Regenerative Therapy 10 (2019) 77–83

Contents lists available at ScienceDirect

Regenerative Therapy

journal homepage: http://www.elsevier.com/locate/reth

Original Article

Allogeneic multipotent mesenchymal stromal cell sheet transplantation promotes healthy healing of wounds caused by zoledronate and dexamethasone in canine mandibular bones

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ARTICLE INFO

Article history:
Received 25 June 2018
Received in revised form
29 September 2018
Accepted 30 October 2018

Keywords:
Osteonecrosis of the jaw
Bisphosphonate
Multipotent mesenchymal stromal cells
Cell sheet
Beagle dogs
Regenerative therapy

ABSTRACT

Introduction: Many cases of bisphosphonate-related osteonecrosis of the jaw (BRONJ), which is an intractable disease, have been reported. Although a general intravenous injection of multipotent mesenchymal stromal cells (MSCs) may be effective for treating BRONJ, it has some severe problems. Therefore, our aim was to develop a treatment of locally administered MSCs. In this study, we investigated the effect of MSC sheet transplantation in the mandibular bone healing in beagle dogs, which were administered zoledronate and dexamethasone.

Methods: MSCs isolated from subcutaneous fat were seeded onto temperature-responsive culture dishes to produce MSC sheets. Zoledronate and dexamethasone were administered to beagle dogs. Then, the parts of mandibular cortical bones were removed, and MSC sheets were transplanted to cover those bone defects (MSC sheet transplant side) or not (Control side). The specimens were evaluated in micro CT, histology, and immunohistochemistry.

Results: Four weeks after surgery, redness and swellings were observed in the mucosal wounds of the control sides of 2 of 3 dogs. In contrast, the mucosal wounds of the MSC sheet transplant sides of all dogs completely healed. Histological images showed some free sequestrums and many bacterial colonies, and Immunohistological analysis showed some cathepsin K-positive multinuclear cells detached from jaw bone surfaces in the control sides.

Conclusions: MSC sheet transplantation promotes healthy healing of wounds caused by zoledronate and dexamethasone in canine mandibular bones. And the injured canine mandibular bones administered zoledronate and dexamethasone showed BRONJ-like findings.

1. Introduction

Bisphosphonates (BPs) are the first-line drugs for osteoporosis because they have been shown to suppress bone resorption [1]. Furthermore, in the cancer treatment field, their effectiveness has been confirmed as a prophylactic agent for bone metastasis and skeletal-related event [2]. However, since bisphosphonate-related osteonecrosis of the jaw (BRONJ) was first reported in 2003 [3], many cases have been reported all over the world. Because its etiology and methods of treatment and prevention are unknown, it is

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Peer review under responsibility of the Japanese Society for Regenerative Medicine.

https://doi.org/10.1016/j.reth.2018.10.008

difficult to treat patients who are administered BPs. Although surgery is performed in the case of advanced stage BRONJ, its success rate is not high [4]. It was reported that the success rate of surgery for BRONJ is only 60–86% [5–8]. The most recent Multicenter Retrospective Study in Japan reported that 32% of surgical cases, and half of high-dose cases in which patients had cancer, did not heal [9]. Therefore, an alternative approach for treating BRONJ is desirable. Although it was reported that a general intravenous injection of multipotent mesenchymal stromal cells (MSCs) was effective for treating BRONJ in animal models [10,11], the non-localized injection of stem cells has some severe problems [12–15]. Therefore, we tried to develop a treatment of locally administered MSCs using cell sheet engineering. The effectiveness of this method has been validated in many clinical trials for various diseases [16–18]. We already reported that local transplantation of MSC sheets can have therapeutic effects in a BRONJ-like rat model [12]. In order to advance this therapy to the clinic, it is important to confirm its safety and efficacy with large animal experiments. Thus, in this study, we investigated the effect of MSC sheet transplantation for the mandibular bone healing in beagle dogs, which were administrated zoledronate and dexamethasone.

2. Methods

2.1. Animals

Four beagle dogs (12–13 months old males) were obtained from Institute for Animal Reproduction (Kasumigaura, Ibaraki, Japan) and used in the present study. All experimental protocols were approved by the animal welfare committee of Tokyo Women's Medical University.

