R.T.R® promotes bone marrow mesenchymal stem cells osteogenic differentiation by upregulating BMPs/SMAD induced cbfa1 expression

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Bone marrow mesenchymal stem cells (BMSCs) of rats were isolated and Bio-Oss® and R.T.R® materials were used in this study. Alkaline phosphatase (ALP) activity in each group was calculated. The ability of adhesion and proliferation of BMSCs on Bio-Oss® and R.T.R® increased with time, but they were significantly higher on R.T.R® than that of Bio-Oss® at all time points (p<0.05). Compared with Bio-Oss®, R.T.R® could promote the expression and activity of ALP in BMSCs, and the expression of bone formation related transcription factors bone morphogenetic protein-1 (BMP-1), cbfa1 and osteoblast marker genes ALP, collagen I, osteopontin, osseomucin and osteocalcin. The expression levels of cbfa1, ALP, collagen I, osteopontin, osseomucin and osteocalcin were inhibited after downregulated expression of BMP-1 in BMSCs and inoculation with R.T.R®. R.T.R® could up-regulate BMP-1 expression and cbfa1 expression through BMPs/SMAD signaling pathway, thereby promoting the expression of ALP, collagen I, osteopontin, osseomucin and osteocalcin and promoting osteoblast differentiation.

Keywords: Maxillary sinus floor augmentation, Bone marrow stromal cells, Bone substitutes, Differentiation

INTRODUCTION

Maxillary sinus floor lifting (MSFL) technique is a surgical treatment for alveolar ridge atrophy of maxillary posterior teeth, maxillary sinus gasification, osteoporosis and other reasons to the lack of vertical bone mass and difficult to use conventional dental implants. Traditional MSFL needs bone transplantation to maintain space and promote bone regeneration1-5. Autogenous bone transplantation is considered to be the first choice for MSFL bone regeneration. However, the operation always requires additional surgical sites for donor bone acquisition, thus increasing the risk of donor site morbidity and prolonging the operation time6-8.

Bio-Oss® is an inorganic bone graft, it is derived from bovine bone, which removes organic components. R.T.R® is a synthetic graft made entirely of β-tricalcium phosphate (β-TCP). Bio-Oss® and R.T.R® are widely used bone substitute materials. Bio-Oss® is a decalcified bovine bone, its properties are similar to human bone, but there may be antigenicity problems of xenogeneic bone. R.T.R® has microporous structures similar to human cancellous bone, it is often used as a bone substitute in place of autologous bone grafts for MSFL and shows high biocompatibility and bone conductivity6-15. However, these substitutes have no cellular components at all and they require longer healing time than autologous bone grafts, the new bone-producing tissue is immature within six months after transplantation16-20.

At present, the most effective solution is to implant bone marrow mesenchymal stem cells (BMSCs) onto these substitutes and then transplant them. However, the mechanism of various materials promoting bone formation is still unclear21-22. Which material is the best for MSFL after inoculation with BMSCs remains uncertain.

In this study, we compared the proliferation and osteogenic differentiation of BMSCs after inoculation with Bio-Oss® and R.T.R® materials, and explored its molecular mechanism.

MATERIALS AND METHODS

Isolation and culture of rat BMSCs
Rats were killed by injection of excessive pentobarbital and immersed in 75% alcohol for 15 min. The femurs and tibia were separated under sterile conditions, soft tissue was removed and the metaphysis was cut off. DMEM medium (10 mL) was used to rinse the pulp cavity completely and to the irrigation fluid was collected to the centrifuge tube, they were centrifuge at 1,000 rpm/min for 5 min and the supernatant was discarded. The cells were re-suspended in DMEM medium containing 10% FBS (10 mL) and placed in a 100 mm cell culture dish. They were cultured in an incubator at 37°C containing 5% CO2 for passage and follow-up experiments.

For stable downregulation of bone morphogenetic proteins (BMPs) expression of BMSCs, we infected BMSCs using lentiviruses expressing BMPs 3’-UTR (Suzhou GenePharma, Suzhou, China) according to MOI=20. After being infected for 72 h, puromycin was continuously used for 2 weeks to screen the stable
expression cells BMSCs-BMPs-KD.

This study was independently reviewed and approved by the ethics committee of our university. Adequate measures were taken to minimize pain or discomfort to the used animals. The experiments were carried out in accordance with the Guidelines laid down by the National Institute of Health (NIH) in the USA regarding the care and use of animals for experimental procedures.

**Cell attachment rate**
The sterile Bio-Oss® and R.T.R® materials were cut into the same size circle and they were placed into 6-well plates. The BMSCs cells (2×10^4) were suspended in DMEM medium containing 10% FBS and seeded onto the surface of different materials. Unadherent cells were washed with PBS at the corresponding time point after inoculation, they were stained with crystal violet, OD570 value was detected to quantify cell number23). The cell attachment rate was determined by the proportion of adherent cells to the total number of cells after culture for 30 min, 1 and 2 h.

