Title

Osteogenesis of cryopreserved osteogenic matrix cell sheets

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Introduction

Bone marrow-derived mesenchymal stem cells (BMSCs) have been widely used for bone tissue regeneration. With rapid advancements in tissue engineering (TE), skeletal diseases including arthritis, bone tumors and osteonecrosis, have been successfully treated using tissue engineered bone (TEB) combined with BMSCs.[1-3] At present, preparation of TEB with BMSCs requires several weeks for cultivation to permit BMSC expansion and colonization into scaffolds, such as hydroxyapatite (HA).[4-9] In many cases, prolonged TEB preparation times likely affect surgery planning owing to timing difficulties. Even planned elective surgeries are often postponed because of patient health, in some cases making pre-prepared TEB unusable. Controlled timing for creation of TEB is therefore of critical importance for its large-scale clinical use.

As cryopreservation technology has advanced,[10,11] cryopreserved cells are expected to become a promising cell source in regenerative medicine. Successful cryopreservation of BMSCs prior to implantation is well documented both preclinically[12-14] and in the clinic[15,16]. In fact, the cryopreservation and thawing process is reported to result in less damage on both proliferation and differentiation of BMSCs compared with non-cryopreserved BMCSs.[10,15,16] Despite these reports, it is still uncommon to create TEB using cryopreserved BMSCs. One reason for this is that BMSCs are unable to differentiate spontaneously into osteoblasts when cultured in basic culture media *in vitro*;[16,17] therefore, cryopreserved BMSCs require additional factors during cultivation after thawing.[16]

We previously developed a novel cell transplantation technique for bone formation using BMSCs in absence of a scaffold.[18,19] BMSCs were cultured in medium containing dexamethasone (Dex) and ascorbic acid phosphate (AscP), and were lifted as single cell sheets (designated osteogenic matrix cell sheets: OMCSs) with high osteogenic potential. OMCSs combined with HA resulted in extensive bone formation *in vivo*,[18,19] supporting the use of this method for hard tissue reconstruction. This study aimed to determine whether cryopreserved OMCSs combined with HA constructs, maintain sufficient osteogenic potential after *in vivo* transplantation, which could significantly impact on reduced TEB preparation times in the clinic.

Materials and methods

Bone marrow cell preparation

The method of bone marrow cell preparation has been reported previously.[4,9,18,19] Briefly, bone marrow cells were obtained from the femur shafts of 7 week-old male Fischer 344 rats. Both ends of the femur were cut away from the epiphysis and the bone marrow was flushed out using 10 ml of standard culture medium expelled from a syringe through a 21-gauge needle. Standard culture medium consisted of minimal essential medium (MEM; Nacalai Tesque, Kyoto, Japan) containing 15% fetal bovine serum (FBS, JRH Bioscience Inc., Lenexa, KS, USA) and antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin, Nacalai Tesque).

Harvested cells were transferred into two T-75 flasks (BD Falcon; BD Biosciences, Franklin Lakes, NJ, USA) containing 15 ml of standard culture medium. Cell cultures were maintained in a 95% humidified atmosphere with 5% CO_2 at 37°C. After reaching confluence, cultured cells were released from the culture substratum using trypsin/EDTA (Gibco, Invitrogen, Carlsbad, CA, USA).

Cell sheet preparation and cryopreservation

The cell sheet preparation method has been previously reported.[18,19] Briefly, BMSCs released using trypsin/EDTA, were seeded at 1×10^4 cells/cm^2 in 10 cm dishes (100×20 mm; BD Falcon) for subculture in standard culture medium containing 10 nM Dex (Sigma, St. Louis, MO, USA) and 82 μg/ml AscP (L-ascorbic acid phosphate magnesium salt n-hydrate, Wako Pure Chemical Industries, Kyoto, Japan) until confluent OMCSs were formed (approximately day 14). Cells were rinsed twice with phosphate-buffered saline (PBS; Gibco) and the OMCSs were lifted using a scraper. OMCSs were divided into 3 groups according to storage period of cryopreservation; fresh group (fresh), 4-week cryopreservation (4-week) and 12-week cryopreservation (12-week) groups. For cryopreservation, OMCSs were picked up using tweezers, then placed into freezing tubes (1 OMCS/tube, 2 ml cryogenic vial; BD Falcon) containing 500 μl of cell freeze medium (Cell Banker 1®, Juji Field, Inc., Tokyo, Japan). The freeze medium was sourced commercially, therefore the cryoprotective agent (CPA) concentration and content are not disclosed, except for inclusion of dimethyl sulfoxide (DMSO).

Tubes were then transferred to a freezer (-80°C) without programmed freezing steps and stored at -80°C for 4- or 12-weeks. Cryopreserved OMCSs were rapidly thawed at room temperature and rinsed twice with PBS prior to subsequent experimentation. Temperature changes of cell freeze medium in freezing tube were measured with a thermometer sensor (CENTER 370 RTD thermometer; Center

Technology Group, Taiwan). The sensor was inserted to cryopreservation medium through a hole made on the cryovial cap, then the temperature change was recorded during the cryopreserved and thawing process.

Histologic Examination (OMCS)

Following additional culture for 24 hours in 10 cm dishes containing 10 ml of standard culture medium, three OMCSs were fixed in 10% formalin neutral buffered solution (Wako Pure Chemical Industries, Ltd., Osaka, Japan) for 2 days and embedded in paraffin and cut parallel at the middle prior to staining with hematoxylin and eosin (H&E) for light microscopic observation.

Cell Viability Assay

To investigate the viability of the OMCSs before and after freezing, a method based on tetrazolium reductase activity (Cell Counting Kit-8®; WST-8, Dojindo, Kumamoto, Japan) was employed.[20] Briefly, OMCSs were cultured with 6-, 12-, 24- and 48-well plates (Falcon; n=6 per plate) were used to generate a standard. The different sizes of OMCSs were harvested with a scraper and then incubated in a 95% humidified atmosphere with 5% CO_2 at 37°C for 24 hours. The samples were placed in WST-8 solution (100 µl in 1 ml of cultured medium) in the culture wells. After 3 hours of incubation, the solution obtained from each culture well was measured by a spectrophotometer (450 nm). From the results of these standardization experiments, a linear relationship was obtained between the averaged optical density (OD) and seeded cell number per cultured medium (cell/ml) (correlation $R^2 = 0.9751$). OMCSs cultured in 6-well plate were also cryopreserved using the same protocol for 4 weeks (n=6; 4-week group) and 12 weeks (n=6; 12-week group) groups. Using this standard, the number of viable cells of OMCSs in each group was analyzed before freezing and after thawing. The number of viable cells 24 hours after thawing was calculated as the percentage of that before freezing. For all specimens, the cell viability of samples was measured at 24 hours after initiation of the thawing process. The measurement for the cell viability was the same as applied for the standardization.

