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IN VIVO STUDY OF SEA CORAL MATERIAL AS BONE REPLACEMENT – A CLINICAL HISTOLOGICAL STUDY

Principal Investigator

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Otorhinolaryngology-Head & Neck Surgery

ABSTRACT IN BAHASA MELAYU

OBJEKTIF

Kajian ini dirangka untuk menguji keserasian graf karang laut yang dihasilkan oleh Bank Tisu Negara, Pusat Pengajian Sains Perubatan, Universiti Sains Malaysia, sebagai pengganti tulang pada model arnab. Kesan daripada graf karang laut pada perumah dipelajari dengan kaedah pemerhatian tingkah laku selepas pembedahan, penilaian makroskopi dan penilaian melalui pengimbasan mikroskopi elektron.

KAEDAH

Ini ialah kajian experimen ke atas haiwan yang melibatkan 24 ekor arnab putih "New Zealand'. Graf karang laut diimplan ke tulang rahang untuk jangkamasa satu bulan, dua bulan, tiga bulan dan empat bulan sebelum graf diambil. Graf yang diambil diperiksa secara makroskopi dan dinilai dengan pengimbasan mikroskopi elektron.

KEPUTUSAN

Tiada kesan reaksi imun yang ketara diperhatikan pada arnab. Pemeriksaan makroskopi menunjukkan graf tersebut bergabung elok di dalam defek tulang. Ujian pengimbasan mikroskopi elektron menunjukkan pertumbuhan sel yang baik dan pergabungan sel di dalam graf karang laut.

KESIMPULAN

Kajian ini mengesahkan keserasian karang laut yang diproses sebagai material untuk menganti tulang dengan ketiadaan reaksi imun yang ketara dan pergabungan yang baik antara tulang dan tisu lembut dengan graf.

ABSTRACT

OBJECTIVE

This study was designed to assess the biocompatibility of the coral graft processed by the National Tissue Bank, School of Medical Sciences, Universiti Sains Malaysia as a bone substitute in rabbit models. The effects of coral graft in host were studied by means of behavioral observation, macroscopic evaluation and scanning electron microscopy evaluation.

METHOD

This was an experimental animal study involving 24 New Zealand White Rabbits. The coral graft was implanted into their right mandible for a period of one month, two months, three months and four months before harvesting the graft. The harvested grafts were examined macroscopically and were evaluated using scanning electron microscopy.

RESULT

There was no significant immunological reaction noted in rabbits. The macroscopic evaluation showed that the graft was well incorporated within the bony defect. The scanning electron microscopy evaluation showed a good cellular growth and cellular incorporation within the implanted corals

CONCLUSION

This study confirmed the biocompatibility of the processed coral as a bone material replacement with no significant immunological reaction, and good bone and soft tissue incorporation within the graft.

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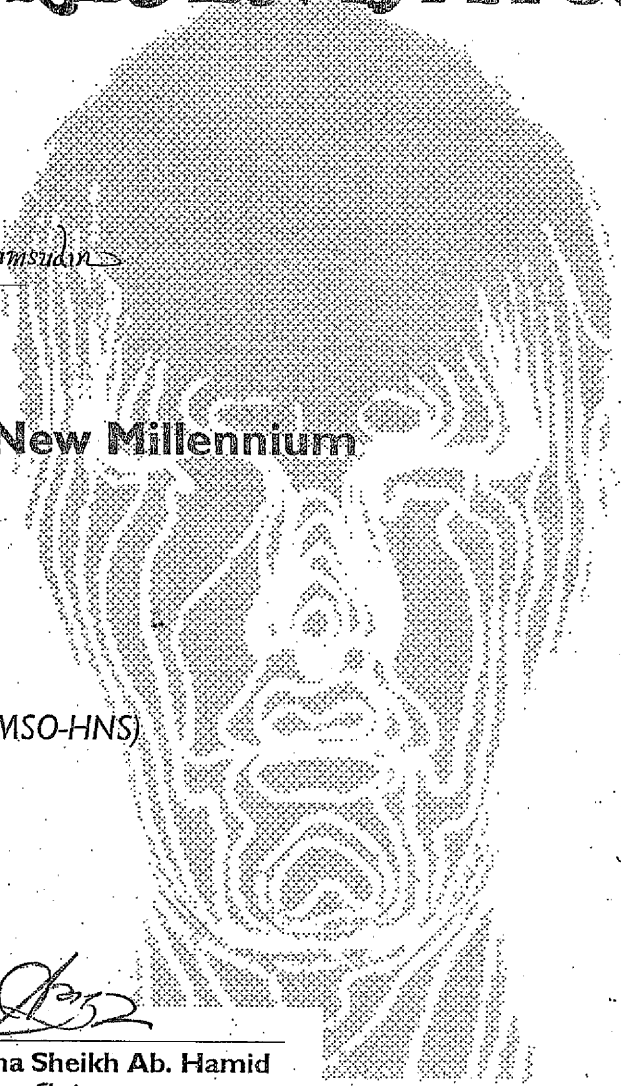