**Cell proliferation rate**
The sterile Bio-Oss® and R.T.R® materials were cut into the same size circle and they were placed into 6-well plates. The cells (2×10^4) were suspended in DMEM medium containing 10% FBS and seeded onto the surface of different materials. They were washed with PBS for 2 times after culture for 3 and 7 days, the 0.25% trypsin (2 mL) was added and incubated at 37°C for 5 min. The complete medium was added for adequate washing and the cells were collected. The number of cells was counted and cell proliferation rate was calculated.

**Alkaline phosphatase (ALP) staining and activity determination**
The sterile Bio-Oss® and R.T.R® materials were cut into the same size circle and they were placed into 6-well plates. The cells were seeded on the surface of different materials and cultured for 10 days and then stained with ALP, the experiment was conducted according to the instructions of the kit (Beyotime, Shanghai, China). For the detection of ALP activity, the cells were harvested at the 3rd, 7th, and 10th days after inoculation and detected by the ALP activity detection kit (Beyotime).

The cells were lysed with Cell Lysis Solution (400 μL, Sigma-Aldrich, St. Louis, MO, USA) and incubated at 30°C for 4 h, and then the cells were collected and incubated for 30 min, they were detected using p-nitrophenyl disodium phosphate (p-NPP) as substrate. ALP activity in each group was calculated according to the standard. At the same time, Bio-Rad protein assay kit (Bio-Rad, Hercules, CA, USA) was used to detect the total protein content of the cells according to the manual. ALP activity was expressed by pNP (mM)/total protein (mg).

**RNA extraction and RT-PCR**
Total RNA was extracted using Trizol RNA extraction kit (Invitrogen, Carlsbad, CA, USA) according to the manual. The purity and content of RNA were detected and evaluated by NanoDrop™ 1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). cDNA was synthesized by reverse transcription using miScript II RT Kit (Qiagen) in accordance with operate specification. Experimental conditions were as follows: total RNA 0.2 μg mixed with DEPC to 12 μL, centrifuged for 35 s, then chilled on ice. Adds 2 μL miScript Reverse Transcriptase Mix, 2 μL miScript Nucleics Mix and 4 μL 5× miScript HiSpec buffer to the mixture, then centrifuged for 35 s and incubated for 60 min at 37°C. Finally, adds MMuLV reverse transcription (200 U/μL) to terminal volume 20 μL. Incubates the mixture for 60 min at 42°C, 10 min at 70°C, then chills on ice. Using ABI StepOne Plus, the amplification reaction system was: 25 μL of PCR reaction mixture containing 10 μL SYBR Green PCR Master Mix, upstream primer (25 μmol/L) 1 μL, downstream primer (25 μmol/L) 1 μL, dNTPs (10 mmol/L) 1 μL, cDNA 2 μL, adding ddH2O to 25 μL. The reaction conditions were as follows: force-denatured the temple cDNA for 4 min at 94°C, then denatured at 94°C for 15 s, primer annealing at 60°C for 10 s, extension at 72°C for 10 s, 40 cycles in total, finally extend at 72°C for

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward primer 5'-3'</th>
<th>Reverse primer 5'-3'</th>
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<tr>
<td>BMP-1</td>
<td>GGTCATCCCATTTGTATCG</td>
<td>CCTCATCTGTGCGCCTCA</td>
</tr>
<tr>
<td>Cbfa1</td>
<td>ATGCCCTCTGGATATTGAA</td>
<td>GGATTTGTGAAGACCGTTA</td>
</tr>
<tr>
<td>ALP</td>
<td>GACGGTGAACGGGAGAACA</td>
<td>GGACAGAAGCCAGTGAAGC</td>
</tr>
<tr>
<td>collagen I</td>
<td>TGACCAGCCTCGCTCACA</td>
<td>CGGGCAGGGTTCTTCTTA</td>
</tr>
<tr>
<td>osteopontin</td>
<td>GTTGGGCTTACGGAACTGA</td>
<td>TGTCCACAGGCCTGTTTC</td>
</tr>
<tr>
<td>osseomucin</td>
<td>CTCGTTCTGGCAAGCCACA</td>
<td>AAGCCTCAGCAATGTGCCGT</td>
</tr>
<tr>
<td>osteocalcin</td>
<td>ACTTGTGCTGGGTGGTCTCT</td>
<td>CAATACGCAATGGCATTAA</td>
</tr>
<tr>
<td>β-actin</td>
<td>TGAGGATGTCAAGGGTCCAG</td>
<td>GTCACCTTCAGGGCTCCAG</td>
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7 min. First, we double diluted and amplified the cDNA to improve that there was a linear relationship between cDNA template amount and Ct value. And we also try to improve the amplification efficiency of target gene is the same with the house-keeping gene. GAPDH gene was used as an endogenous control.

