DEVELOPMENT OF COMPOSITE BUBALINE CANCELLOUS BONE XENOGRAFTS
BY SEEDING GUINEA PIG FETAL OSTEOBLASTS

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ABSTRACT: Xenografts are considered as an alternative strategy to restore a critical sized bone defect. The present
study was conducted to standardize the technique for seeding and cryopreservation of decellularized bubaline cancellous
bone with Guinea pig fetal osteoblast for the development of a composite bone xenograft. The composite bone grafts were
prepared by seeding the expanded osteoblasts on the acellular bubaline cancellous bone matrix. The foetal osteoblast
seeded scaffold, acellular bone scaffold, and native bovine bone were analyzed using scanning electron microscopy (SEM)
and histological examination. Composite bone xenograft was cryopreserved in 10% glycerol at -80°C for three months
and evaluated for post-thaw viability. Findings from the in vitro study suggested that the seeding of acellular scaffold was
adequate, and osteoblasts had good adhesion and proliferation inside pores of acellular bone matrix. Histologically the
integrity of collagen matrix was best preserved in the acellular group as compared to the freshly seeded and cryopreserved
scaffold. The osteoblast seeded decellularized bubaline cancellous bone xenografts can be preserved for three months
with adequate cell viability in the post-thaw evaluation at –80°C in sterile tubes containing 10% glycerol as the
cryoprotectant.

Key words: Bubaline cancellous bone, Xenografts, Composite scaffolds, Scanning electron microscopy, Osteoblasts, Segmented
bone defect.

INTRODUCTION

Bone tissue engineering has emerged as a major approach for the restoration of bone defects (Petite et al. 2000). Although autografts are time-tested method, the limited availability and donor site morbidity restricts the use of autografts. Allogeneic bone has been used as bone graft, but risk of pathogen transmission from donor to host, immunogenicity and limited availability limit the use of such grafts (Lucarelli et al. 2005). Therefore, the use of alloplastic biomaterials or xenografts could be useful to restore critical sized bone defects (Mucalo and Worth 2008). Xenogenic bone, such as bovine cancellous bone, can act as an alternative to the autogenous bone since they offer several beneficial properties (Kneser et al. 2006, Meyer et al. 2008). Bovine cancellous bone desired xenografts possess a porous architecture, which allows bone tissue in-growth (Johnson et al. 2000, Murugan et al. 2006), and has excellent osteoconductive and osteogenic properties (Camelo et al. 2001).

The advent of bone tissue engineering has brought new ideas and the development of innovative biomaterials for bone repair (Yang et al. 2008). The principle of this method is to apply functionally active cells on supporting scaffolds under controlled stimulation with growth factors in order to produce biologic substitutes as functional tissue replacement (Schieker et al. 2006). A tissue engineered “composite bone graft” thus contains osteogenic cells and osteo-inductive growth factors along with an osteoconductive matrix. The advantage of composite over simple scaffold is that it will not depend on local recruitment of the osteo-competent cells for new bone synthesis and could be particularly useful in clinical cases in which the host tissue cannot provide these cells (Logeart-Avramoglou et al. 2005). Osteogenicity may be
added to bone bio-scaffold by means of incorporating osteoblasts, mesenchymal stem cells, periosteal cells or other osteogenic cell types (Trentz et al. 2003, Kneser et al. 2006). Osteoblasts are polyhedral cells which synthesize ECM proteins including type I collagen (90%), and non-collagenous proteins such as osteocalcin, osteopontin and bone sialoprotein (BSP) (Papagerakis et al. 2002).

Though the benefits of using the cell composites of bone tissue substitute are considerable, but the availability (allogenic/xenogenic) of ready to use scaffolds is a great challenge to the orthopaedic surgeons. Therefore, there is a need to develop cryopreserved tissue engineered bone scaffolds. The aim of bone cryopreservation is not only to store the tissue effectively but also to conserve the mineral and phosphor-calcium framework of the bone. The present study was therefore, conducted to standardize the technique of seeding and cryopreservation of decellularized bubaline cancellous bone with Guinea pig fetal osteoblast for the development of composite bone xenografts.