2.2. Isolation and culture of MSCs from adipose tissue

Subcutaneous fat was taken from the abdomens of one beagle dog and enzymatically digested with phosphate-buffered saline (PBS, Thermo Fisher Scientific, Waltham, MA, USA) containing 0.1% type A collagenase (Roche Diagnostics, Indianapolis, IN, USA) under shaking for 1 h at 37 °C. The stromal-vascular fraction (SVF) was extracted after centrifugation at 700×*g* for 5 min at room temperature. Single cell suspensions of SVF were passed through a 70-mm strainer (Falcon, BD Labware, Franklin Lakes, NJ, USA) and cultured in complete medium [α-MEM GlutaMAX (Invitrogen, Thermo Scientific, Carlsbad, CA) with 20% fetal bovine serum (FBS, Moregate Biotech, Bulimba, Australia) and 1% penicillin/streptomycin (Sigma–Aldrich, St Louis, MO, USA)] in a humidified atmosphere of 95% air and 5% CO_2 at 37 °C. After 24 h, the floating cells were removed, and the medium was replaced with fresh medium. The adherent cells (adipose-derived MSCs) were subcultured using trypsin-ethylenediaminetetraacetic acid (trypsin-EDTA, Life Technologies) every 3 days until passage 5. All used MSCs taken from one dog.

2.3. Flow cytometry assay

MSCs isolated at passage 5 were suspended in 500 μL of PBS supplemented with 2% FBS and 10 μg/mL of each specific antibody. To detect the surface markers, fluorescein isothiocyanate (FITC)-coupled antibody against CD29 (SM3016F, Acris Antibodies, San Diego, CA, USA), FITC-coupled antibody against CD34 (FAB3346F, R&D Systems, Minneapolis, MN, USA), FITC-coupled antibody against CD44 (11-5440-41, Invitrogen), FITC-coupled antibody against CD45 (11-5450-41, Invitrogen) were used. For the isotype control, FITC-coupled mouse IgG1κ (IC002F, R&D Systems) and FITC-coupled rat IgG2bκ (11-4031-81, Invitrogen) were used.

2.4. Colony-forming assay

MSCs isolated at passage 5 were seeded onto collagen-coated (TMTCC-050, Cell Applications, San Diego, CA, USA) 100-mm culture dishes at a density of 1000 cells/dish and cultured in MSCs growth medium (TMMFM-001, Cell Applications). Seven days after seeding, the MSCs were stained with 0.5% crystal violet (Kanto Chemical, Tokyo, Japan) in methanol for 5 min and washed twice with distilled water.

2.5. Differentiation assay

To examine osteogenesis, MSCs at passage 5 were seeded onto 100-mm culture dish at a density of 1000 cells/dish and cultured for 7 day as previously described [19]. The medium was replaced with osteoinductive medium (CACn417D250, Cell Applications) for an additional 21 days. The MSCs were fixed with 4% paraformaldehyde and stained with 1% alizarin red S solution (Wako pure chemical). To examine adipogenesis, MSCs at passage 5 were seeded onto 100-mm culture dish at a density of 1000 cells/dish and cultured for 7 days, as previously described [19]. The medium was replaced with adipoinductive medium (CACn811D250, Cell Applications) for an additional 21 days. The MSCs were fixed with 4% paraformaldehyde and stained with fresh Oil Red O solution (Wako pure chemical).

2.6. Preparation of MSC sheets

MSCs isolated at passage 5 were seeded onto temperature-responsive culture dishes (35-mm diameter, UpCell, Cell Seed, Tokyo, Japan) at a density of 2 × 105 cells/dish. The MSCs were cultured in complete medium with 82 μg/mL ascorbic acid (Wako Pure Chemical Industry, Osaka, Japan) for a week. The temperature of the culture dishes was reduced to room temperature, and subsequently the medium was removed to produce MSC sheets (1.5106 cells/one sheet) (Fig. 1).

