The construction of a novel xenograft bovine bone scaffold, (DSS) 6-liposome/CKIP-1 siRNA/calcine bone and its osteogenesis evaluation on skull defect in rats

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ABSTRACT

Background: Xenograft bone scaffolds have advantages such as mechanical strength, sufficient source and safety. Combined with siRNA properly targeting CKIP-1, a negative regulator of osteogenesis, may contribute to the repair result of calcine bone alone.

Methods: Herein, we constructed a novel xenograft bovine bone scaffold namely (DSS)6-liposome/CKIP-1 siRNA/calcine bone, the characteristics of which were investigated by confirming the effect of (DSS)6-liposome, observing the appearance and testing mechanical strength of calcine bone, and observing the combined result of CKIP-1 siRNA by FAM immuno-fluorescence. In addition, cytotoxicity by CCK-8 and LDH activity of L929 cells and MC3T3-E1 osteoblasts cultured with the scaffold were tested in vitro, primary osteoblasts proliferation, the mRNA expressions of CKIP-1, ALP, COL1-α and OCN, the protein expressions of CKIP-1, BMP-2, COL-1 and Runx2 and calcium nodules were also determined by CCK-8, RT-qPCR, western-blot and Alizarin Red staining in vitro. Then, we successively established the skull defect model for evaluating the repair result of the novel scaffold by HE staining of 2, 4, 8 and 12 weeks, immunohistochemical stainings of 2, 4, 8 and 12 weeks such as ALP, COL-1α and OCN, Micro-CT scanning of 4 and 12 weeks and the relative parameters and so on in vivo.

Results: It indicated that (DSS)6-liposome/CKIP-1 siRNA/calcine bone could successfully knock down the CKIP-1 mRNA and protein expressions, promote osteoblasts proliferation with the little cytotoxicity in vitro, increase the protein expressions of BMP-2, COL-1 and Runx2 and calcium nodules were also determined by CCK-8, RT-qPCR, western-blot and Alizarin Red staining in vitro. Then, we successively established the skull defect model for evaluating the repair result of the novel scaffold by HE staining of 2, 4, 8 and 12 weeks, immunohistochemical stainings of 2, 4, 8 and 12 weeks such as ALP, COL-1α and OCN, Micro-CT scanning of 4 and 12 weeks and the relative parameters and so on in vivo.

Conclusion: Our research indicates (DSS)6-liposome/CKIP-1 siRNA/calcine bone could repair skull defects well in rats, and it may lay the foundation of applying the novel xenograft bone scaffold in the clinical.

The Translational potential of this article: These findings provide evidence that (DSS)6-liposome/CKIP-1 siRNA/calcine bone could be used as a novel xenograft bone scaffold for osteogenesis with the good safety.

1. Introduction

As a kind of bone graft material with natural bone structure, xenograft calcine bone is made by degreasing and deproteinizing animal bone and then forging and burning at the high temperature. Its three-dimensional structure is very suitable for the growth of new bone. After the high temperature, its antigenicity can be completely eliminated, and immune rejection will not occur. It is rich in sources and the tissue, cell, blood compatibility and biological safety are also good. However, when it was implanted into organisms alone, it was difficult to become an ideal bone biological scaffold due to its limited surface area and lack of active factors [1,2]. The composite material can complement each other in terms of their properties. The preparation and application of composite material are currently active fields in the study of tissue engineering biomaterials, so calcined bone should be surface-modified in order to play a better role in osteogenesis [3,4].

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2.2. Loading of (DSS)6-liposome/CKIP-1 siRNA complexes

The (DSS)6-liposome was prepared according to the reference [11]. The CKIP-1siRNA (Sense: 5'-GGACUUGGUAAGAAGGAAAdTdT-3'; Anti-sense: 5'-UUCUUCUAGCAGAGCGAATdTdT-3'; according to the references [13], Shanghai GenePharma.) was incubated with the liposome at 1 μg: 1 ml for 20 min at room temperature, and the screened 1g calcined bone particles were added and combined overnight at room temperature. Then, 10% (w: w) sucrose was added and placed at 4 °C for 1 h, − 8 °C for 5 h, and then freeze-dried for 24 h. The compound efficiency could be detected by fluorescently labeled siRNA (FAM-siRNA) and quantitatively analyzed by Image-Pro Plus 6.0.