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In Vivo Study of CORAGRAF: A Preliminary Results

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Summary

The main objective of the study was to determine the biodegradability, resorption and osteoconductivity potency of coral implant. Coral blocks (CORAGRAF) were prepared from sea coral *Porites species*. The blocks were implanted in the right mandible of rabbit model. Implants were harvested at 2 and 4 weeks interval for the undecalcified for light and scanning electron microscopy. Dental hydroxyapatite (DHA) was implanted in the left mandible as control. The results of this study demonstrated that CORAGRAF is a good implant material that may receive bone healing and be resorbed in an acceptable rate. The mechanism of the resorption seem to be due to the crumbling process. Particles were the edge of the coral become powdery then second step which is cell mediated phagocytosis and dissolution in extracellular fluid.

Introduction

Natural coral is a bone graft substitute, which has been widely used in maxillofacial, orthopaedic, ORL and periodontal surgery. The capacity of coral to disappear and to be substituted by new bone distingulshes it from non-resorbable materials extensively used in these surgeries. An optimal clinical utilization of coral requires thorough knowledge of factors influencing resorption, particularly regarding the interface between implant and connective tissue, which is larger than the surface in contact with the bone. This study was designed to evaluate coral as bone substitute for reconstruction of critical mandible bone defect in rabbit using histological and scanning electron microscopy (SEM) observations.

Materials and Methods

Coral blocks (CORAGRAF) 4mm x 4mm x 4mm from sea coral *Porites species* are produced by the National Tissue Bank, Universiti Sains Malaysia (USM). They were immersed in hypochlorite solution, cleaned with ultrasound and rinsed with distilled water before final drying. DHA was prepared in the same size and used as control implant. Eight New Zealand male rabbits at 2 months old were anaesthetized by intramuscular injection of Ketamine and Xylazine. Muscle was blunt dissected to reach the mandible. The defects were created on both sides of the mandible. CORAGRAF was placed in the right side while the left side was implanted with DHA as control. Then the area was closed with resorbable suture. The implants were retrieved at 2 and 4 weeks later. For the undecalcified method, the implants were fixed in neutral buffered formalin solution, dehydrated with alcohol and infiltrated by alcohol/technovit solution. All samples were embedded and polymerized in plastic fixation medium at 450 nm wavelength and sectioned using Exakt band cutting machine. The final thin section (8µm) were grinded and stained with Mayer's H&E. For SEM method, coral implants were dried at 150°C for 24 h and dehydrated. The implants were coated with gold and examined with scanning electron microscopy (Leica Cambridge S360 at 10 KV).

Results

None of the control DHA implants showed bone formation at 2nd and 4th week or implant –bone integration at 2nd week. However, there was good integration border to the host bone at 4th week. The

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Dissertation Submitted In
Partial Fulfillment Of The Requirement
For The Degree Of Master Of Medicine
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CHAPTER 1: INTRODUCTION

Bone grafting is well-recognized in various surgical fields especially in orthopedic surgery. It is estimated about 500,000 bone graft procedures are performed in United States yearly. Bone graft has been used to provide mechanical support and to fill the osseous voids after trauma or surgical resection. Autograft has been considered the gold standard for grafting operation for the last 50 years (McAuliffe J.A, 2003).