2.7. Transplantation of MSC sheet into bisphosphonate treated beagle dogs

Zoledronate (Zometa, 66 μg/kg; Novartis Pharma, Basel, Switzerland) were intramuscularly administered to 3 beagle dogs every 2 weeks and dexamethasone (5 mg/kg; Fuji Pharma, Tokyo, Japan) were intramuscularly administered once a week for 4 weeks. Two weeks after first administration, incisions and opened flaps of mandibular gingiva were made, and mandibular cortical bones, 5 mm in diameter, were removed using bone trephine bar. Same defects were created bilaterally in 3 beagle dogs. MSC sheets combined with polyglycolic acid (PGA) sheets (Neoveil, Gunze, Tokyo, Japan), were transplanted to cover the bone defects (MSC sheet transplant side) or only PGA sheets were transplanted to the bone defects (Control side). Then, wounds were completely sutured and the sutures were removed 2 weeks later (Fig. 2).

2.8. Histological analysis and immunohistochemical analysis

The mandibular tissues were removed, decalcified with decalcifying liquid (K-CX; Falma, Tokyo, Japan) for 3 days, fixed in 10% paraformaldehyde, and paraffin-embedded. Next, sections (5 μm thick) were cut longitudinally using a microtome (SM2000R; Leica Microsystems, Wetzlar, Germany), and stained with hematoxylin

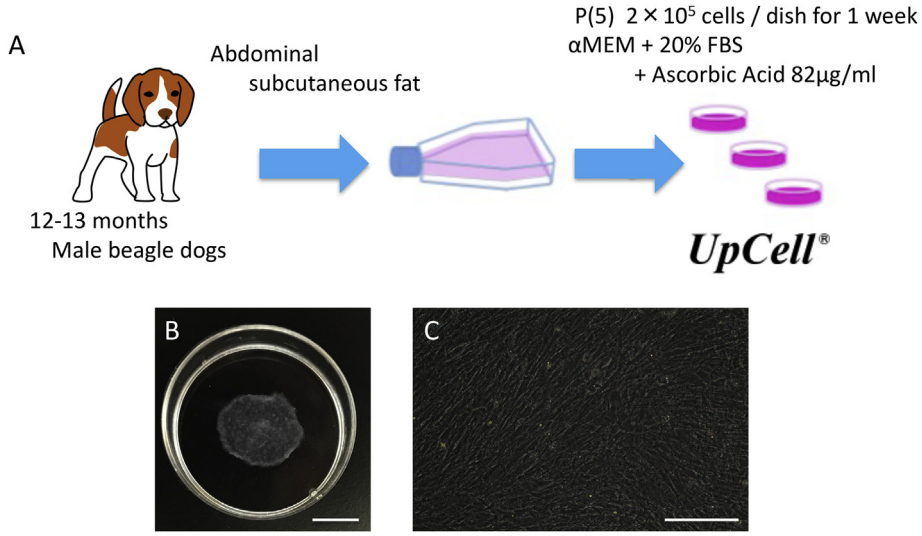

Fig. 1. (A) Multipotent mesenchymal stromal cells (MSCs) were isolated from abdominal subcutaneous fats in beagle dogs. MSCs at passage 5 were seeded onto temperature-responsive culture dishes at a cell density of 2×10^5 cells/dish and cultured in complete medium with 82 μg/mL ascorbic acid for a week. (B) Morphology of abdominal subcutaneous fat derived MSC sheet. Scale bar: 500 μm (C) Macro images of abdominal subcutaneous fat derived MSC sheet. Scale bar: 10 mm.

and eosin. For immunohistochemical analysis, after deparaffinization, the endogenous peroxidases of the sections were blocked with 3% hydrogen peroxide (H_2O_2). The sections were then immunostained with anti-cathepsin K antibody (NCL-CATH-K, Novocastra, Newcastle, UK) for 60 min in room temperature. After primary antibody staining, the sections were washed with tris-buffered saline and incubated with a secondary antibody (EnVisionTM Reagent; K4000, Dako, Glostrup, Denmark) for 30 min in room temperature. The sections were developed using diamino benzidine substrate.

2.9. Micro-CT analysis

Quantitative analysis of new bone in the bone defects was performed using an in vivo micro-computed tomography system (R_mCT2; Rigaku, Tokyo, Japan). The mandibular bones were scanned using micro-CT with an X-ray source of 90 kV/160 micro A after sacrifice. For each defect, we measured the new bone area using Image J version 1.49v (NIH, Bethesda, MD, USA).