2.3. Cytotoxicity testing

Scaffolds were weighed and immersed in the α-MEM solution at 0.2 g/ml in a constant temperature incubator for 48 h to obtain the extraction solution of scaffolds. L929 cells (mouse fibroblasts) or MC3T3-E1 osteoblasts were replaced with the extraction liquid of scaffolds. After the seventh day, 10 μl CCK-8 dye was added to the update culture medium for 4 h, detected at the wavelength of 450 nm. And the relative cells growth rate (RGR) was calculated to evaluate the cytotoxicity. After 72 h, the LDH activity was detected by the LDH kit and calculating the LDH cytotoxicity.

2.4. Real-time quantitative polymerase chain reaction

Total RNA was isolated from the cells using the TRizol reagent. Then it was reverse-transcribed to the complementary DNA by RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific Fermentas, Burlington, Canada). Real-time quantitative polymerase chain reaction (RT-qPCR) was performed as described previously [17]. Expression levels of CKIP-1, ALP, COL-1α and OCN (primers: referring to Refs. [10,13]) were corrected by normalization to the expression level of GAPDH, and relative expression levels were calculated with the 2 ΔΔCt rule [18].

2.5. Western-blot

In a short word, protein samples from cells were separated by SDS-polyacrylamide gels, then transferred to PVDF membranes (0.45 μm) for the semiquantitative analysis. Membranes were blocked with 1% BSA-T-TBS solution, then incubated with the primary antibodies (CKIP-1 antibody #sc-376060, BMP-2 antibody #sc-6895, COL-1 antibody #sc-59772 and Runx2 antibody #sc-390351, Santa Cruz Biotechnology, Inc., USA.), followed by incubation with the secondary antibodies conjugated with horseradish peroxidase. The antibody labelling was detected by ECL kit (Pierce) according to the manufacturer’s instructions, and the images were put down by Gel analysis system (Tanon 5200 ECL Detection System, Tanon Science & Technology Co.).

2.6. Primary osteoblasts proliferation and differentiation

The primary rat osteoblasts were cultured according to our previous study [19]. Then, the primary cultured rat osteoblasts were seeded on the surface of the scaffolds (the thickness of 1 mm) at a certain density, 72 h later, cells proliferation was detected by CCK-8, the mRNA expressions of CKIP-1, ALP, COL-1α and OCN were detected by RT-qPCR, and the protein expressions of CKIP-1, BMP-2, COL-1 and Runx2 were detected by western-blot. And after 7 days, the primary cultured rat osteoblasts seeded on the surface of the scaffolds were stained by Alizarin Red and quantitatively analyzed by Image-Pro Plus 6.0.

2.7. Experimental animals

Experimental male CD-1 nude mice and male SD rats approved by the Animal Ethics Committee of the Fourth Medical Center of the PLA General Hospital. The environment was free of pathogens, the ambient temperature was 22 °C, 12 h light/12 h dark cycle was guaranteed every day, the indoor humidity was 50–55%, and adaptive feeding was carried out for one week before the experiment. NIH guidelines (or for non-U.S. residents similar national regulations) for the care and use of laboratory animals (NIH Publication #85-23 Rev. 1985) have been observed.

2.8. Bone induction in nude mice

The scaffolds were prepared into the same standard-size according to the same procedure, then implanted into the triceps muscle bags of bilateral hind limbs of nude mice, and the fascia layer and skin were sutured. The mice were randomly divided into A-D groups: calcine bone,
CKIP-1 siRNA/calcine bone, liposome/CKIP-1 siRNA/calcine bone, and (DSS)6-liposome/CKIP-1 siRNA/calcine bone. Hematoxylin-eosin (HE) staining was performed on scaffolds and the surrounding muscle tissues of postoperative 4 weeks to evaluate the osteoinductive activity of the scaffold.

