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Growth of Bone Marrow Derived Osteoblast-Like Cells into Coral Implant Scaffold: Preliminary Study on Malaysian Coral

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Abstract: Problem statement: Biomaterial fabrication in Malaysia started as a consequence of the demand for cheaper implant materials. Various biomaterials have been developed utilizing local resources like Malaysian coral. Locally processed Malaysian coral need to be complemented with proper evaluation and testing including toxicology, biocompatibility, mechanical properties, physicochemical characterization and in vivo testing. The present study was carried out to assess natural coral of porites species as scaffold combined with in vitro expanded Bone Marrow Derived Osteoblast-Like cells (BM-DOL), in order to develop a tissue-engineered bone graft in a rat model. **Approach:** Coral was used in a block shape with a dimension of 10 mm length × 5 mm width × 5 mm thickness. BM-DOL cells were seeded into porous coral scaffold in a density of 5×10^6 mL^{-1}. After 7 days of in vitro incubation in osteogenic medium, one block was processed for light (LM) and Scanning Electron Microscopy (SEM) observations while the other blocks were implanted subcutaneously in the back of 5 weeks-old Sprague-Dawely rats for 3 months. Coral blocks without cells were implanted as a control. The implants harvested and processed for gross inspection, histological and scanning electron microscopy observations. **Results:** Both LM and SEM showed attachment of well arrangement multilayer cells inside the pores of in vitro seeded coral scaffolds. Gross inspection of all in vivo coral-cell complexes implants revealed vascularized like bone tissue formation. Histological sections revealed mature bone formation occurred in the manner resemble intramembraneous bone formation. SEM observations revealed multi-layer cellular proliferation with abundant collagen covered the surface of coral implants. Control group showed resorbed coral block. **Conclusion:** This study demonstrated that Malaysian coral can be use as a suitable scaffold material for delivering bone marrow mesenchymal stem cells in tissue engineering and therefore, offers a great potential to enhance bone healing around implants in a compromised bone bed.

Key words: Malaysian coral, osteoblasts, bone tissue engineering, extra cellular matrix

INTRODUCTION

Considerable attention has been directed to use biomaterial because of limitation in the use of biological grafts. The use of coral as a bone graft substitute dates back to the early 1970's[1,2]. Natural coral has a porous structure that offers a substantial surface exchange area. The size and interconnectivity of the coral pores have been shown to be critical factors in the rate of coral resorption and in the role of coral in the bone regeneration[3-5]. As reported by Chen *et al*.[6] the interconnected porous coral exoskeletons allow bone infiltration to the Centrum of the implant while other biomaterials, lacking adequate pore interconnections and limit bone formation to the periphery. The osteoconductive capacity of porous coral allows cell attachment and growth through the scaffold of the material, characteristic of a good support of cells[7,8]. Research has clearly demonstrated coral to be only an osteoconductive and not an osteoinductive material[9]. Augmentation of the natural osteoconductivity biomaterials with agent such as bone marrow aspirates[3] and cultured osteoblasts[1,4] has yielded promising results. Tissue engineering has been developed for the reconstruction of living tissue[10]. It is based on principles of developmental and molecular biology, signal transduction and cell biology. Mesenchmal Stem Cells (MSCs) investigated as progenitor cells for the engineered fabrication of specific mesenchymal tissues for repair or regeneration of various connective tissue sites throughout the body. The aim of this preliminary study is to assess the cellular proliferation and attachment of BM-DOL cell *in vitro* seeded on the locally processed Malaysian coral scaffold in order to develop a tissue-engineered bone graft at an extra skeletal site through the transplantation of culture-expanded marrow-derived osteoblasts into porous coral.

MATERIALS AND METHODS

Coral scaffolds: Natural Malaysian coral of porites species with a pore size of 66.5-186.2 µm and porosity of about 42% based on a prior study[11] was used in this study. Briefly, dead Sea coral of porites species had been harvested from Malaysian biodiversity, processed it using innovative techniques. Coral skeleton material were cleaned from any debris and washed with distilled water. Coral was cut into blocks with a dimension of 10 mm length × 5 width × 5 mm thickness. The material was then chemical treated followed by freeze-drying. The samples were triple packed and radio sterilized using gamma irradiation (Fig. 1).