2.10. Photographic image analysis

Area of inflammation in the mandible mucosa was measured on the photographic images of gingiva using Image J version 1.49v (NIH, Bethesda, MD, USA).

2.11. Statistics

The mean differences between the two groups were analyzed by an unpaired two-tailed Student's t-test using JMP Pro 11.0.0 (SAS,

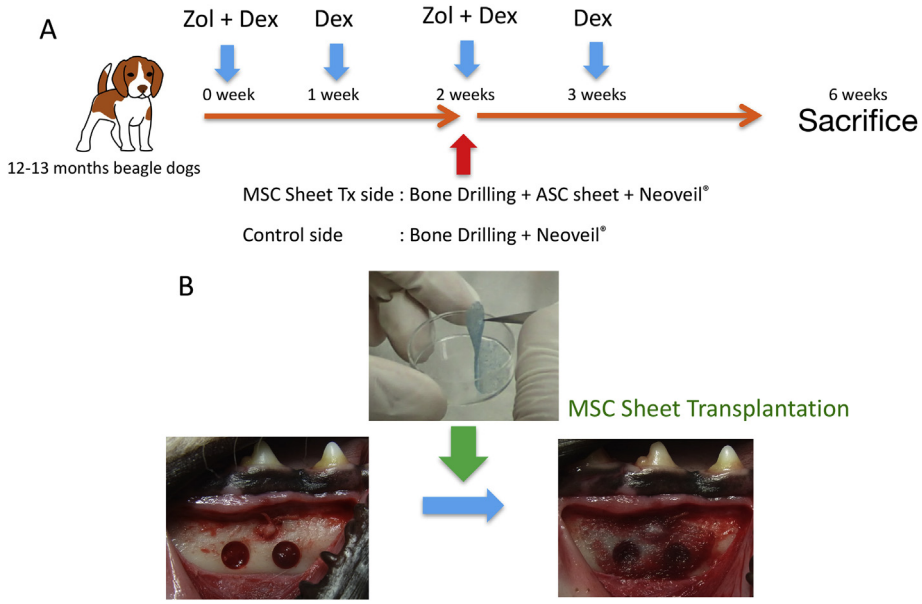

Fig. 2. Zoledronate (Zol) were intramuscularly administered to beagle dogs every 2 weeks and dexamethasone (Dex) were intramuscularly administered once a week for 4 weeks. Two weeks after first administration, mandibular cortical bones, 5 mm in diameter, were removed using bone trephine bar and MSC sheets, combined with polyglycolic acid (PGA) sheets (Neoveil, Gunze, Tokyo, Japan), were transplanted to cover the bone defects (MSC sheet transplant side) or only PGA sheets were transplanted to the bone defects (Control side).

Cary, NC, USA). A p-value of less than 0.05 was considered significant.

3. Results

3.1. MSC markers, colony-forming assay, and differentiation assay

Flow cytometric analysis revealed that MSCs derived from subcutaneous fats were positive for the MSC-related markers CD29 and CD44, and were negative for the endothelial cell markers CD34 and CD45 (Fig. 3A). The colony-forming assay showed that the MSCs formed many colonies stained with crystal violet (Fig. 3B). The MSCs cultured with osteoinductive medium for 21 days showed alizarin red S-positive calcium deposits and the MSCs cultured with adipoinductive medium for 21 days had oil red O-positive lipid droplets. Therefore, MSCs derived from subcutaneous fats had both adipogenic and osteogenic potentials (Fig. 3B).