The relative expression of mRNA was calculated by 2^ΔΔCT method. The premier sequences are shown in Table 1.

Protein extraction and western blotting method
The cells in the logarithmic growth period were harvested and lysed with Cell Lysis Solution (Sigma-Aldrich), then they were centrifuged with 10,000 rpm at 4°C for 5 min. The extract supernatants were loaded on 10% SDS-polyacrylamide gels and electrotransferred onto the polyvinylidene fluoride membranes (PVDF; Amersham Biosciences, Piscataway, NJ, USA). The membrane containing the proteins was used for immunoblotting with required antibodies. The first antibodies were anti-ALP, anti-FAK, anti-CBFA1, anti-BMP-1, anti-collagen I, anti-osteopontin, anti-osseomucin, anti-osteocalcin (1:500; Abcam, Cambridge, UK) and anti-β-actin antibodies (1:2000; Abcam). The second antibodies were anti-rabbit and anti-mouse antibody with horseradish peroxidase. The protein bands were observed using Imagequant LAS4000 (GE Healthcare Life Sciences China, Beijing, China) and quantified as a ratio to β-actin.

Statistical analysis
The data were expressed as mean±SD. Statistical analysis was performed using one-way ANOVA or Student’s t-test with SPSS 17.0 software (SPSS, Chicago, IL, USA). p<0.05 was considered to be statistically significant.

RESULTS

Compared with Bio-Oss®, R.T.R® has stronger ability to promote BMSCs adhesion and proliferation
Comparing the adhesion ability of BMSCs on Bio-Oss® and R.T.R®, it was found that the adhesion ability of BMSCs on both of them increased with time, but the adhesion rate on R.T.R® was significantly higher than that on Bio-Oss® at all time points (p<0.05, Fig. 1A). BMSCs cells with the same number were inoculated on Bio-Oss® and R.T.R® respectively, the number of cells increased significantly at 3rd and 7th days after inoculation (5.2±0.3×10⁴ and 9.6±0.6×10⁴ in Bio-Oss®; 7.4±0.1×10⁴ and 14.7±0.7×10⁴ in R.T.R®, respectively), the proliferation rate of R.T.R® group was significantly higher than that of Bio-Oss® group (p<0.01, Fig. 1B). These results suggested that R.T.R® has a stronger role in promoting adhesion and proliferation of BMSCs than that of Bio-Oss®.

Compared with Bio-Oss®, R.T.R® can enhance the expression and activity of ALP in BMSC
BMSCs were inoculated on Bio-Oss® and R.T.R®, respectively. ALP staining results showed that ALP was highly expressed in BMSCs on 7th and 14th day, and the expression level of ALP in R.T.R® group was higher than that in Bio-Oss® group (p<0.05, Fig. 2A). The cells were harvested at the 3rd, 7th, and 10th days after inoculation and the ALP activity was detected. It was found that ALP activity increased significantly in both of them, and it was significantly higher in R.T.R® than that in Bio-Oss® (p<0.01, Fig. 2B).

Compared with Bio-Oss®, R.T.R® can enhance the expression of bone formation related transcription factors and marker genes
The BMSCs cells were harvested at the 7th day after inoculation, RT-PCR and western blotting results showed that BMP-1, CBFA1, ALP, collagen I, osteopontin, osseomucin, and osteocalcin expression levels increased significantly in R.T.R® and Bio-Oss® groups, and they were significantly higher in R.T.R® than that in Bio-Oss® (p<0.01, Fig. 3).
Fig. 2  Alkaline phosphatase staining and activity analysis.  
A: ALP staining results; B: Analysis of ALP activity in different groups of cells. **p<0.01 vs. Control

Fig. 3  Detection of bone formation related transcription factors and marker genes by RT-PCR and western blotting methods. 
A: RT-PCR results; B, C: western blotting results. BMP-1, CBFA1, ALP, Collagen I, osteopontin, osseomucin and osteocalcin expression levels increased significantly in R.T.R® and Bio-Oss® groups, and they were significantly higher in R.T.R® than that in Bio-Oss®. *p<0.05 vs. Bio-Oss®, **p<0.01 vs. Bio-Oss®, ***p<0.001 vs. Bio-Oss®
Fig. 4 Detection of bone formation related transcription factors and marker genes by RT-PCR and western blotting methods after down regulation of BMP-1. A: RT-PCR results; B, C: western blotting results. BMP-1, Cbfa1, ALP, collagen I, osteopontin, osseomucin and osteocalcin expression levels were significantly down regulated in R.T.R® than that in control group.