Rat bone marrow cell isolation and culture: BM-DOL cells obtained from the femora of 5 weeks old Spruge-Dawely rats. The cells were isolated and cultured as described previously[12]. Cells morphology and growth were evaluated using phase contrast microscopy. After 7 days of primary culture in α-MEM supplemented with 10% fetal calf serum, 50 µg mL^{-1} ascorbic acid, 50 µg mL^{-1} gentamycin, 10 mM β-glycerophostate and 10 nM dexamethasone, the cells were trypsinized and resuspended in supplemented medium. The cells were counted and the density was adjusted to 5×10^6 mL^{-1} with medium. Experiments were conducted during the exponential phase of growth and viability was examined by trypan blue exclusion.

Seeding of cells on coral scaffold: Coral scaffold blocks were placed in six-well plates. Culture medium was added to pre wet the scaffolds. After 30 min, medium was sucked out and cells in 500 µL were precisely seeded into scaffold and maintained at 37°C in a humidified atmosphere consisting 5% CO_2 for 2 h, then 3 mL of medium was added. The medium was changed every 2 days. The scaffolds were incubated *in vitro* for 7 days. Two coral scaffolds were cultured in the same environment but without cells as control. One specimen from each treatment was harvested at 7 days for light and scanning electron microscopy observations.

***In vivo* implantation of coral scaffold-cell complexes:** This study was approved by the animal ethical committee/University Sains Malaysia. Five weeks old, Sprague dawely male rats, anaesthetized by intraperitoneal injection of ketamine (100 mg kg^{-1}) and xylazine (10 mg kg^{-1}). One small incision made in the back of each rat and 1 cm subcutaneous pouches were then created. Coral scaffold-cells complex was place 5 mm from the incision, which was then close with resorbable thread. One rat acted as control, was implanted with coral without cells. The implants were retrieving 3 months later and observed grossly.

Fig. 1: Growth of bone marrow: Shows processing procedures of coral

Preparation of specimens: Each specimen was divided into two parts. The first part was fixed for 48 h in 10% neutral buffered formalin solution. Undecalcified sections were prepared and stained with hematoxylin and eosin staining for histological observations. The second part was processed for SEM, fixed with 2.5% glutaraldehyde post fixed with 0.5% OsO_4, dehydrated through a graded series of alcohol, immersed in HMDS for 10 min 3 times then air-dried at room temperature. After sputtercoating with gold the specimens were examined using scanning electron microscopy (Leica Cambridge S360 at 10 KV).

RESULTS

BM-DOL cells culture: Primary bone marrow cells revealed proliferation of a typical spindle-shaped osteoblastic morphology in culture. Seven days after the BM-DOL cells were seeded and cultured on coral scaffold, phase contrast microscopy revealed well attachment and spreading of the cells on coral scaffolds, which were appeared as rounded in shape at 3days and became more elongated, with long cellular process extended in different directions at 7 days (Fig. 2). Histological Sections from coral-cells complexes at seven days revealed attachment of the cells inside coral pores. SEM revealed the characteristic spindle-like morphology of the MS-DOL cells anchored, attached, spread, proliferated and well arranged on coral scaffold. The cells migrate into pores with elaboration of multilayered filopodia extended in different direction. The attachment of cells gave the granular appearance of coral surface and the extracellular matrix synthesis was abundant on the scaffold surface (Fig. 3a-c).

Grafts properties: No visible inflammatory reactions, infection or extrusions observed at the site of implantation of scaffold-cell complexes graft and the control.