3.2. Effects of MSC sheet transplantation in the dog mandibular bones administered with bisphosphonate and dexamethasone

Four weeks after surgery, redness and swellings, which represented serious inflammation, were observed in the mucosal wounds of the control sides of 2 of 3 dogs. In contrast, the mucosal wounds of the MSC sheet transplant sides of all dogs completely healed (Fig. 4 and Table 1). Micro CT analysis revealed that there was no significant difference between the two sides in the radiopaque rate of bone removal area (Fig. 4 and Table 1). Histological images showed normal cortical bone structure was maintained in the transplant sides, whereas bone structure was disordered in the control sides (Fig. 5A and C). Moreover, there were some free sequestrums (black arrow in Fig. 5B) and many bacterial colonies (yellow arrow in Fig. 5B) in the control sides. In contrast, the MSC sheet transplant sides showed histologically healthy mucosal condition (Fig. 5C and D). Immunohistological analysis showed some cathepsin K-positive multinuclear cells detached from jaw bone surfaces and existed between bone and bone (black arrow in Fig. 6B) in the control sides. In MSC sheet transplant sides, many cathepsin K-positive multinuclear cells remained on the bone surfaces as normal histological finding (black arrow in Fig. 6D).

4. Discussion

Since the first report in 2003 [3], BRONJ has become a major problem in dentistry, oral and maxillofacial surgery, and clinical oncology and osteology. As the mechanism, treatment, and prevention of BRONJ are still unknown, the situation around this disease has not changed [9,20,21]. It has also been reported that osteonecrosis of the jaw (ONJ) caused by denosumab, an anti-RANKL monoclonal antibody that suppresses bone resorption, occurs at the same frequency of BRONJ [4,21]. Moreover, ONJ due to anti-cancer drug suppression of angiogenesis has been reported at a low frequency [21]. From these facts, such ONJ were named BRONJ, anti-resorptive agents-related osteonecrosis of the jaw (ARONJ), or medication-related osteonecrosis of the jaw (MRONJ). Although these ONJ should not be discussed all together, they may have some similar pathological findings [4,20].

Some etiological mechanisms of BRONJ have been proposed, including over-suppression of bone turnover, anti-angiogenesis, oral bacterial infection, soft tissue toxicity, and immune dysfunction [4,21]. However, because patients receiving BPs have the variety of underlying diseases, defining the etiology of BRONJ may be difficult. The research so far has shown that the pathology of BRONJ are the same as osteomyelitis, and characteristics exclusive to BRONJ have not been identified. It has been reported that chronic suppurative osteomyelitis infected with actinomyces and sequestrum are observed in many clinical pathological specimens of BRONJ [21–23]. Also, it was reported that large osteoclasts detached from the bone surface and osteoclasts with irregular nuclei counts were observed in many pathological specimens [24]. As shown in the results of this study, immunostaining of cathepsin K revealed similar findings to that in the control sides (Fig. 6).

In terms of the prevention of BRONJ, necessity of drug withdrawal prior to dental surgery, antibiotics, and an appropriate