**MATERIALS AND METHODS**

**Experimental animals**

Two adult pregnant female Dunkin Hartley guinea pigs (Cavia porcellus) (5-month-old) were used for the study (n=2). The animals were housed in individual steel cages, maintained on green grass commercial diet, and ad libitum water. They were maintained on a 12 hours light-dark schedule at a temperature of 23±2°C and humidity of 45% to 55%. The animals were acclimatized for a period of two week prior to the experiment. The experimental animals were provided with humane care, and procedures were performed according to National Institute of Health (NIH) guidelines for the care and use of laboratory animals (NIH Publications, eighth edition, 2011). The work was reported in line with the Animal Research: Reporting In Vivo Experiments (ARRIVE) statements (Percie du Sert et al. 2020).

**Isolation, culture, and expansion of primary guinea pig foetal osteoblasts**

The isolation, culture, expansion, and characterization of the osteoblasts were performed by the methods as previously described (Cao et al. 2006, Rashmi et al. 2017). General anaesthesia was induced with intramuscular injection of xylazine at the dose rate of 6 mg/kg bodyweight followed by ketamine at the dose rate of 60 mg/kg bodyweight after 10 minutes (Rafee et al. 2017). Full term developed foetuses were collected from the healthy pregnant guinea pig after performing caesarean section (Fig. 1a). The foetuses were euthanized by intracranial injection of 0.2 ml xylazine (Fig. 1b) and collected in sterile 1% PBS. The calvarial and long bones were harvested and washed with 1% PBS containing three times the usual concentration of antibiotics (penicillin - 100 U/ml and streptomycin - 100 µg/ml).

After washing, the bones were sectioned into small pieces (Fig. 2a). They were subjected to enzymatic digestion using 0.25% trypsin at 37°C for 20 min in a magnetic stirrer. The bone pieces were then washed with culture medium (DMEM) and dipped in DMEM having 20% fetal bovine serum (FBS) in a T-25 culture flask. The flasks were then incubated overnight in a humidified atmosphere with 5% CO at 37°C. On the next day, fresh DMEM supplemented with 10% FBS, penicillin (100 U/ml), and streptomycin (100 µg/ml) was added. The medium was changed twice a week, thereafter.

The cells were trypsinized and harvested on attaining 80-90% confluency after a period of 14-15 days. The culture medium was removed from the flask and the cells were washed with sterile PBS followed by the treatment with 0.25% trypsin-ethylenediamine tetra acetic acid (EDTA) (2 ml) for 15 min. The enzymatic activity was stopped by adding 3 ml of culture medium and the contents of the flask were collected in a centrifuge tube, which was then centrifuged at 2500 rpm for 10 min. The supernatant was discarded, and the pellet was resuspended in culture medium and mixed by gentle pipetting to get single cell suspension. The cells were further sub-cultured in new T-25 flasks by layering them at the rate of 5 x 10³-10⁴ cells/cm². The culture was maintained at 37°C and 5% CO₂ in a humidified atmosphere. After 15 days, when the cells reached in full confluency, further passages were done following the same procedure.