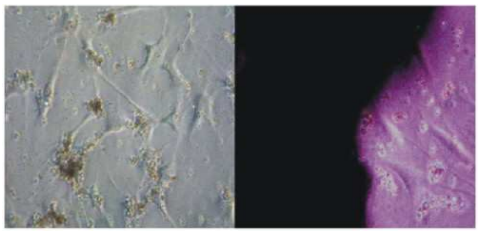


Fig. 2: Growth of bone marrow: Phase contrast microscopy micrograph shown morphology of BM-DOL cells seeding on coral discs

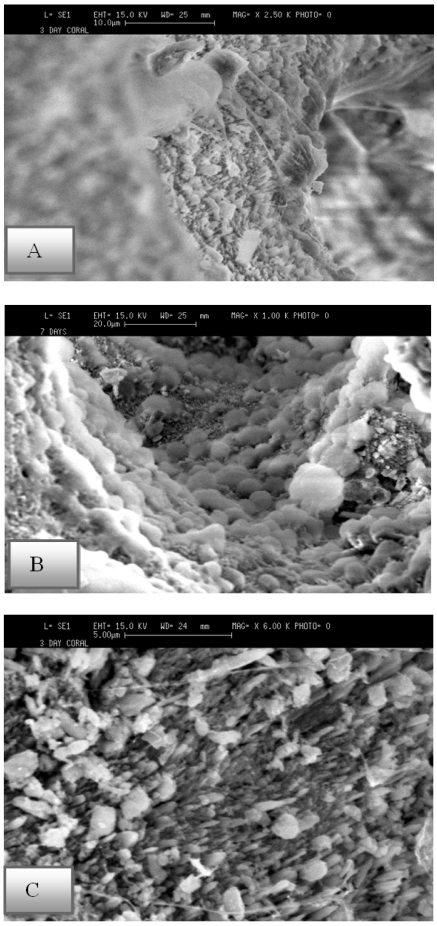


Fig. 3: (a-c): Growth of bone marrow: SEM photomicrographs shown BM-DOL cells grown in the surface and inside the pores of coral discs

On macroscopical examination, the surface of the grafts in coral scaffold-cells complexes was cover with smooth vascular look like bone tissue, while control group, coral alone was partially absorbed and had rough surface. In all specimens of coral scaffold-cells complex group, the undecalcified sections showed abundant amount of mature dense bone formation (Fig. 4).

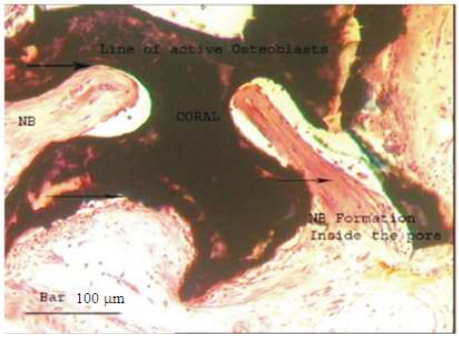


Fig. 4: Growth of bone marrow: Dense bone formation invade the coral pores, line of active osteoblasts (arrow), New Bone (NB) (Masson Trichome stain Bar = 100 µm)

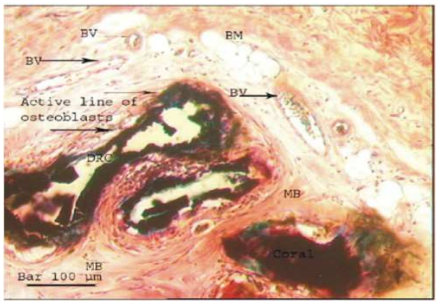


Fig. 5: Growth of bone marrow: Coral surface replaced by trabecular structure like osseous tissue (MB: Mature Bone, BM: Bone Marrow, DRC: Deformed Remnant Coral, BV: Blood Vessel) Bar = 100 µm

A line of active osteoblasts was seen in coral pores and an active seam of secretory osteoblasts. A plenty of blood vessels were observed in the pores as well as surrounded the scaffold. Multinucleated giant cells found close to the surface of coral possible contributing to its biodegradation. The new bone formations resemble intramembraneous bone formation. Most coral implant had disappeared, but only few scattered deformed fragments remained embedded as remnant crystals-like structure in some region within bone and adipose-fibrous tissue (Fig. 5 and 6). Scanning electron microscopy revealed abundant bone formation, on the surface. Coral scaffold appeared occupied with cells, cellular matrix and collagen fibbers. The pores were gradually tapered and replaced with bone like-osteon (Fig. 7a and b).