Attachment and survival of cells within the OMCSs was also assessed (n=1 per group) using light microscopy and confocal laser microscopy (CLM, Zeiss LSM Axiovert 200, Carl Zeiss, Germany). Following additional culture for 24 hours in 10 cm dishes (100 × 20 mm; BD Falcon) containing 10 ml of

standard culture medium, viable cells were stained with green fluorescent Calcein AM dye and dead cells with red fluorescent Ethidium homodimer-1 dye (LIVE/DEAD® Viability/Cytotoxicity Assay Kit, Invitrogen) following the manufacturer's protocol.

Implantation of HA constructs combined with cell sheets

Porous HA ceramics (50% average void volume, 5 mm diameter by 2 mm thick, Cellyard HA scaffold, Pentax Co, Tokyo, Japan) were used. Both solid and porous components of the scaffold microstructure were interconnected. OMCSs from each group (fresh, 4- and 12-week groups) were combined with the HA ceramics just after scraping off or thawing to make the HA/OMCS constructs. Control HA constructs without OMCSs were also included. Each group comprised six constructs, which were implanted subcutaneously into the back of recipient rats (n=6 HA disks per rat) to assess the osteogenic potential of cryopreserved OMCSs.

Radiographic and histological analysis of harvested constructs

Four weeks after implantation, all samples were harvested to evaluate osteogenesis. Two disks from each group were fixed in 10% formalin neutral buffered solution (Wako Pure Chemical Industries, Ltd., Osaka, Japan) for 2 days and analyzed radiographically. After X-ray images were obtained, the samples were decalcified with K-CX solution (Falma Co., Tokyo, Japan), embedded in paraffin and cut parallel at the middle prior to staining with H&E. The remaining disks were stored at -80°C until alkaline phosphatase (ALP) activity and osteocalcin (OC) content were measured.

Biochemical analysis

Assays for ALP activity and OC content were carried out according to a method described previously.[9] Briefly, each HA disk was crushed, homogenized in 1 ml of 0.2% Nonidet P-40/50 mM Tris-HCl buffer (pH 7.6) containing 1 mM $MgCl_2$, and centrifuged at 13,000 rpm for 10 minutes at 4°C. Supernatants were assayed for ALP activity using *p*-nitrophenylphosphate (pNPP) substrate. An aliquot (10 μl) of the supernatant was added to 1 ml of 50 mM pNPP containing 1 mM $MgCl_2$ and the mixture was incubated for 30 minutes at 37°C. Then 2 ml of 0.2 N NaOH was added to stop the enzymatic reaction and the absorption at 410 nm was measured by spectrophotometry. ALP activity was represented as

p-nitrophenol release (μmol) per HA disk after 30 minutes of incubation at 37°C.

OC in the HA disk was extracted from the sediment of the Nonidet P-40 extract with 3ml of 20% formic acid for approximately 2 weeks at 4°C. An aliquot of the formic acid extract was applied to a prepacked Sephadex G-25 column (NAP-25 column; Amersham Pharmacia Biotech AB, Uppsala, Sweden) and eluted with 10% formic acid. The eluted protein fractions were collected, lyophilized and subjected to enzyme-linked immunoassay (Rat osteocalcin ELISA kit DS; DS Pharma Biomaterial Co., Ltd., Osaka, Japan) for rat OC. All experiments in the present study were performed in duplicate.

Statistical analysis

ALP activity and OC content values were represented as mean ± standard deviation (SD) for each HA disk. Statistically significant differences between experimental groups were evaluated using the Kruskal-Wallis test followed by a *post hoc* test with Scheffe's procedure. Values of $p<0.05$ were considered statistically significant.

Results

Temperature changes of cryopreservation/thawing process

Figure 1 shows the temperature changes of freezing OMCS with cryopresevation medium in freezing tube. It took approximately 5 minutes for cryopresevation from 0°C to -15°C (Figure 1A) and approximately 2.5 minutes for thawing (Figure 1B).

Macroscopic appearances and Histologic Examination (OMCS)

Macroscopic appearance of the three OMCS groups after thawing (fresh, 4-week, 12-week groups). The 4- and 12-week cryopreserved OMCSs appeared larger in size compared with the fresh OMCSs (Figure 2A, B, C). Histology of OMCS revealed that the fresh OMCS is folded over and over results in thick structure of the cell layer. (Figure 2a). On the other hand, the structure of cell layer becomes thin with the increased cryopreservation period and the sheet structure was nearly monolayer in the 12-week cryopreserved OMCS groups (Figure 2b and c).

Cell Viability Assay

Cell viabilities of OMCSs cryopreserved for 4- and 12 weeks compared to fresh group were 61.0 ± 9.8 % and 50.5 ± 7.8 %, respectively. Figure 3 shows representative images of the three OMCS groups after LIVE/DEAD staining (fresh, 4-week, 12-week groups). Although a small amount of red staining was observed, representing dead cells, fresh OMCSs were predominantly filled with clusters of green fluorescent viable cells (Figure 3A: fresh group). Numbers of red stained cells increased after the thawing in both the 4- and 12-week cryopreserved OMCS groups (Figure 3B: 4-week group and 3C: 12-week group). A very similar cell staining pattern was observed for OMCSs in all three groups (Figure 3A, B, and C).

Radiographic and histological analysis of harvested constructs

Figure 4 shows X-ray photography of the harvested HA disks. Obvious calcification surrounding the disks was observed in all experimental groups (Figure 4A, B, C), except the control group (Figure 4D). The OMCS transplanted in conjunction with the HA disks had already turned to a hard tissue and no longer existed in the form of a soft sheet when the HA disks were harvested.

Figure 5 shows representative histological sections of the harvested constructs stained with H&E. Low and high magnification images show abundant bone formation in the pores and on the surface of the HA disks in all experimental groups, except the control group. Osteocytes and osteoblasts were observed in the bone tissue at high magnification. A similar bone formation pattern was observed for all three experimental groups (Figure 5A/a, B/b, C/c). No bone tissue was observed in the pores or at the surface of the control group (Figure 5D/d).

