the control and DMEM groups in the present study exhibited increase in DAOCs and decrease in OCABS, indicating that apoptosis is promoted in OCs. In contrast, the CM group showed a decrease in DAOCs and increase in OCABS, indicating the suppression of apoptosis. In addition, the number of OBs adjacent to OCABS increased, suggesting that DPSC-CM administration improved the bone remodeling ability.

MSC-CM contains various growth factors secreted from stem cells involved in osteogenesis, angiogenesis, and cell differentiation. The effectiveness of the medium in treating MRONJ has been documented. However, MSC-CM preparation requires bone marrow fluid collection, which is a highly invasive process. Such invasiveness is considered to be a serious problem for elderly patients with MRONJ. In contrast, DPSC is easier to collect than MSC, and according to some reports, the former may possess high proliferative and pluripotent capabilities. The DPSC-CM used in this study expressed MCP-1. OC precursors are reportedly derived from monocytes. The MCP-1 is a monocyte, chemotactic protein that promotes monocyte and lymphocyte chemotaxis and increases IL-1 and IL-6 production. The protein may induce OC formation by accelerating the accumulation and induction of OC precursors.

The present study demonstrated that DPSC-CM administration to MRONJ model rats improved osteonecrosis. A future challenge was to evaluate the action and effect of DPSC-CM during dental extraction for proactively preventing MRONJ. Therefore, further analysis of the growth factors involved in DPSC-CM is considered necessary.
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Conflict of Interest
The authors have declared that no COI exists.

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