2.9. Surgical procedures

Adult male SD rats weighing about 300 g were selected. The rats were anesthetized by intraperitoneal injection of 3% pentobarbital sodium, and were placed in the prone position. An incision of about 2 cm was made at the midline of the parietal skull to separate the periosteum, and a 5 mm diameter defect was made at the left and right sagittal suture with a bone trephine (Supplementary Fig 1 and 2). The scaffold was implanted into the defect and the periosteum and skin were sutured. Postoperative intramuscular injection of penicillin 80,000 units, continuous injection for 3 days, routine feeding. The operation was strictly aseptic and the defect was completed by the same group of researchers. The model rats were randomly divided into A-D groups: calcine bone, CKIP-1 siRNA/calcine bone, liposome/CKIP-1 siRNA/calcine bone, and (DSS)6-
liposome/CKIP-1 siRNA/calcine bone.

2.10. Histology analysis

**HE staining**

At the 2, 4, 8, 12 weeks after operation, the implant scaffolds and nearby bones were removed and immersed in 10% neutral buffered formalin fixative for fixation. After fixation, dehydration and decalcification, the cut surface was placed at the bottom for paraffin embedding, and sliced at a certain thickness, and then stained with HE for observation. The histological scoring criteria proposed by Nilsson were used [20]. Semi-quantitative analysis was performed by two experimenters who were unclear about the experimental group.

**Immunohistochemical staining**

At the 2, 4, 8, 12 weeks after operation, the implant scaffolds and nearby bones were removed and immersed in 10% neutral buffered formalin fixative for fixation. After fixation, dehydration and decalcification, the cut surface was placed at the bottom for paraffin embedding, and sliced at a certain thickness, and then blocked and incubated with the primary antibodies of anti-ALP, anti-COL1α and anti-OCN (1:100, Santa Cruz Biotechnology, Santa Cruz, CA, USA) overnight at 4 °C. After incubation with the secondary antibodies (Santa Cruz) for 1 h at room temperature, the sections were washed and then visualized using diaminobenzidine. Staining quantity and intensity of positive cells were scored under a microscope in 3 randomly fields per slice. The scoring standard was referred to the reference [21]. Semi-quantitative analysis was performed by two experimenters who were unclear about the experimental group.

2.11. Micro-CT observation

After the fourth and eighth weeks, the skull was taken out from the rat. Micro-CT (Inveon MM CT) was used to scan and observe. Inveon Acquisition Workplace was selected as the scanning and analysis software.

2.12. Statistical analysis

GraphPad Prism 7.00 software were used for statistics and plotted. The measurement data were expressed as mean ± standard deviation. The statistical difference was tested by unpaired t-test or one-way ANOVA. *P* < 0.05 indicated that there was a statistical difference.