Biochemical analysis

Figure 6 shows ALP activity and OC content for each group (Figure 6A, B). ALP activity of the three groups (fresh, 4-week, 12-week groups) was significantly higher than the control group, indicating a high level of osteoblast activity 4 weeks after transplantation. There was no significant difference in ALP activity between the fresh, 4-week and 12-week groups. OC content of the three groups was significantly higher compared with the control group. However, OC content was decreased with increased cryopreservation period ($P<0.05$ for fresh vs 12-week group; NS for fresh vs 4-week group and 4-week vs 12-week group).

196

197 **Discussion**

198 This study demonstrates that the structural integrity of OMCSs is maintained during
199 cryopreservation and thawing and the TEB produced using cryopreserved OMCS shows extensive
200 osteogenic ability. Cryopreserved/thawed OMCSs were capable of producing a mineralized matrix on HA
201 scaffolds. Cells likely migrate from the OMCSs, which are initially wrapped around the HA scaffolds,
202 into the scaffold pores where they begin to form bone. It is clear that the bone formation in the HA pores
203 is derived from the OMCS due to the fact that no bone formation was observed in control group. Our
204 previous studies using Sry gene also demonstrated the neonatal bone formation were derived from
205 OMCSs.[19] Therefore, the simply cryopreserved OMCSs could be applied in hard tissue reconstruction to
206 minimize cell preparation requirements prior to the time of use.

207 The application of TEB to hard tissue reconstruction currently requires a stepwise process,
208 including cell isolation and culture expansion, scaffold preparation, cell seeding onto the scaffold to form
209 a cell/scaffold construct and implantation of the construct into the bone defect.[4-9] Many hard tissue
210 reconstruction elective surgeries require precise timing for the cell preparation to coincide with the
211 operation; therefore, shortening the cell preparation time is currently an unmet clinical need. Although
212 prior BMSC cryopreservation is one technique to shorten the process, further cultivation time for the
213 thawed BMSCs to make the cell/scaffold construct is still required before implantation.[12,16] Consequently,
214 cryopreserved TEB is a favorable solution,[13,21-24] to enable rapid preparation and immediate application of
215 TEB in surgery. Previous studies have reported an approximate 50% deterioration in cell viability after
216 cryopreservation of TEB.[5,21] Cryopreservation using traditional methods may result in loss of tissue
217 function and viability caused by several factors, of which ice crystal formation in the cells or constructs is
218 the most significant. Additionally, a secreted extracellular matrix may prevent the CPA from diffusing
219 uniformly into the cells, resulting in exposure of surface cells to toxic CPA levels.[21] Adhesion between
220 cells and constructs is also challenged during cryopreservation because of mechanical stress, extracellular
221 ice formation and differences in thermal contraction between the cell and substrate.[23] Vitrification is a
222 method reported to prevent the formation of ice crystals during cooling and warming[4,24] and has
223 superiority over other conventional freezing methods.[22] However, TEB has a 3-dimentional (3D) structure
224 with a larger volume; therefore, complete immersion into freeze media might compromise success of this

process, both logistically and biologically, leading to deterioration in cell viability.[25,26] The preferred method for cryopreserving TEB is still debatable. Different outcomes can be expected depending on cell type, biomaterial type, cell/biomaterial interaction and size of the construct, to name a few.

Based on this, novel techniques to shorten the cell preparation period or preparation technique of TEB just before its application are required, and we believe cryopreserved OMCSs are a solution. Protection by the freezing media is of critical importance to prevent cell damage during the cryopreservation/thawing process. Unlike TEB, in which the structure is 3D, OMCSs are suitable for cryopreservation because they comprise a layered cell sheet structure, so freezing media elicits less damage and enables maintenance of osteoblastic ability. This study shows cryopreserved OMCSs maintained osteogenic ability, and therefore pose a solution to shorten the time needed to make osteogenic tissue engineered HA/BMSCs constructs. Some studies have reported the osteogenic ability of cryopreserved TEBs;[13,21-23] therefore, an experimental study to compare the osteogenic ability between cryopreserved TEB and cryopreserved OMCS is necessary in the future.

Previous studies have reported that the cryopreservation/thawing process for human MSCs has no effect on their growth or osteogenic differentiation.[10,15,16] Whilst other studies have demonstrated the ability of cryopreserved/thawed MSCs to differentiated into other cell types, including cardiac myoblasts,[27] vascular endothelial cells,[28] hepatocytes[29] and neural cells.[30] It has been reported that DMSO is not only cytotoxic, but also induces differentiation of MSCs into neuron-like cells[31] or cardiac myocytes[32] when added to culture medium after thawing. Therefore, DMSO in freeze medium may decrease the osteoblastic differentiation potential of BMSCs. The present study shows that fresh and 4-week OMCS groups have a similar ALP activity and OC content, indicating that cryopreservation of OMCSs does not affect their ability to undergo osteoblast differentiation after transplantation. The majority of cells in the OMCSs have already undergone osteoblast differentiation during the sheet creation process, thus the negative influence of DMSO will be minimal from this respect. At this point, further studies to assess differentiation of MSC cell sheets into other cell types are needed to optimize the cryopreservation protocol.

Our cryopreservation protocol, consisting of rapidly freezing at -80°C, is very simple compared with previous methods[13,21-23] and could maintain OMCS osteogenic ability for at least 4 weeks. Therefore, we believe OMCSs cryopreserved using our simple protocol are an option for hard tissue reconstruction,

and their advantages include reduced processing steps and cryopreservation costs. However, cryopreservation at -80°C may cause chemical reactions as thermal energy and unfrozen water.[33] In the present study, OC content tended to decrease with increased cryopreservation period until 12 weeks, indicating that osteogenic ability of cryopreserved OMCSs deteriorated during cryopreservation. Kito *et al.*[20] demonstrated that the corneal epithelial cell sheets stored at -196°C showed a higher cell survival rate than those at -80°C. Therefore, an alternative method, such as liquid nitrogen cryopreservation at -196°C, may be preferable to prevent this deterioration[15,21,23]. Using this method, molecular motion is significantly reduced and thermally driven reactions are negligible over time. In cases where the patients threaten delayed or nonunion of the fracture due to deteriorated osteogenic ability, surgeon could harvest their MSCs at the initial operation for fracture treatment and create the OMCSs followed by cryopreservation for future use. As such clinical cases, cryopreservation with liquid nitrogen is considered to be desirable because the preservation period would be more than 6 months. Future studies will elucidate the effect of short- and long-term preservation with liquid nitrogen.