Harvesting bone graft is considered a relatively benign procedure, however various studies have described major and minor complications arising from the harvesting procedure. It was found that 9 % of patients had suffered from major complications, whereas 10 % to 39 % of patients were found to have minor complications. Major complications include injury to the artery, nerve and urethra, deep infection; chronic pain; herniation of abdominal contents; peritoneal perforation, iliac fracture, and subluxation of the hip. Minor complications vary from wound healing problems and hematoma formation to gait disturbance and cosmetic deformity. The effects of harvesting the graft may be chronic in which most of patient experience continuous pain at the donor site six months after surgery (McAuliffe J.A, 2003).

Because of the morbidity that arises from the graft harvesting procedures, it has triggered the scientists to find another substitute for bone graft. Several studies have been conducted to find suitable bone graft alternative. The principle of being

suitable bone alternative is that the material should possess one or more of these qualities:

1. An osteoconductive matrix; the materials which provide scaffolds for bone ingrowth.
2. Osteoinductive factors; the biochemical agents that induce bone regeneration and repair, by recruiting and stimulating host parenchymal cells
3. Osteogenic cells; the cells that survive transplantation with potential to differentiate and facilitate the various stage of bone formation and fracture healing
4. Materials that provides structural integrity to match those tissues at the site of implantation, such as titanium implants.

In this research, coral has been chosen as bone substitute in the grafting operation. Its osteoconductive properties has been shown in few studies (Kuhne, Bartl et al. 1994; Ashby, Rudkin et al. 1996; Preidler, Lemperle et al. 1996; Ayers, Simske et al. 1998; Moreira-Gonzalez., I.T. et al. 2001; Thalgott J.S. et al. 2001, 2002; Stubbs, Deakin et al. 2004). In this study, the coral block was implanted in the rabbit's mandible for the purpose of macroscopic and scanning electron microscopy evaluation. It is hope that in future, coral graft will be used as bone replacement and provides a wide spectrum of usage in surgical field.

CHAPTER 2: OBJECTIVES

The objectives of the study can be divided into;

2.1 GENERAL OBJECTIVE

To study the biocompatibility of sea coral graft as bone replacement material in rabbits

2.2 SPECIFIC OBJECTIVES

To study the effects of coral on the host;

- Behavioral observation
- Macroscopic evaluation
- Scanning electron microscopic evaluation

CHAPTER 3: CONCEPT OF BONE GRAFTING

3.1. STRUCTURE OF BONE

Bone is a complex and highly organized connective tissue. Physically, it is hard, rigid and strong. Microscopically, there is presence of relatively few cells and much intercellular substances in the form of collagen fibers and stiffening substances. Microscopic components of bone generally can be divided into three; the cellular components, the bone matrix and the marrow components (Nather A et al, 2005).

3.1.1 THE CELLULAR COMPONENTS

The cellular components consist of;

- Osteoblasts

Osteoblasts are the bone forming cells, located on the surface of bone like simple cuboidal epithelium. These cells are responsible for the synthesis of organic components of the bone matrix.

- Osteocytes

These cells occupy the lacunae in the bone matrix. They possess long thin cytoplasmic process (filopodia) located in the canaliculi.

- Osteoclasts

Osteoclasts are large multinucleated cells formed by fusion of monocytes. They live in shallow depressions on the bone surface called Howship's lacunae (Nather A. et al, 2005).

3.1.2 THE BONE MATRIX

The bone matrix is composed of:

- Organic matter.

Organic matter consists of type I collagen fibres embedded in the ground substance containing proteoglycans and glycoproteins. The collagen fibres are made up of bundles of fibrils to resist pulling forces.

- Inorganic matter (mineral)

Inorganic matter is made up of stiffening substances to resist bending and compression. It is an analogue of calcium phosphate-hydroxyapatite Ca10(PO4)6(OH)2 crystals. Its association with collagen fibres is responsible for the hardness of bone (Nather A. et al, 2005). The degree of integration and orientation of the mineral and organic components gives the bone its mechanical strength.