**Seeding of osteoblasts on decellularized bovine cancellous bone scaffold**

The acellular bone xenograft was prepared from bubaline femur as per the methods described by Pathak et al. (2012). The femur bone of a buffalo was procured from the slaughter house and was cut into small pieces using an osteotome. The bone pieces were thoroughly washed in tap water to remove the marrow contents and finally washed in 1x PBS with 0.1% EDTA at room temperature. These bone pieces were decellularized by five freeze and thaw cycles. In each cycle, the bone pieces were put into a metal beaker and placed in liquid nitrogen for one minute and thereafter were thawed at 56°C for 5 min. The bone pieces were then kept in 3% H₂O₂ for 45 min to remove residual antigenic material (DePaula et al. 2005).
The decellularized bovine cancellous bone scaffolds were cut into small pieces in the shape of (contents) guinea pig radius bone (length of 1 cm, height 0.25 cm and width 0.25 cm) and were dipped in 70% ethanol for 1 hr on a magnetic stirrer. Scaffolds were washed twice with sterile 1x PBS for 1 hr. Finally, the bone pieces were sterilized using UV treatment (6 hr). To confirm whether the scaffolds are sterile, they were dipped in T-25 flask filled with DMEM and incubated to see whether any bacterial or fungal growth occurred.

The tested scaffolds were incubated in the culture medium for 4 hr to pre-wet the scaffold surface (Fig. 2b). The foetal osteoblast cells in second or third passage were washed with sterile 1x PBS and detached through trypsinization (Trentz et al. 2003). After centrifugation, cell pellet was resuspended in culture medium and a suspension of 4 x10^6 cells was prepared in 100 µl culture medium and seeded on a scaffold (1 cm x 0.25 cm x 0.25 cm). The cells were transferred to the core of scaffold with the help of a syringe. Multiple injections were made to bring about uniform seeding throughout the scaffold surface and its core. The scaffolds were incubated after seeding for 4 hr in an incubator and then culture medium was added. These scaffolds were cultured in 6-well plates for up to 2 weeks (Kneser et al. 2006). The cultures were maintained in a humidified atmosphere consisting of 5% CO₂ at 37°C, and the media were changed twice a week (Liao et al. 2004). The adhesion and penetration of osteoblasts in acellular bubaline cancellous bone matrix were investigated by Scanning Electron Microscopy (SEM), and Haematoxylin and Eosin (H&E) staining.

**Scanning electron microscopy (SEM)**

The foetal osteoblast seeded scaffold, acellular bone scaffold, and native bovine bone were examined by using SEM. The samples were washed in 1x PBS and were fixed overnight using 2.5% glutaraldehyde in PBS at 4°C and were dehydrated in a series of ethanol solutions. The samples were first incubated in 30%, 50% and 70% ethanol for 10 min each, then in 90% and 100% ethanol for 15 min each. The tissue samples were then dried in a critical point dryer using CO₂ as the transitional fluid and mounted on the scanning electron microscope specimen holder. After that, a thin layer of gold/palladium ion sputtering was done on Jeol ion sputter Model JFC 1600 at 7-10 mA and 1-2 KV for 15 min. Finally, the specimens were examined under scanning electron microscope at appropriate acceleration voltage and magnification range to test the adhesion, anchorage to pore, proliferation, and matrix secretion of osteoblast cells on acellular bubaline bone scaffold.

**Histology**

Osteoblast seeded and unseeded acellular bone scaffolds were cut into small pieces and fixed in 10% formalin for 48-72 hr. The scaffolds were then decalcified using Goodling and Stewart’s fluid (Wallington 1972). The solution was stirred daily and changed once in every three days. The sections were checked regularly for the status of decalcification. The completion of decalcification was assessed by flexibility, transparency and pin penetrability of the bone sections. The tissues were then processed in a routine manner and 4 µm thick sections were cut and stained with H&E stain as per the standard procedure (Luna 1968).

**Cryopreservation**

The composite bone scaffolds were transferred into sterile tubes containing 10% glycerol as the cryoprotectant (Fig. 2c). The tubes were kept at 4°C for 30 min in order to reduce the toxicity of glycerol and to
equilibrate extra- and intracellular concentrations of glycerol. After this period, the tubes were transferred into -80°C deep freezer for 3 months period. In order to check viability of these composite grafts after 3 months, the samples were thawed directly in a 37°C water bath. The glycerol was removed step-by-step by immersion, with 5 min in each step in DMEM medium containing 0.5 M sucrose followed by 0.25 M sucrose and then subsequently decreased till it reaches zero. The thawed bones were washed twice with DMEM without sucrose and then evaluated for viability of osteoblast-like cells present in the composite graft (Reuther et al. 2005).