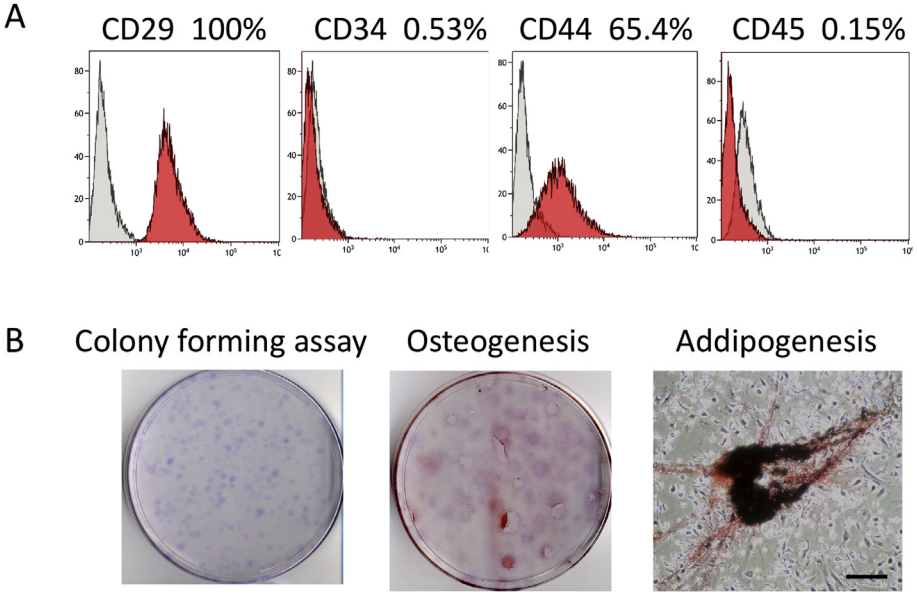

Fig. 3. (A) Flow cytometric analysis revealed that MSCs derived from subcutaneous fats were positive for the MSC-related markers CD29 and CD44, and were negative for the endothelial cell markers CD34 and CD45. (B) The colony-forming assay showed that the MSCs formed many colonies stained with crystal violet. The MSCs cultured with osteoinductive medium for 21 days showed alizarin red S-positive calcium deposits and the MSCs cultured with adipoinductive medium for 21 days had oil red O-positive lipid droplets. Therefore, MSCs derived from subcutaneous fats had both adipogenic and osteogenic potentials.

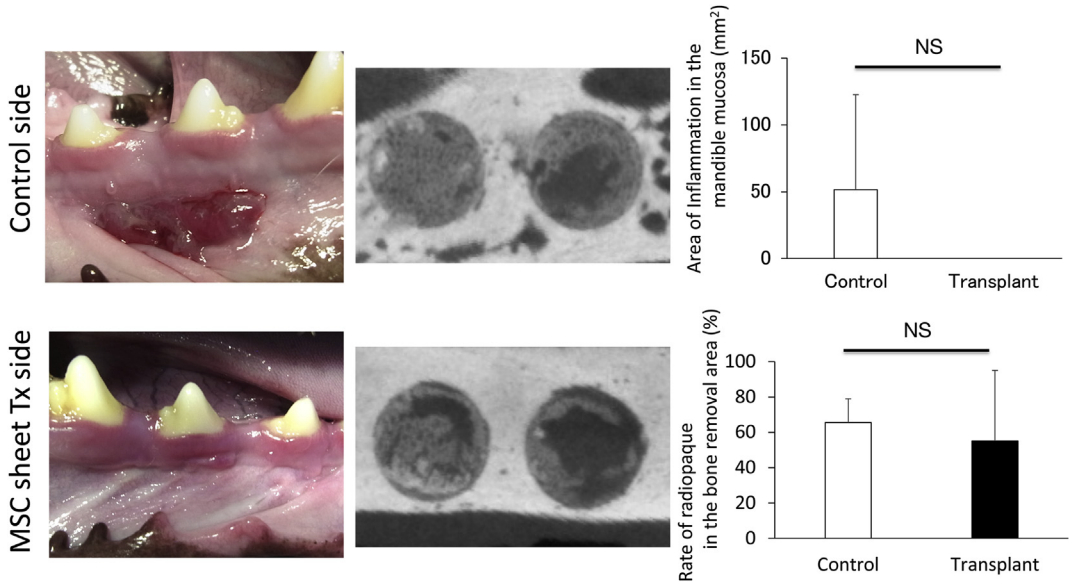

Fig. 4. Four weeks after surgery, redness and swellings which represented a serious inflammation were observed in the mucosal wounds of the control sides of 2 of 3 dogs. In contrast, the mucosal wounds of the MSC sheet transplant sides of all dogs completely healed. Micro CT analysis revealed that there is no significant difference between the two sides in the radiopaque rate of bone removal area.

Table 1
Area of inflammation in the mandible mucosa and rate of radiopaque in the removal area.

	Area of inflammation in the mandible mucosa (mm^2)			Rate of Radiopaque in the removal area (%)		
	Control	Transplant	*p*-value	Control	Transplant	*p*-value
Dog 1	132.6	0	0.273	61.39	61	0.691
Dog 2	22.3	0		54.57	12.49	
Dog 3	0	0		80.49	91.77	

surgical procedure have not been established [4,21]. In the treatment, stage 1 cases are treated conservatively, but treatment for stage 2 cases are aggressively changing to extended surgery [9,21]. For some patients, surgery fails to cure BRONJ symptoms and jaw resection is necessary, which results in a substantial decrease in quality of life. Thus, development of less invasive and more reliable therapy for BRONJ is desired. Alternative treatments that have been reported so far include transitioning from bisphosphonates to teriparatide [25], hyperbaric oxygen therapy [26], platelet-rich plasma [27], and low-level laser irradiation [28]. Even in the field of cell therapy, it has been reported that bone exposure can be treated by intravenous injection of MSCs in a BRONJ-like mouse or pig model [10,11].