The macroscopic findings show that OMCS size seems to become a little bit larger after cryopreserved/thawed process. We consider that the folded sheet structure which revealed by histology was spread to monolayer in 12-week cryopreservation OMCS, close to the size of the OMCS before scraping. In 12-week cryopreserved OMCS, osteogenic capacity was decreased after the implantation. The following could explain the reasons; the folded structure of OMCS may prevent permeation of the cryopreservation medium into the folded sheet structure and cause the freezing damage followed by the decreased number of survival cells. Decreased cell number in the OMCS resulted in reduction of osteogenic ability in the 12-week cryopreserved OMCS after cryopreserved/thawed process. The culture dishes with a thermoresponsive polymer graft[33], poly (N-isopropylacrylamide) or collagen membranes[20] may avoid the formation of the folds and result in increased number of survival cells in cryopreserved OMCSs. Previous study reported that it was possible to cryopreserve a corneal monolayer sheet (8 mm in thickness) created with a collagen membrane using 1.5 ml of cryopreservation medium[20], while we used 0.5ml cryopreservation medium in the present study. Therefore, in the cryopreservation of the OMCS, it may be possible to further increase the viability by using those grafted culture dishes or enough amount of cryopreservation medium. Concerning this point, further study will be required.

The technique for cryopreservation of cell sheets has already been reported,[20] however, to our

knowledge, this is the first report of cryopreserved cell sheets forming bone tissue after implantation. In the experiment of cryopreservation of a corneal sheet,[20] the form of the cell sheet was maintained immediately after thawing, but the form was no longer maintained after 24 hours of culture after the thawing. In contrast, our cell sheet maintained its form after thawing. Even with the simple method used in the present study, the extracellular matrix formed by osteoblasts maintained sufficient durability to the cryopreservation and we can easily produce TEB cell/biomaterial constructs immediately after thawing, which supports the cryopreservation of the OMCS to be useful. Because few hospitals have facilities for cell processing, future TE approaches for hard tissue reconstruction will require more simplistic treatment option. Transportation of BMSCs and OMCSs is of critical importance for large-scale production and clinical use. Although we need to conduct similar experiments using human cells and long-term preservation at -196°C in future, cryopreserved OMCSs fit the clinical need of providing availability of TEB in all hospitals.

Conclusion

The present study clearly indicates that the structural integrity of OMCSs was maintained during cryopreservation and thawing, and that cells were capable of producing a mineralized matrix on HA scaffolds, resulting in bone formation. This simple cryopreservation technique can be used for the application of OMCSs in hard tissue reconstruction.

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Conflicts of interest statement

The authors state that they have no conflicts of interest to disclose

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313 **References**

314 1. Wakitani S, Imoto K, Yamamoto T, Saito M, Murata n, Yoneda M. Human autologous culture
315 expanded bone marrow mesenchymal cell transplantation for repair of cartilage defects in
316 osteoarthritic knees. Osteoarthritis Cartilage 10 (2002) 199-206.
317 2. Ohgushi H, Kotobuki N, Funaoka H, Machida H, Hirose M, Tanaka Y, et al. Tissue engineered
318 ceramic artificial joint – ex vivo osteogenic differentiation of patient mesenchymal cells on total
319 ankle joints for treatment of osteoarthritis. Biomaterials 26 (2005) 4654-4661.
320 3. Kawate K, Yajima H, Ohgushi H, Kotobuki N, Sugimoto K, Ohmura T, et al. Tissue-engineered
321 approach for the treatment of steroid-induced osteonecrosis of the femoral head: transplantation of
322 autologous mesenchymal stem cells cultured with β-tricalcium phosphate ceramics and free
323 vascularized fibula. Artif Organs 30 (2006) 960-962.
324 4. Ohgushi H, Yoshikawa T, Nakajima H, Tamai S, Dohi Y, Okunaga K. AL2O3 doped
325 apatite–wollastonite containing glass ceramic provokes osteogenic differentiation of marrow stromal
326 stem cells. J Biomed Mater Res 44 (1999) 381-388.
327 5. Akahane M, Ohgushi H, Yoshikawa T, SempukuT, Tamai S, Tabata S, et al. Osteogenic phenotype
328 expression of allogeneic rat marrow cells in porous hydroxyapatite ceramics. J Bone Miner Res 14
329 (1999) 561-568.
330 6. Kotobuki N, Ioku K, Kawagoe D, Fujimori H, Goto S, Ohgushi H. Observation of osteogenic
331 differentiation cascade of living mesenchymal stem cells on transparent hydroxyapatite ceramics.
332 Biomaterials 26 (2005) 779-785.
333 7. Okamoto M, Dohi Y, Ohgushi H, Shimaoka H, Ikeuchi M, Matsushima A, et al. Influence of the
334 porosity of hydroxyapatite ceramics on in vitro and in vivo bone formation by cultured rat bone
335 marrow stromal cells. J Mater Sci Mater Med 17 (2006) 327-336.
336 8. Matsushima A, Kotobuki N, Tadokoro M, Kawate K, Yajima H, Takakura Y, et al. In vivo osteogenic
337 capability of human mesenchymal cells cultured on hydroxyapatite and on beta-tricalcium phosphate.
338 Artif Organs 33 (2009) 474-481.
339 9. Nakamura A, Dohi Y, Akahane M, Ohgushi H, Nakajima H, Funaoka H, et al. Osteocalcin secretion
340 as an early marker of in vitro osteogenic differentiation of rat mesenchymal stem cells. Tissue Eng
341 Part C Methods 15 (2009) 169-180.