The control group reveals disappearance and resorption of coral particles due to non-uniform erosion of coral, some of the remnant pores were occupied by fibrous tissue and no evidence of bone formation appeared in SEM observations of the graft.

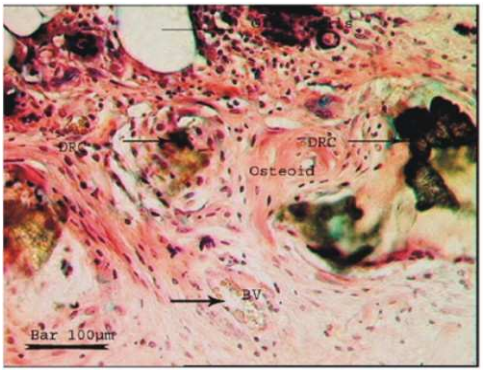
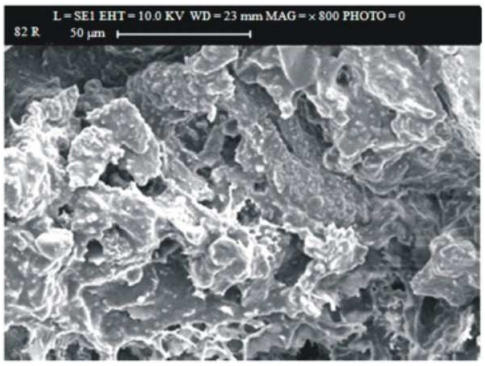
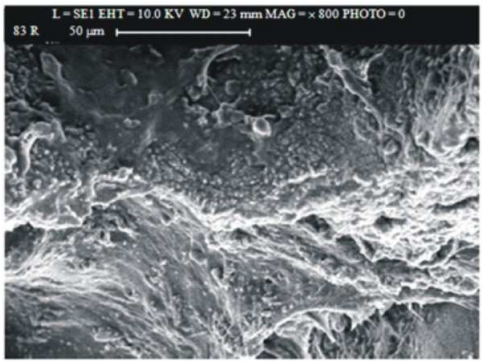


Fig. 6: Growth of bone marrow: Multinucleated giant cells (arrow) closet to the surface of coral remnant, Blood Vessel (BV), (DRC) (Masson Trichome stain, Bar = 100 µm)



(a)



(b)

Fig. 7: Growth of bone marrow: SEM photomicrograph, reveal coral implant (a) revealed a modification of pore morphology (b) the pores surface were almost covered by rough surface or round cells as well as a dense collgenous extracellular matrix. In addition to extensive packed mineralized collagen fiber bundle found inside pores. (× 800, Bar = 50 µm)

DISCUSSION

Bone grafting mediated via tissue engineering of stem cells for repairing defects represents a new direction towards bone regeneration in this millennium. Now, the goal is to provide stem cells an appropriate environment to proliferate and differentiate to the specific lineage for ultimate regeneration of the lost or damaged tissue.