3.1.3 THE MARROW COMPONENTS

The marrow tissue fills the cavities of long bones and occupies the spaces of cancellous bone. In long bones, the marrow is yellowish consisting of mainly fat cells and marrow cells. In flat bones, the marrow is reddish containing connective tissue, blood vessels and numerous marrow cells, which is the organ of haematopoiesis.

Macroscopically, there are two types of bone: cortical (compact) bone or cancellous (spongy) bone. Cortical or compact bone is made up of a structure of Haversians systems or osteons. Osteon is a cylinder parallel to the axis of

diaphysis. In the centre, there is Haversian canal lined by endosteum. The canal is oval or round in cross section and it runs in longitudinal direction. Each canal is surrounded by four to 20 concentric lamellae. Each osteon communicates with the others through transverse canals known as the Volkmann's canals. Each osteon is separated from neighbouring osteons by a cement line. Cancellous bone consists of a series of interconnecting plates of bone known as trabeculae. Each trabeculae contains collagen fibers arranged in the parallel lamellae (Nather A. et al, 2005).

The bone porousity between cortical and cancellous is different. Bone pore sizes (porosity) in normal cortical bone range from 1 to 100 μm whereas the trabecular bone has pores ranging from 200 to 400 μm. The size range, extent, and interconnectivity of the pores are the critical factors affecting the diffusion of nutrients, cell attachment, migration and expression, and tissue ingrowths that are necessary for bone formation and repair or regeneration (LeGeros R.Z., 2002).

3.2 OSTEOGENESIS

Process of normal bone formation (osteogenesis) involves three main steps:

1. Production of the extracellular organic matrix (osteoid)
2. Mineralization of the matrix to form bone
3. Bone remodeling by resorption and reformation

In the initial phase of the extracellular organic matrix (osteoid) production, the mesenchymal cells will undergo condensation, and then differentiate into the

osteoblasts. The osteoblasts secrete a product known as osteoid. The osteiod contains type I collagen which comprises 90 – 95 % of organic bone matrix. The collagen I is deposited in parallel or concentric layers to produce lamellar bone. It also contains proteoglycans and various glycoproteins. One of the important glycoprotein is osteocalcin, which is involved in binding the calcium ions. The osteoblasts also release membrane-bound vesicles (matrix vesicles) which contain alkaline phosphatase and pyrophosphatase. The osteoblasts activity is affected by parathormone, estrogen, growth factors and physical activity.

In mineralization phase, the osteocalcin and matrix vesicles will drive the mineralization formation along the collagen fibres. The hydroxyapatite crystals will be deposited under the action of alkaline phosphatase and pyrophosphatase. Initially only small foci of mineralization present, later they fuse together to form a larger bone. As the bone formation progress, the osteoblasts come to lie within the lacunae and are called osteocytes. The osteocytes have cell processes which occupy the canaliculi to permit the circulation of tissue fluids.

As the blood vessels invade the bone, it will bring in the osteoclasts for bone remodeling. The osteoblasts adhere to the bone surface for the resorption process. Bone growth occurs simultaneously with resorption in which, the osteoblasts continue to produce lamellae of new bone which is soon remodeled by osteoclasts.

There are two mechanisms of bone formation in human, which are intramembranous and endochondral ossification. Intramembranous ossification

[redacted]urs in flat bones, such as skull and clavicle whereas the endochondral [redacted]ification occurs in long and short bones.

[redacted] intramembranous ossification, the mesenchymal cells migrate to the site of [redacted]ventual bone formation. The cells condense, align and differentiate into the [redacted]steoblasts. The osteoblasts secrete an organic framework of extracellular matrix, [redacted]he osteoid. The osteoid is laid down in long strands. The osteoblasts will line the [redacted]steoid and begin to deposit calcium salts (mineralization) contributing to the bone matrix formation. Matrix is a mixture of organic extracellular matrix and the inorganic components of the developing bone. Together these two components give the strength, flexibility and ability to hold a defined structure. Once the organic strands are mineralized they are termed trabeculae.