342 10. Spurr EE, Wiggins NE, Marsden KA, Lowenthal RM, Ragg SJ. Cryopreserved human
343 haematopoietic stem cells retain engraftment potential after extended (5-14 years) cryostorage.
344 Cryobiology 44 (2002) 210-217.
345 11. Bruder SP, Jaiswal N, Haynesworth SE. Growth kinetics, self-renewal, and the osteogenic potential
346 of purified human mesenchymal stem cells during extensive subcultivation and following
347 cryopreservation. J Cell Biochem 64 (1997) 278-294.
348 12. Yoshikawa T, Nakajima H, Takakura Y, Nonomura A. Osteogenesis with cryopreserved marrow
349 mesenchymal cells. Tissue Eng 11 (2005) 152-160.
350 13. Yin H, Cui L, Liu G, Cen l, Cao Y. Vitreous cryopreservation of tissue engineered bone composed of
351 bone marrow mesenchymal stem cells and partially demineralized bone matrix. Cryobiology 59
352 (2009) 180-187.
353 14. Lippens E, Cornelissen M. Slow cooling cryopreservation of cell-microcarrier constructs. Cells Koto
354 Tissues Organs 192 (2010) 177-186.
355 15. Kotobuki N, Hirose M, Machida H, Katou Y, Takakura Y, Ohgushi H. Viability and osteogenic
356 potential of cryopreserved human bone marrow-derived mesenchymal cells. Tissue Eng 11 (2005)
357 663-673.
358 16. Liu G, Shu C, Cui L, Liu W, Cao Y. Tissue-engineered bone formation with cryopreserved human
359 bone marrow mesenchymal stem cells. Cryobiology 56 (2008) 209-215.
360 17. Yoshikawa T, Ohgushi H, Uemura T, Nakajima H, Ichijima K, Tamai S, et al. Human marrow
361 cells-derived cultured bone in porous ceramics. Biomed Mater Eng 8 (1998) 311-320.
362 18. Akahane M, Nakamura A, Ohgushi H, Shigematsu H, Dohi Y, Takakura Y. Osteogenic matrix
363 sheet-cell transplantation using osteoblastic cell sheet resulted in bone formation without scaffold at
364 an ectopic site. J Tissue Eng Regen Med 2 (2008) 196-201.
365 19. Akahane M, Ueha T, Shimizu T, Shigematsu H, Kido A, Omokawa S, et al. Cell Sheet Injection as a
366 technique of osteogenic supply. International Journal of Stem Cells 3 (2010) 138-143.
367 20. Kito K, Kagami H, Kobayashi C, Ueda M, Terasaki H. Effects of cryopreservation on histology and
368 viability of cultured corneal epithelial cell sheets in rabbit. Cornea 24 (2005) 735-741.
369 21. Kofron MD, Opsitnick NC, Attawia MA, Laurencin CT. Cryopreservation of tissue engineered
370 constructs for bone. J Orthop Res 21 (2003) 1005-1010.

22. Kuleshova LL, Gouk SS, Hutmacher DW. Vitrification as a prospect for cryopreservation of tissue-engineered constructs. Biomaterials 28 (2007) 1585-1596.

23. Liu BL, McGrath JJ. Effects of freezing on the cytoskeleton, focal adhesions and gap-junctionsin murine osteoblast cultures. Conf Proc IEEE Eng Med Biol Soc 5 (2005) 4896-4899.

24. Wen F, Magalhães R, Gouk SS, Bhakta G, Lee KH, Hutmacher DW, et al. Vitreous cryopreservation of nanofibrous tissue-engineered constructs generated using mesenchymal stromal cells. Tissue Eng Part C Methods 15 (2009) 105-114.

25. Mehl PM. Nucleation and crystal growth in a vitrification solution tested for organ cryopreservation by vitrification. Cryobiology 30 (1993) 509-518.

26. Rabin Y, Bell E. Thermal expansion measurements of cryoprotective agents. Part II: measurements of DP6 and VS55, and comparison with DMSO. Cryobiology 46 (2003) 264-270.

27. Makino S, Fukuda K, Miyoshi S, KonishiF, Kodama H, Pan J, et al. Cardiomyocytes can be generated from marrow stromal cells in vitro. J Clin Invest 103 (1999) 697-705.

28. Reyes M, Dudek A, Jahagirdar B, Koodie L, Marker PH, Verfaillie CM. Origin of endothelial progenitors in human postnatal bone marrow. J Clin Invest 109 (2002) 337-346.

29. Schwartz RE, Reyes M, Koodie L, Jiang Y, Blackstad M, Lund T, et al. Multipotent adult progenitor cells from bone marrow differentiate into functional hepatocyte-like cells. J Clin Invest 109 (2002) 1291-1302.

30. Deng W, Obrocka M, Fischer I, Prockop DJ. In vitro differentiation of human marrow stromal cells into early progenitors of neural cells by conditions that increase intracellular cyclic AMP. Biochem Biophys Res Commun 282 (2001) 148-152.

31. Chu Q, Wang Y, Fu X, Zhang S. Mechanism of in vitro differentiation of bone marrow stromal cells into neuron-like cells. J Huazhong Univ Sci Technolog Med Sci 24 (2004) 259-261.

32. Young DA, Gavrilov S, Pennington CJ, Nuttall RK, Edwards DR, Kitsis RN, et al. Expression of metalloproteinases and inhibitors in the differentiation of P19CL6 cells into cardiac myocytes. Biochem Biophys Res Commun 322 (2004) 759-765.

33. Memon IA, Sawa Y, Fukushima N, Matsumiya G, Miyagawa S, Taketani S, et al. Repair of impaired myocardium by means of implantation of engineered autologous myoblast sheets. J Thorac Cardiovasc Surg 130 (2005) 1333–1341.

401 **Figure Captions**

402 Figure 1. Temperature changes of the cryopreserving/thawing process. (A): cryopreserving phase, (B):
403 thawing phase.

404

405 Figure 2. Macroscopic and microscopic (H&E stained) appearance of OMSCs after thawing. (A) and (a):
406 fresh group, (B) and (b): 4-week group, (C) and (c): 12-week group. Micrographs show that the OMCS is
407 considerably folded over in the fresh group (a), on the other hand, the folded lesion were reduced in both
408 the 4-(b) and 12-week groups (c). Black bar indicates 50 μm.

409

410 Figure 3. Confocal laser microscopy images of OMCSs stained with fluorescent dyes, Calcein AM
411 (Green; Live) and Ethidium homodimier-1 (Red; Dead), after thawing; (A) fresh group, (B) 4-week group
412 and (C) 12-week group (A, B, C; OMCSs was stained after 24 hours culture after scraping-off or thawing).
413 White bar indicates 200 μm.

414

415 Figure 4. X-ray photographs of harvested disks; (A) fresh group, (B) 4-week group, (C) 12-week group
416 and (D) control group. Obvious calcification surrounding the disks was observed in all experimental
417 groups, except the control group.