3. Results

3.1. Construction and characterization of (DSS)6-liposome/CKIP-1 siRNA/calcine bone

Targeting molecule (DSS)6 (sequence: DSSDSSDSSDSSDSSDSC5H8NO3S) was synthesised by Sangon Biological Engineering (Shanghai) Co., LTD. The quality control test report showed that it had high purity and its sequence could be coupled with liposomes (Supplementary Fig 3 and 4). FAM immunofluorescence modification was used to verify the transfection effect of (DSS)6-liposome/CKIP-1 siRNA, the transfection efficiency of (DSS)6-liposome was the highest, followed by the liposome alone and the siRNA alone in turn, as expected (Fig. 1A). The calcine bone was shown as Fig. 1B. The porosity of calcine bone was 84.9% ± 0.6%, pore size distribution was between 200–850 μm detected by SEM. The nitrogen content of the sample detected by metal sodium solution method was none, indicating no protein content. The main components of calcine bone were calcium phosphate and HA, detected by XRD (Fig. 1C) and IR (Fig. 1D). The bone density at both ends of the spine was 0.81–0.97 g/cm³, and the compressive strength was 2.6–2.9 Mpa. The force and displacement curves were shown in Fig. 1E. The bone density in the middle part of the spine was 0.59–0.64 g/cm³.
Figure 3. Osteoblasts proliferation and differentiation. A. The CKIP-1 mRNA expression, n = 3; B. The CKIP-1 protein expression, n = 4; C. The osteoblasts proliferation, n = 5; D. The mRNA expressions of ALP, COL1-α and OCN, n = 3; E. The protein expressions of BMP-2, COL-1 and Runx2, n = 4. F. Alizarin Red staining, n = 3. *P<0.05.
Figure 4. Pathological evaluation of defect repair. A. HE staining of 2, 4, 8 and 12 weeks; B. Histological scorings of 2, 4, 8 and 12 weeks, n = 2. *P<0.05. 200 μm, scale bar. A-D groups: calcine bone, CKIP-1 siRNA/calcine bone, liposome/CKIP-1 siRNA/calcine bone, and (DSS)6-liposome/CKIP-1 siRNA/calcine bone.

Figure 5. Immunohistochemistry analyze of osteoblast-related proteins. A. ALP staining of 2, 4, 8 and 12 weeks, and the histological scoring of 2, 4, 8 and 12 weeks, n = 3; B. COL1-α staining of 2, 4, 8 and 12 weeks, and the histological scoring of 2, 4, 8 and 12 weeks, n = 3; C. OCN staining of 2, 4, 8 and 12 weeks, n = 3. *P<0.05. 100 μm, scale bar. A-D groups: calcine bone, CKIP-1 siRNA/calcine bone, liposome/CKIP-1 siRNA/calcine bone, and (DSS)6-liposome/CKIP-1 siRNA/calcine bone.
the compressive strength was 1.2–1.5 Mpa, and the force and displacement curves were shown in Fig. 1F. The compressive strength of bone powder in different particle sizes was shown as Fig. 1G. And the compressive strength of 0.10.9 mm, 0.10.2 mm and 0.20.3 mm was better than that of other groups. The compound effect of calcined bone with CKIP-1 siRNA was shown as Fig. 1H, (DSS)6-liposome/CKIP-1 siRNA was better than liposome/CKIP-1 siRNA, and the latter was also better than CKIP-1 siRNA alone.

3.2. Cells activity and toxicity

The cell morphology of L929 cells was spindle or polygon, with a full cell body and the large and clear, round or oval nuclei, and each cell contained 1 or 2 nucleoli. During the growth process, the cells were attached to adjacent cells and grow in flakes (Fig. 2A). The RGR by CCK-8 test of L929 cells cultured with calcine bone alone was higher than that of (DSS)6-liposome/CKIP-1 siRNA/calcine bone, however, the difference was not significant (Fig. 2B). Similarly, the relative LDH cytotoxicity of L929 cells cultured with calcine bone alone was lower than that of (DSS)6-liposome/CKIP-1 siRNA/calcine bone, and the difference was also not significant (Fig. 2C). In addition, MC3T3-E1 osteoblasts with the typical morphology (fusiform, triangular, or polygon with 1 or 2 nucleoli in each cell) (Fig. 2D) were also cultured to test the RGR and the relative LDH cytotoxicity. Consistent with the results of L929 cells, there were also no significant differences between calcine bone alone and (DSS)6-liposome/CKIP-1 siRNA/calcine bone (Fig. 2E and F).