The treatment using MSCs is roughly divided into two strategies; replacement therapy, in which the disordered tissues are replaced by taking advantage of the multipotency of MSCs, and cell therapy using immunomodulatory, anti-inflammatory, and angiogenesis activities by various humoral factors secreted from MSCs

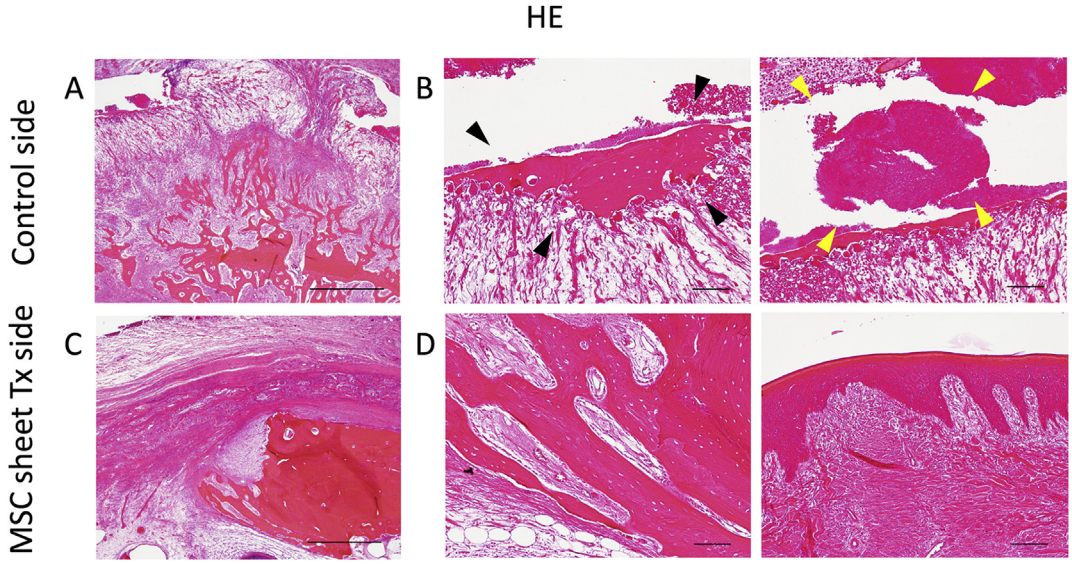

Fig. 5. Histological images showed normal cortical bone structure was maintained in the transplant sides, whereas bone structure was disordered in the control sides (Fig. 5A and C). Moreover, there were some free sequestrums (black arrow in Fig. 5B) and many bacterial colonies (yellow arrow in Fig. 5B) in the control sides. In contrast, the MSC sheet transplant sides showed histologically healthy mucosal condition (Fig. 5C and D). A, C Scale bar: 1000 μm; B, D Scale bar: 100 μm.