418

419 Figure 5. H&E stained sections of harvested constructs. Lower magnification images; (A) fresh group,
420 (B) 4-week group, (C) 12-week group and (D) control group. Bone formation in the HA pores and the
421 surface of the HA disk can be observed in all experimental groups, except the control group. High
422 magnification images; (a) fresh group, (b) 4-week group, (c) 12-week group and (d) control group.
423 Similar bone formation patterns, comprising osteocytes and osteoblasts in the bone tissue, were observed
424 in the former three groups. No bone tissue was observed in the pores or at the surface of the control group.
425 Black bar indicates 200 μm.

426

427 Figure 6. ALP activity (A) and OC contents (B) in the control, fresh, 4-week and 12-week groups. No
428 significant difference was found for ALP activity between the fresh, 4- and 12-week groups. The OC
429 content of the three groups was significantly higher compared with control. OC content decreased with

increased cryopreservation period.

Figure 1.

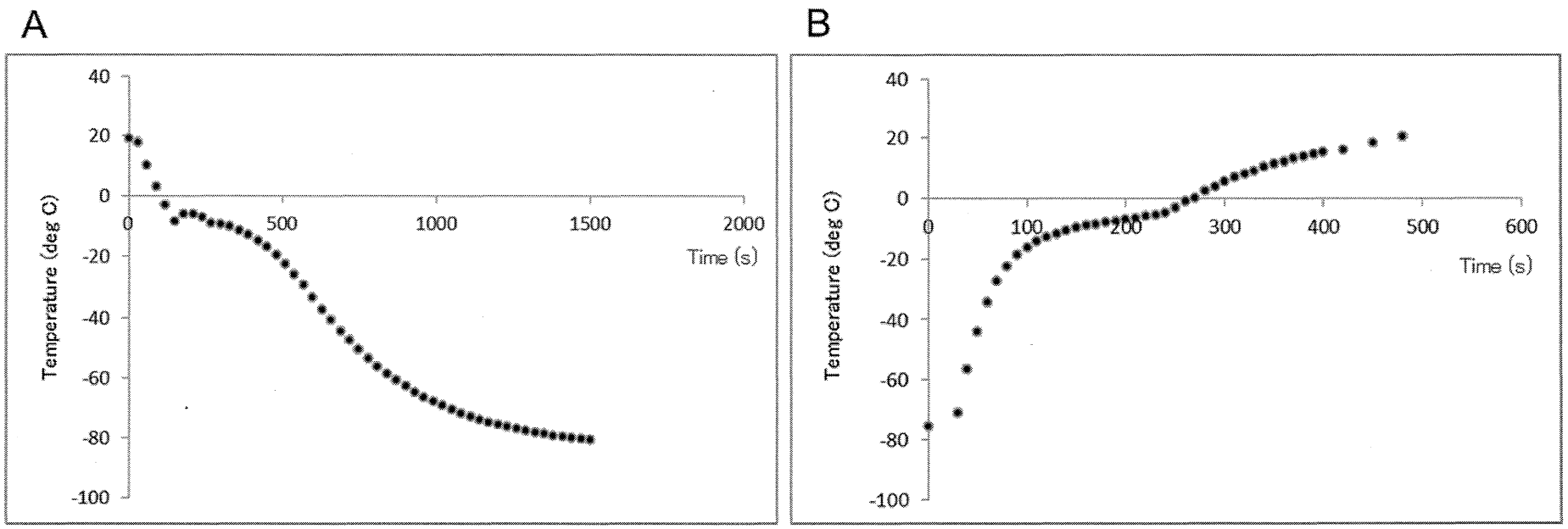

A
B
Temperature (deg C)
Time (s)
40
20
0
-20
-40
-60
-80
-100
0
500
1000
1500
2000
100
200
300
400
600

Figure 2.