3.3. Osteoblasts proliferation and differentiation

In primary rat osteoblasts, both the CKIP-1 mRNA (Fig. 3A) and protein (Fig. 3B) expressions of (DSS)6-liposome/CKIP-1 siRNA/calcine bone were obviously reduced than those of (DSS)6-liposome/NC siRNA/calcine bone or calcine bone alone, and the osteoblasts proliferation of (DSS)6-liposome/CKIP-1 siRNA/calcine bone was significantly promoted than that of (DSS)6-liposome/NC siRNA/calcine bone or calcine bone alone (Fig. 3C). Further, we also compared the mRNA expressions of ALP, COL1-α and OCN, which represented the osteogenesis differentiation, the mRNA expressions of ALP, COL1-α and OCN in the group of (DSS)6-liposome/CKIP-1 siRNA/calcine bone were all increased than those of (DSS)6-liposome/NC siRNA/calcine bone or calcine bone alone, while the OCN was not significant (Fig. 3D). In line with the above results, the protein expressions of BMP-2, COL-1 and Runx2 in the group of (DSS)6-liposome/CKIP-1 siRNA/calcine bone were also all increased than those of (DSS)6-liposome/NC siRNA/calcine bone or calcine bone alone (Fig. 3E). In addition, to ulteriorly confirm the osteoblast differentiation, Alizarin Red staining was performed and the result showed that (DSS)6-liposome/CKIP-1 siRNA/calcine bone had the better osteoblast differentiation than others (Fig. 3F).

3.4. Bone induction in nude mice

The results showed that there was no significant difference in osteogenic induction among the groups after 8 weeks (Supplementary Fig 5), indicating (DSS)6-liposome/CKIP-1 siRNA/calcine bone would not cause the ectopic ossification.

3.5. Pathological evaluation of defect repair

HE staining results showed that the repair of defects in each group at 2 weeks and 4 weeks was mainly about the coverage and filling of fibrous...
than other groups (Fig. 6C). At 12 weeks, the BV/TV of Group D was (Fig. 6B) while the trabecular thickness of Group A was obviously smaller

bone and percentage of bone formation 4 and 12 weeks after surgery

3.7. Micro-CT observation of defect repair

Micro-CT scanning was performed to compare the amount of new bone and percentage of bone formation 4 and 12 weeks after surgery (Fig. 6A). At 4 weeks, the BV/TV of all groups was nearly the same (Fig. 6B) while the trabecular thickness of Group A was obviously smaller than other groups (Fig. 6C). At 12 weeks, the BV/TV of Group D was significantly bigger than that of Group C, which of Group C was bigger than Group B, and Group B was also bigger than Group A (Fig. 6B). In the meantime, the trabecular thickness of Group D was obviously bigger than other groups (Fig. 6C).

3.8. Pathological evaluation of major tissues and organs

After 12 weeks, the rats were sacrificed and the main organs were taken: heart, liver, spleen, lung, kidney, brain and muscle. HE stainings were performed to observe the abnormal tissue morphology and evaluate the safety of the materials. The results showed that there were no obvious abnormalities in the main organs of the rats in each group (Supplementary Fig 6).

4. Discussion

Small interference RNA/calcined bone composite scaffold, a new type of gene intervention bone replacement holder, would inevitably face the safety test of siRNA in vivo experiments. After siRNA was introduced into the body, it was difficult to ensure that siRNA was pooled in the target tissue to play a therapeutic role locally. Due to the extensive presence of RNA degrading enzymes in body tissue to play a therapeutic role locally. Due to the extensive presence of RNA degrading enzymes in body fluids a lot, instead, targeting the adja-
cent osteogenesis distribution area, which could be captured and utilized by the local osteogenesis interface and osteoblasts or BMSCs. After CKIP-1 siRNA entering cells, the function of local osteoblasts and BMSCs could be regulated to improve the local osteogenic activity and obtain better quality and strength of bone repair [6,9]. In agree with this point, the effect of CKIP-1 siRNA on the primary rat osteoblasts had also been investigated in vitro, and it indicated that (DSS)6-liposome/CKIP-1 siRNA/calcine bone could promote osteoblasts proliferation and increase the mRNA expressions of ALP, COL1-α and OCN and the protein expressions of BMP-2, COL-1 and Runx2, the actions mechanism of which was understood and controlled, and the last barrier to the application of scaffold in the clinical.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jot.2021.02.001.

Declaration of competing interest

The authors declare no conflict of interest.