**Cell culture**

The bone fragment was washed and incubated with DMEM containing 10% fetal bovine serum at 37°C in a humidified atmosphere of 95% air and 5% CO2. Explants were subsequently maintained by replacing the medium every 3-4 days until cell density reached 70-80% confluency, and then cells were detached with 0.25% trypsin and sub-cultured.

**MTT assay**

The changes in the number of viable cells on the substrates were quantitatively assessed by using MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) test. MTT is a yellow tetrazolium salt, which can be enzymatically converted by a living cell to a purple formazan product. The intensity of the colour produced was therefore, directly proportional to the number of viable cells in culture, and thus to their proliferation in vitro. The proliferation of cryopreserved composite graft was determined by using the MTT assay. The appropriate cell numbers were incubated in a 96-well plate, and then 20 µL of MTT was added for 4 h to each well. The cells were lysed with 150 µL DMSO, and the conversion of MTT to formazan by metabolically viable cells was determined by using a 96-well microtiter plate reader at 490 nm (Wang et al. 2011).

**RESULTS AND DISCUSSION**

**Culture characteristics of primary guinea pig foetal osteoblasts**

During the primary culture, round or polygonal cells were observed after 2-3 days in the culture flasks migrating from the bone pieces under phase contrast microscope (Fig. 3). Initially this culture represented a mixture of heterologous cell population. The cells were with protuberances adhered to the culture flask after 5 days of culturing. The floating non-adherent cells got discarded while changing the medium. The adhered cells started to change their morphology from round to various shapes. The morphology ranged from triangular, polygonal to short spindle shapes (Fig. 3). After 12-15 days of culture, approximately 80% confluency was observed. After the first passage, triangular cells were prevalent and were packed closely in the culture flask. The cell proliferation was uniform throughout the bottom of the culture flask.

**Seeding of osteoblasts on decellularized bovine cancellous bone scaffold**

After the multiple freeze-thaw cycles, the gross appearance of bone scaffolds changed and became porous and white. The treatment with 3% H2O2 and 70% ethanol sterilized the scaffolds and improved its clarity. The active seeding of osteoblasts on scaffolds by multiple injections was found technically easy and simple. The cells were

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![Fig. 2. (a) Collected calvarium and long bone from the Guinea pig fetus. (b) Incubation of scaffolds in DMEM before seeding. (c) Preparation of scaffold for cryopreservation (sterile tubes containing 10% glycerol).](image-url)
found adhered to ECM after 2-3 days of seeding. Furthermore, the cells proliferated and grew into the inner pores of the scaffold as observed under phase contrast microscope. Grossly, it was found that the colour of the medium changes every 3-4 days, thereby indicating cell proliferation and viability. No contamination was observed in culture up to 14 days post-seeding.

**Scanning Electron Microscopy (SEM)**

The SEM technique was used to observe the architecture of matrix, pores and interconnectivity of pores within the decellularized scaffolds. The adhesion of osteoblasts, and proliferation on matrix was also evaluated in case of seeded scaffold, and composite cryopreserved scaffold. In acellular scaffolds under lower magnification (200x-70x), many pores were visible having pore diameter ranging between 300 µm and 779 µm (Fig. 4). The acellular bone appeared as a highly porous scaffold under lower magnification. SEM image of the seeded scaffold and composite cryopreserved scaffold, revealed that the cells proliferated and encroached into the inner pores of the scaffold. The cells were found to spread and anchored onto the pore walls. Under lower magnification, numerous pores filled with cellular matrix material were also observed (Fig. 4).