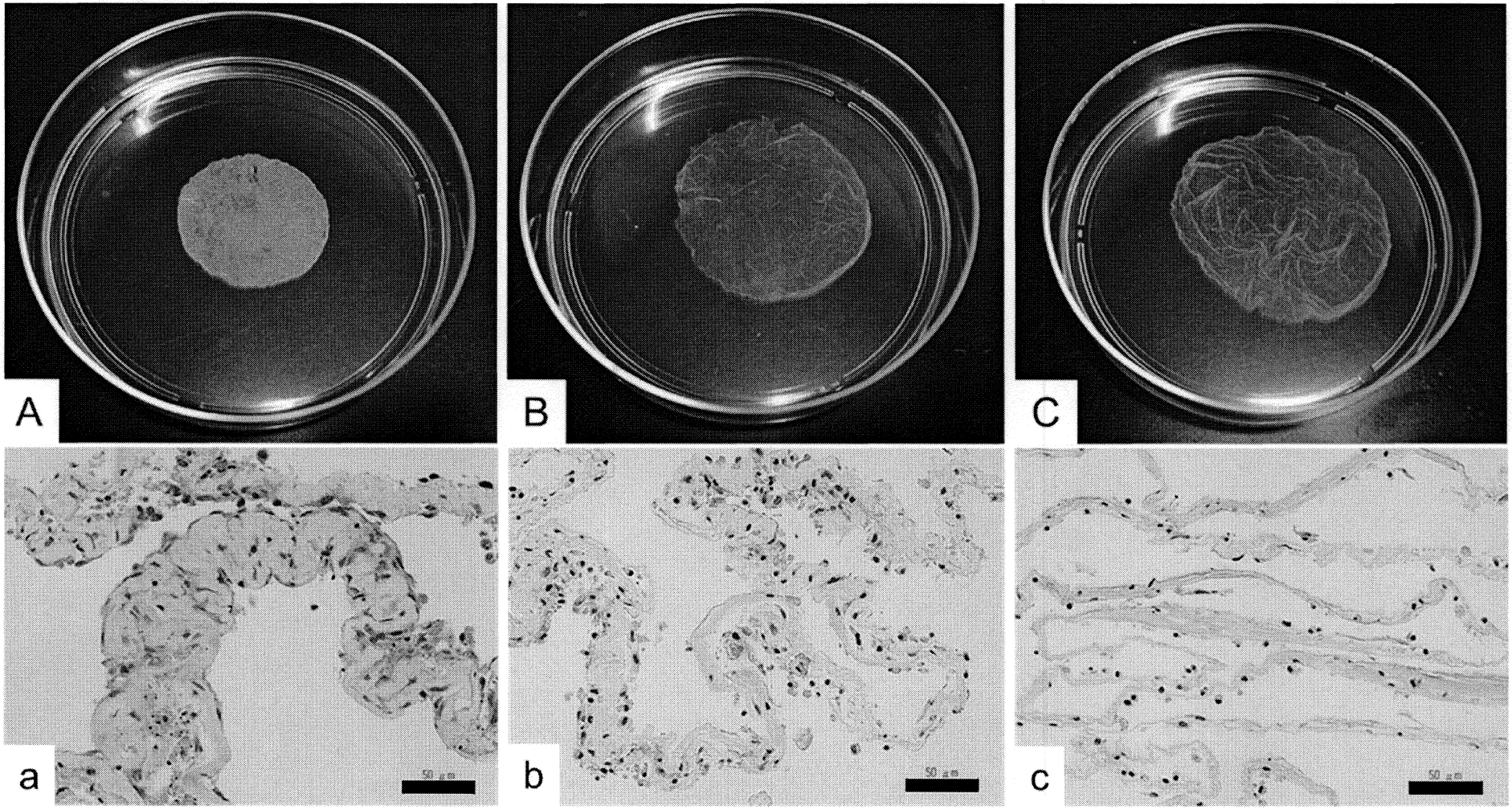


Figure 3.

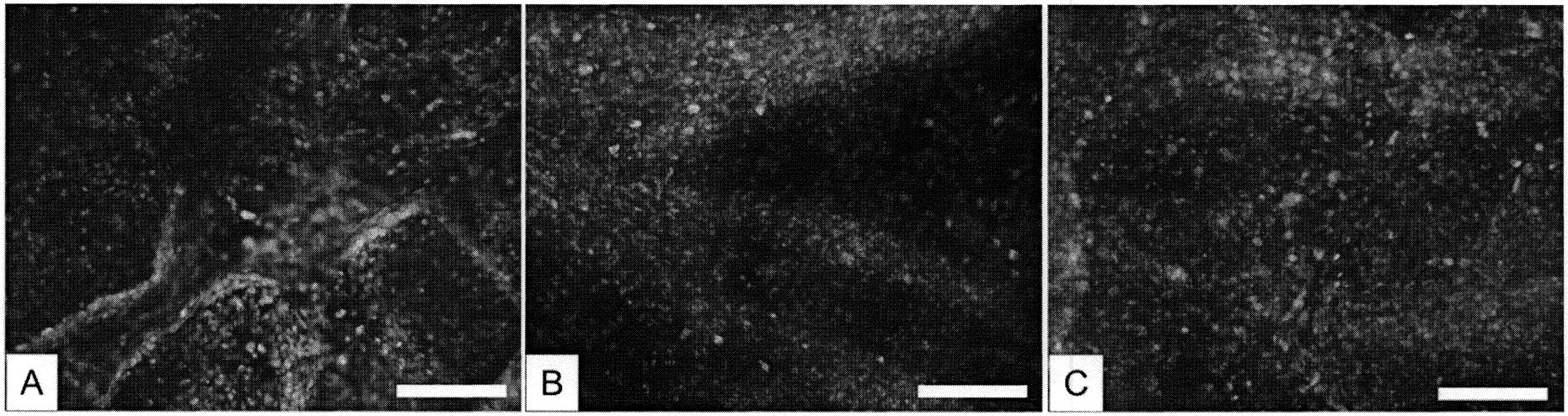


Figure 4.

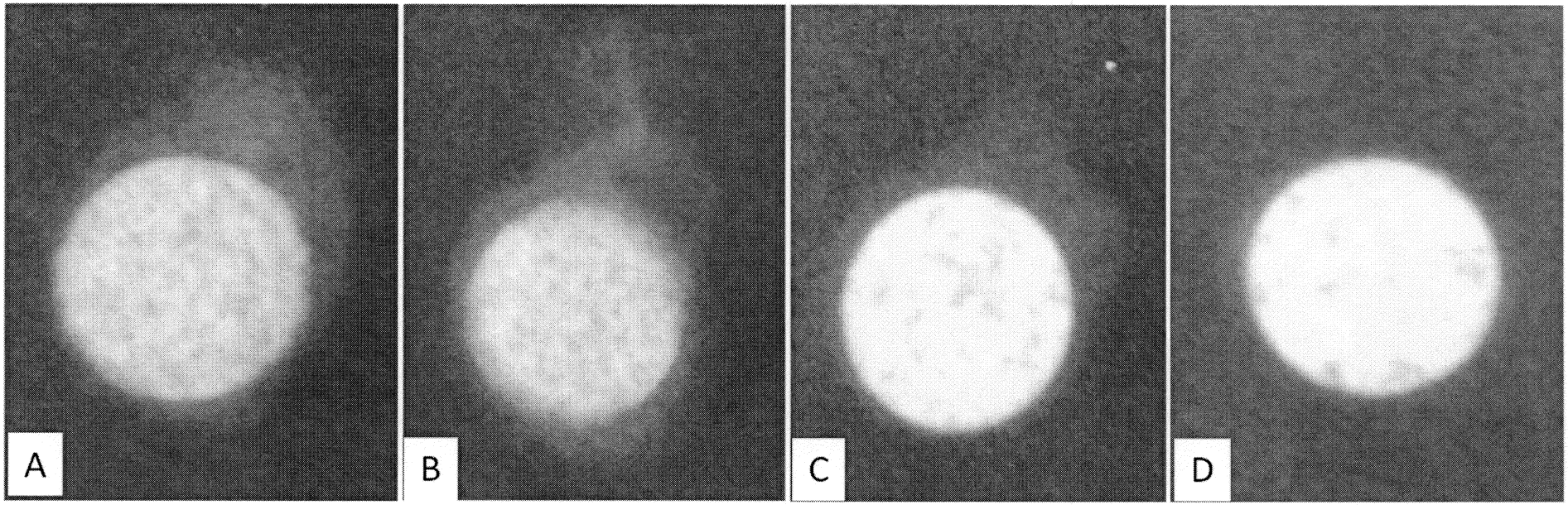


Figure 5.