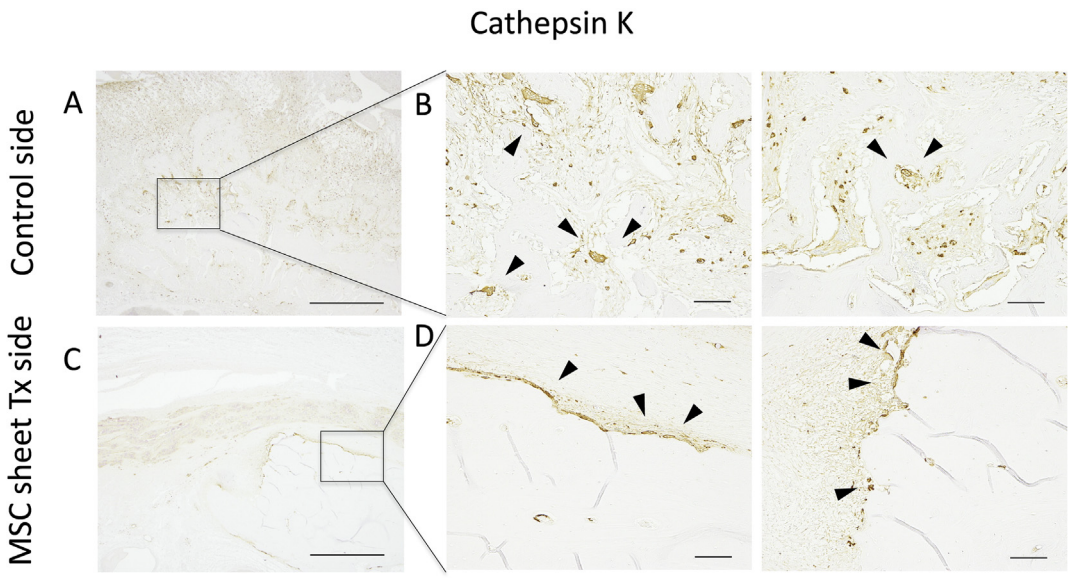

Fig. 6. Immunohistological analysis showed some cathepsin K-positive multinuclear cells detached from jaw bone surfaces and existed between bone and bone (black arrow in Fig. 6B) in the control sides. In MSC sheet transplant sides, many cathepsin K-positive multinuclear cells remained on the bone surfaces as normal histological finding (black arrow in Fig. 6D). A, C Scale bar: 1000 μm; B, D Scale bar: 100 μm.

[29–32]. We reported that MSCs may cure BRONJ-like rat model by differentiation into pericytes or osteoblasts and secretion of VEGF, HGF, RANKL [12].

Given the route of administration of MSCs, intravenous administration is difficult because the low adherent rate to the transplanted area can promote the proliferation and metastasis of cancer cells [12,13,15,33–35]. Therefore, we investigated a local administration method of MSCs using a cell sheet, which has been proven effective for the treatment of various diseases [16–18]. In addition, we reported that MSC sheet transplantation can treat bone exposures in a BRONJ rat model [12]. In order to advance this method with MSC sheets to clinical trials, we investigated to confirm the efficacy and safety in beagle dogs. As shown in the results of this study, the control sides one month after the surgery presented inflammatory characteristics and pathological findings close to clinical BRONJ. In contrast, the MSC sheet transplant sides showed normal healing findings (Figs. 4 and 5). We reported that promotion of angiogenesis by secretion of VEGF and HGF from transplanted MSC sheets and differentiation of osteoclasts by RANKL from them may cure bone exposures in a BRONJ rat model [12]. In this study, MSC sheet transplantation possibly suppressed the development of BRONJ by similar mechanism. However, detailed mechanisms were not able to be confirmed in this study because experimental reagents for dogs were not commercially available. There was no significant difference of bone regeneration between two groups in this study. That may be because MSC sheets, which were not intentionally differentiated into osteoblasts before transplantation, were used. We will develop a large animal model with the aim of showing pathology closer to clinical BRONJ and conduct MSC sheet transplantation experiments to confirm whether this therapy has a definite therapeutic effect for the clinical trial.

5. Conclusion

MSC sheet transplantation promotes healthy healing of wounds caused by zoledronate and dexamethasone in canine mandibular bones. And the injured canine mandibular bones administered zoledronate and dexamethasone showed BRONJ-like findings.

Conflicts of interest

Teruo Okano is the founder and director of CellSeed, Inc. and holds technical licenses and patents from Tokyo Women's Medical University. He is a stakeholder in CellSeed, Inc. Tokyo Women's Medical University receives research funds from CellSeed, Inc. The other authors disclose no financial relationships relevant to this publication.

Acknowledgments

The authors are very grateful to Ms. Hallie Thorp for carefully proofreading the manuscript. The authors also thank Dr. Ryo Takagi for valuable technical support of beagle dog's anesthesia. This study was financially supported by the Creation of Innovation Centers for Advanced Interdisciplinary Research Areas Program of the Project for Developing Innovation Systems “Cell Sheet Tissue Engineering Center (CSTEC),” funded by the Ministry of Education, Culture, Sports, Science, and Technology (MEXT) of Japan, and a bilateral research project between Japan and Korea funded by the Japan Society for the Promotion of Science (JSPS) Grant number 14544639. This work was supported by JSPS KAKENHI Grant Number JP18K17181.

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