**Histological evaluation**

Histological examination of the acellular, freshly seeded graft, and cryopreserved graft was done after cutting the sections. In case of freshly seeded scaffolds, the pink matrix along with osteocytes and nuclei were clearly visible as blue dots within the osteoid matrix. Further, only little osteoblastic activity was observed along with the deposition of fresh collagen in the pores of the seeded scaffold (Fig. 5). The acellular matrix was observed as intact porous matrix of concentric layer of collagen fibres with almost complete removal of cells and nuclei. The matrix fibres were also found to be distorted (Fig. 5). The histological examination of composite cryopreserved matrix revealed the preserve of cells and nuclei in the same fashion as described for freshly seeded group (Fig. 5). However, a greater number of cells were observed at the outer margin of the scaffold. The collagen matrix was observed to be more distorted as compared to freshly seeded and acellular group.

**In vitro post-thaw evaluation of cryopreserved composite scaffold**

Cryopreservation of composite grafts was successfully performed using 10% glycerol as the cryoprotectant for three months at -80°C. The bone matrix was preserved following the cryopreservation. The composite bone graft appeared rigid and was not amorphous or brittle but showed increase in porosity. The biomechanical and osteoconductive properties seemed to be unaltered. The in vitro post-thaw evaluation of cryopreserved composite scaffold was performed using cell culture and MTT assay.

![Fig. 3. Primary explant culture of foetal Guinea pig bone tissue.](image)

[(a) Cells migrating from bone pieces after 3 days of culture. (b) Cells after 5 days of culture, started to elongate becoming spindle shape. (c) Foetal osteoblast cells showing trigonal, spindle to polygonal morphology after 10 days of culture (inverted phase contrast microscope, 10x).]


Cell culture

After three days of incubating the bone fragments with standard DMEM culture medium containing 10% fetal bovine serum, no changes were observed. However, after 10 days of incubation, few cells were found to creep out from the explants (Fig. 6). The cell density reached to 40% confluency after a period of 15 days.

MTT assay

Cell viability of osteoblast cells under standard condition was measured by MTT assay. Mitochondrial dehydrogenase was reduced by MTT and there was gradual increase in the absorbance at 490 nm on day 7 and 14 as compared to day 1. The outcome of cell viability assay is depicted in Fig. 7. There was a significant (p < 0.05) increase in the optical density (OD) value on day 14 when compared to day 7 and day 0. Furthermore, significant increase in OD value was also observed on day 7 as compared to day 0.

Xenogenic bone grafts are often required for reconstruction of large bone defects as the quantity of bone harvested from autologous skeletal donor site is limited and often results in donor site morbidity. The reason for superiority of autologous bone is the presence of osteoblasts in the graft, which makes it osteogenic. In the present study, guinea pig foetal osteoblasts were seeded on the decellularized bovine cancellous matrix in vitro and thus a composite scaffold was created with the aim of producing a suitable bone graft that is compatible and acceptable for treating segmental bone defects. The cryopreservation of bioactive scaffold material not only preserves the aseptic condition, mechanical properties, and biocompatibility, but also the biological activity of the scaffold material (Jiao et al. 2014).

An explant culture method was adopted in this study to obtain osteoblasts from the foetal calvarial and long bones. This method of culturing is simple and causes less damage to the cells although the number of cells harvested is limited (Rashmi et al. 2017). Furthermore, the method was slightly modified in our study by digesting the bone pieces with 0.25% trypsin for 20 min to loosen the bone tissue and enable osteoblasts to creep out easily. The enzymatic treatment used in the explants culture took less time (12-15 days) for obtaining confluent primary osteoblasts than with the direct culture (Rashmi et al. 2017).