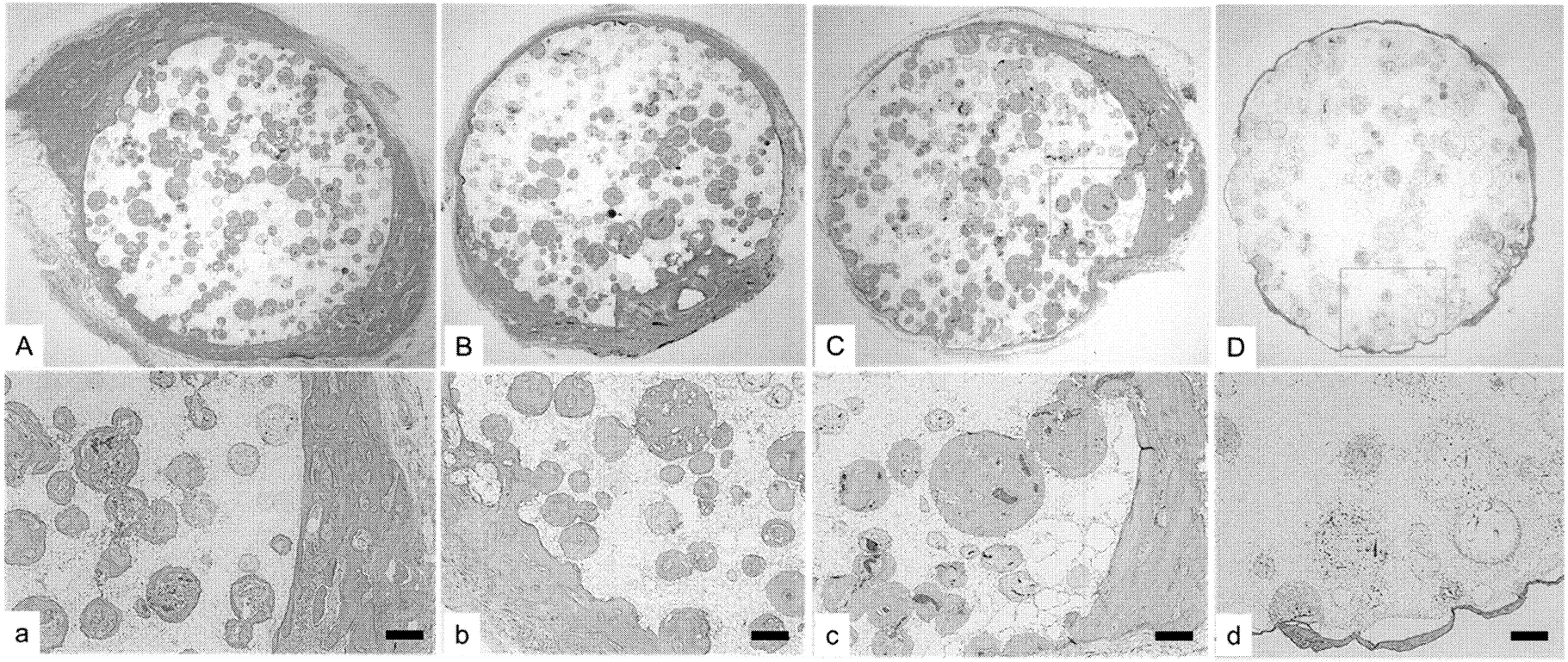
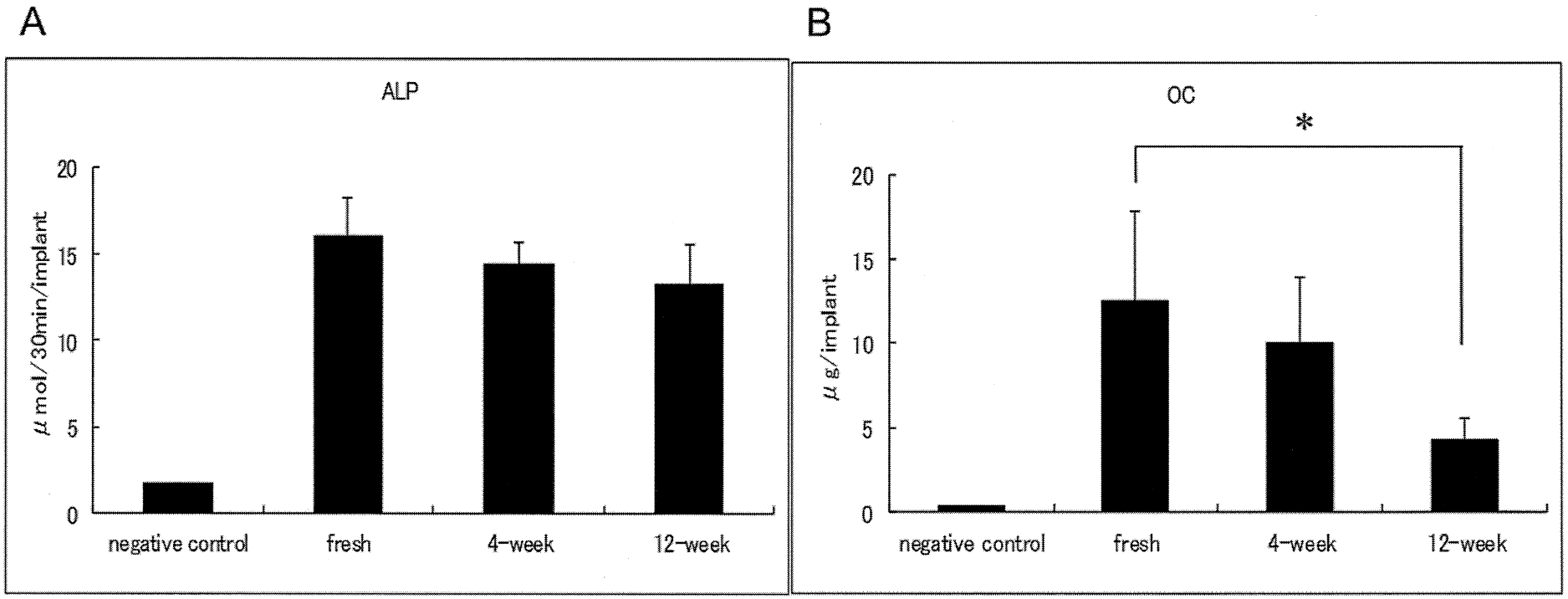


Figure 6.


A
ALP
μ mol/30min/implant
20
15
10
5
0
negative control
fresh
4-week
12-week
B
OC
*
μ g/implant
20
15
10
5
0
negative control
fresh
4-week
12-week