A variety of material based on synthetic and naturally occurring polymers, ceramic and bioglass have been investigated for bone tissue engineering[13-15] Nevertheless, there is still an urgent need for scaffolds with characteristics that are properly fitted to the special demands in tissue engineering[16,17]. Natural coral has found considerable interest as scaffold materials[18]. This study showed that coral seeded with BMSC-DO, subcultured in osteogenic medium was capable of forming bone in vivo. Coral scaffold combined with BMSC-DO enhanced biomechanics, low absorbability and good osteogenic activity however, the incubation of coral without BMSC-DO as control did not lead to bone formation. The gross specimens inspection and histological observation showed that coral scaffold seeded with BM-DOL, subcultured in osteogenic medium were successfully develop bone graft restoration *in vivo*. Natural coral has been used with certain success, particularly in studies reported in the periodontology[16,19]. These studies approved possibility of use natural coral scaffold because of its biocompatibility, osteoconductivity and absorbability at a rate commensurate with bone formation. In addition, coral has cross- linked 3-dimensional pores with physical characteristic meets most requirements for a cell-seeding scaffold[20]. Further, more, it could easy be shaped into the required shape and size. We believe that coral is an ideal material for use in the field of bone engineering. Mesenchymal Stem Cells (MSCs) are being investigated as progenitor cells for the engineered fabrication of specific mesenchymal tissues for the repair or regeneration of various connective tissue sites throughout the body. There have been considerable advance in the application of MSCs in tissue-engineering to regenerate or repair bone, cartilage, tendon, adipose and muscle. In our study, light microscopy revealed extensive bone formation in specimens of coral scaffold seeded with BM-DOL through intramembraneous bone formation. This bone formation demonstrate that coral is bioactive and osteoconductive biomaterial. The result of this study is compatible with previous studies, which described that bone formation within the implant initially occurs directly against the surface of the implant indicated the bioactive ability of coral implants. If the osteoblasts then

proliferate on the surface, the implant is osteoconductive. During the process of osteoconduction, osteoblasts are initially identified typically on the surface of the implant. Rarely are chondroblasts seen within the porosity. Therefore this process is more akin to membraneous bone formation than to osteochondral bone formation[21]. In view of this, researcher postulated a mechanism for healing of critical sized defects. They postulated that the release of tissue factors from the edge of the wound causes differentiation of cells into osteoblasts in the defect. These cells create and mineralize extracellular matrix and form bony islands. These islands provided a scaffold with new bone growth[22]. It has been well documented that vascularization is an essential requirement for bone formation, but not for cartilage formation[23]. On the other hand, direct bone formation without chondrogenesis definitely occurred using the Porous Particles of Hydroxyapatite (PPHAP) when combined with BMP as the carrier[23]. This direct bone formation was explained by rapid vascularization through the interconnected pores in the PPHAP, which did not provide the hypoxic microenvironment necessary for chondrogenesis[24,25]. Bassett[26] first proposed the higher oxygen and nutrient requirements of osteoblasts. He showed that low oxygen tension (5%) favors chondrogenesis in organ cultures of chick embryo tibial cortex, while high oxygen tension (35%) favors bone formation. Our interpretation for the intra-membraneous bone formation is due to the high vascularization in coral scaffold-cells complex group suggesting that the higher supply of oxygen and nutrients in the porous scaffold favored osteogenesis. The intra-membraneous bone formation observed in this study is incompatible with the result of Chen *et al*.[20] who, found that the bone formation occurred through endochondrol bone formation and cartilage islands could be observed in some regions of the specimens. In our study we could not find any cartilage island, but sections revealed plenty of new blood vessel formation which could lead to high oxygen tension therefore, we have to notice that bone formation inside coral scaffold-cells complex is a kind of osteogenic induction by the seeded cells and perhaps could be also due to controlled differentiation of MSCs into osteoblasts by modulating the cell culture medium.

In control group, disappearance and resorption of coral particles and some remnant pores were occupy by fibrous tissue with no evidence of bone formation were observed in histological sections, is compatible with other study[18].

CONCLUSION

In conclusion, our results through tissue engineering by combined coral with BM-DOL incubated in osteogenic medium to produce tissue-engineered bone graft, demonstrate that coral can be used as feasible biocompatible, osteoconductive scaffold provide cross-linked 3-dimension structure to the cells to adhere, proliferate and which guarantees that osteogenesis will occur simultaneously in all coral pores. Further more coral has large interconnecting paths similar to cancellous bone making it easier for blood vessels to invade the centre of material, which approved in this study. These results provide a basis for further future studies to use of tissue engineering to prove whether the coral becomes vascularized due to expansion of BM-DOL mass.

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