**p<0.01 vs. R.T.R®

Down regulating the expression of BMP-1 gene can inhibit the differentiation of BMSCs inoculated on R.T.R® to osteoblast

Lentivirus was used to construct BMP-1 gene downregulated BMSCs, they were inoculated on R.T.R® with control cells at the same time. The BMSCs cells were harvested at the 7th day after inoculation, RT-PCR and western blotting results showed that BMP-1, CBFA1, ALP, collagen I, osteopontin, osseomucin and osteocalcin expression levels were significantly down regulated in R.T.R® than that in control group (p<0.01, Fig. 4). It indicated that BMP-1 was the key regulator of R.T.R® induced BMSCs differentiation into osteoblasts.

DISCUSSION

Bio-Oss® and R.T.R® show excellent biocompatibility and bone conduction ability [7-11]. However, these traditional bone grafts have only osteoconductivity but no osteoinduction. In MSFL surgery, these materials always require longer healing times to obtain sufficient new bone formation and osseointegration than in vivo grafts. In the case of large defect sites, the use of these materials alone may lead to unpredictable or even adverse results without the cellular activity necessary to promote bone regeneration in the host [24]. Adding BMSCs into bone substitute can replace autologous graft as graft material. Studies have shown that transplanting MSCs from iliac crest into Bio-Oss® can produce new bone in a shorter time than implanting Bio-Oss® alone [25,26]. Another clinical study also confirmed that implantation of BMSCs on hydroxyapatite/β-TCP or HA/TCP enhanced bone formation in maxillary sinus region [27]. However, the osteogenic induction of allografts on BMSCs and its molecular mechanism have not been reported.

Bone formation is a complex process in which MSCs differentiate into osteogenic progenitor cells, differentiate into pre-osteoblasts and mature osteoblasts, and eventually lead to accumulation and mineralization of extracellular matrix (ECM). BMPs is a member of the transforming growth factor (TGF) superfamily, it can promote the replication of osteoblasts (OB) in the embryo and induce the differentiation of BMSCs into OB [28]. Bone morphogenetic protein-1 (BMP-1) does not belong to the TGF-β family of proteins, it is a zinc dependent metalloprotease. BMP-1, astacins, the matrix metalloproteinases (MMPs), and the ADAMs/ADAMTSs belong to astacin family. BMP-1 acts on procollagen I, II, and III, it is involved in cartilage development. BMP-1 activation of TGF-β1 by digesting latent TGF-β binding
proteins (LTBPs) is the key step to activate TGF-β1 by proteolysis pathway.25,26 Activation of TGF-β1 by BMP-1 can promote SMAD-1 phosphorylation, and p-SMAD-1 can promote the expression of transcription factor cbfa1. cbfa1 is a bone specific transcription factor in the differentiation of MSCs into osteoblasts. cbfa1 can bind to OSE2 cis-acting elements in the promoter region of osteocalcin, collagen type I, osteopontin and osteomucin to activate the expression of these genes and promote osteogenic differentiation30). We hypothesized that bone substitutes may promote the expression of transcription factor cbfa1 and induce bone formation by affecting the expression of BMP-1 in BMSCs adhered to it and by BMP/TGF-β1 signaling pathway. Osteoblast differentiation in Cbfa1 knockout mice is completely blocked and can not be osteogenic31).

In this study, we found that the ability of adhesion and proliferation of BMSCs on Bio-Oss® and R.T.R® increased with time, but they were significantly higher on R.T.R® than that of Bio-Oss® at all time points. ALP content and activity were higher in R.T.R® group than that of Bio-Oss® group. It indicated that R.T.R® may have the ability to induce BMSCs to differentiate into osteoblasts. Compared with Bio-Oss®, R.T.R® could promote the expression of bone formation related transcription factors BMP-1, cbfa1 and osteoblast marker genes ALP, collagen I, osteopontin, osseomucin and osteocalcin. The expression levels of p-SMAD-1, CBFA1, ALP, collagen I, osteopontin, osseomucin and osteocalcin were inhibited after BMPs siRNA was transfected in BMSCs and inoculated in R.T.R®. It is not clear the molecular mechanism of BMP-1 differentially expressed in BMSCs grown on Bio-Oss® and R.T.R®. It is possible that the difference of BMP-1 expression level caused by the difference of zinc ion content in the two materials may lead to the obvious difference of BMSCs differentiating into osteoblasts32).

In a word, we found that different commercial grafts have different abilities to induce BMSCs osteoblast differentiation in this study. R.T.R® could up-regulate BMP-1 expression and cbfa1 expression through BMPs/SMAD signaling pathway, and then promote the expression of ALP, collagen I, osteopontin, osseomucin and osteocalcin and promote osteoblast differentiation. It will be further validated in animal experiments.

CONFLICT OF INTEREST
None

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REFERENCES