Bovine cancellous bone has a porous architecture which assist bone tissue in growth (Murugan et al. 2006). Cancellous bone has a sufficient pore size and an interconnecting pore structure essential for osteogenesis (Karageorgiou and Kaplan 2005). The freeze and thaw (FT) conserve osteoconductive properties and the process was very effective in devitalizing the cells and generation of acellular cancellous scaffold without affecting the mechanical property for use at load bearing sites (Rashmi et al. 2020). The bone pieces after freeze and thaw were subjected to 3% H2O2 for 45 min as hydrogen peroxide is well known for antiviral and antibacterial effects. Various studies have shown that with exposure to 3% H2O2, the effect on osteo-inductive and mechanical property is negligible with a 1 hr exposure, and the gain in viral clearance is substantial (Beebe et al. 2009). The scaffolds used for bone tissue engineering should have enough mechanical properties to provide structural support for new tissue formation upon implantation of the construct (Salgado et al. 2004). In decellularized bone matrix, components of ECM are generally conserved and well tolerated by xenogenic recipient (Exposito et al. 1992). Decellularization protocol efficiently removes all cellular and nuclear material, while minimizing any...
adverse effect on the composition, biological activity, and mechanical integrity of the remaining ECM (Gilbert et al. 2006).

After the freeze-thaw treatment, the cells were removed leaving vacant space in the matrix that contributed to the porous appearance (Pathak et al. 2012). This was evident when the acellular matrix was stained using H&E stain since only the porous matrix remained (no cells and nuclei). The bovine cancellous bone decellularized using anionic detergents will lack cellular portion from matrix on H&E staining (Chen et al. 2010). However, histological evaluation of the seeded scaffold and composite cryopreserved scaffold identified newly laid down matrix inside the pores of acellular matrix and osteoblastic activity on the scaffold margins. The histological images reflected that the integrity of collagen matrix was best preserved in the acellular group as compared to the freshly seeded and cryopreserved scaffolds. However, when the freshly seeded and cryopreserved scaffold were compared, it was observed that the concentric layer of collagen fibers was preserved in the former group and was highly disintegrated in the latter group (Pathak et al. 2012, Rashmi et al. 2017).

The SEM results of seeded graft and composite cryopreserved scaffold showed numerous cells adhered to scaffold material. The cells proliferated well and grew in the pores of scaffold as shown in previous studies involving scanning electron microscopy of osteoblasts.
seeded synthetic ceramics (Liao et al. 2004, Rath et al. 2012). Under lower magnification, we could notice that the pores were filled with calcified white matrix material as in one study abundant calcification associated with collagen bundles was observed after 4 weeks of culture of bone marrow derived stem cell in osteogenic media onto the polycaprolactone scaffold in scanning electron microscopy (Yoshimoto et al. 2003). Acellular scaffold appeared as highly porous material having pores under lower magnification and no cellular materials were visible on scaffold and on higher magnification when surface collagen was compared with fresh bovine matrix, revealed closely and densely packed collagen matrix. Collagen fibres were less disoriented and densely arranged in comparison to fresh bone sample (Chan et al. 2013). The freeze and thaw process might have caused shrinkage of scaffold as collagen fibers appeared to be compressed into larger bundles that was not evident in the fresh bone (Lumpkin et al. 2008). The results of SEM revealed that seeding of osteoblast was appropriate and acellular matrix supported cell growth very well over a period of 14 days. Similar finding has been reported by Rashmi et al. (2017). Overall, the histological and SEM studies revealed that cryopreservation did not affect the scaffold and the morphology was comparable with the freshly seeded composite scaffolds.

CONCLUSION
In the present study, composite bone graft was prepared by seeding foetal osteoblasts of guinea pig on the acellular bubaline cancellous bone matrix. The acellular bone xenograft used for seeding was prepared from the bubaline femur by decellularization using freeze and thaw technique. Multiple injections of the osteoblasts were found to be an easy and effective technique for seeding decellularized bubaline cancellous bone xenografts. Bubaline composite scaffolds can be preserved for three months with adequate cell viability in the post-thaw scaffolds.

Ethical approval
All the methods and experimental protocols used in this study were approved by the Institute Animal Ethics Committee (IAEC) of ICAR-Indian Veterinary Research Institute, Izatnagar, Bareilly, Uttar Pradesh, India vide order No. F.1-53/2012-13/JD(R).

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