Lamellas are consecutive growth rings added to the trabeculae to increase thickness. The lamellas are added onto by the mesenchymal cells and osteoblasts by cycles of osteoid secretion and mineralization. When multiple trabeculaes within the developing bone make contact with one another, a lattice structure is formed. Areas of bones may be completely filled in with mineralized osteoid. Bones that do not completely filled-in and contain lattice structures are called primary cancellous bones. Bones that completely filled-in are called compact bones.

n the endochondral ossification, mesenchymal cells migrate to the site of bone ormation. The cells are induced to become chondrocytes. These chondrocytes proliferate into a very dense mass of cells devoid of blood vessels. Cartilage forms n the shape of the ensuing bone. Chondrocytes secrete extracellullar matrix

containing primarily collagen fibres and mucopolysaccharides. The extracellular matrix is at first a loose matrix. With continued extracellular matrix secretion, chondrocytes are forced apart and the cartilage grows (interstitial Growth). The chondrocytes then become encapsulated and the extracellular matrix thickens. Due to the physical entrapment of the chondrocytes within the extracellular matrix, cell proliferation decreases.

The cartilage is also surrounded by layer of connective tissue cells derived from mesenchyme (perichondrium). The mesenchymal cells of the perichondrium also secrete extracellular matrix and add to cartilage formation by adding more layers (appositional growth). Within the body of cartilage, the encapsulated cells die and the matrix erodes. At this point, the cartilage is then replaced with bone. There is an invasion of blood vessels into the cartilage which bring in additional cells. Invasion is a sign of impending bone development. Thus, the cartilage which was once avascular is now vascularized.

The outer layer of mesenchymal cells which support apppostional bone growth is now called the periosteum. The periosteum is identical to the perichondrium except for its location. As the cartilage is degraded, strands of remaining cartilage act as templates for osteoblasts. Osteoblasts secrete additional extracellular material which is subsequently calcified. Hence there are strands of calcified bone. Trabeculae also formed by this process. Trabeculae extend by appositional growth via the osteoblasts, and they also fused. Area of bone which is not completely filled is the cancellous bone. The completely filled bone is the compact bone. Most

bones, however not all bones are mixtures containing of compact bone exteriorly and cancellous bone interiorly.

3.3 OSTEOGENESIS IN BONE GRAFTING

Skeletal defect have been replaced by bone grafts more than 300 years (Long P.K and Ibrahim S., 2005). Autologous bone grafts has remain the gold standard in bone transplantation. The autologous bone grafts contain hydroxyapatite and collagen as an osteoconductive scaffold while stromal cells have osteogenic potential. The cancellous bone and surrounding haematoma contains bone morphogenetic proteins and transforming growth factor-beta which induce and augment the regenerative process.

A successful graft is a biomechanically competent graft resulting from graft incorporation. Graft incorporation implies a viable graft that successfully unites with the host bone and attaches to the surrounding soft tissue. Healing and incorporation of autogenous graft is very much similar to fracture healing. When any graft is implanted surgically, a sequence of events occurs at the site of graft. These include haemorrhage, inflammation, revascularization of tissue, substitution and remodeling of graft with locally derived tissue.

The first phase of healing of autogenous grafts is the vascular ingrowth and progenitor mesenchymal cell invasion. These occur during the first three weeks. The vascularisation is slower in cortical graft than cancellous graft which may take

eight weeks in canine model. The second phase occurs between three to twelve weeks. The involved process is the combination of osteoblastic new bone formation and osteoclastic resorption of the necrotic graft. Creeping institution occurs together with osteoclastic resorption, preceeding the osteoblastic new bone formation. The third phase occurs between three to six months after grafting; is the remodeling of the trabeculae to a mature pattern. In humans, the process may take twice the duration. The segmental cortical grafts loose approximately half of the biomechanical strength during the first six months. This is due to osteclastic resorption and is slowly reversed during the second year. The highest incidence of mechanical graft failure occurs between six to eight months after transplantation.

3.4 BASIC CONCEPT OF BONE GRAFTING

The need to replace missing bones and teeth with other materials was evident in prehistoric times. From fifth or fourth centuries BC until the first or second century AD, archeological findings showed that several materials were used to replace missing human teeth including ox teeth, shells, coral, ivory (elephant tusk), wood, human teeth from corpses, and metals; gold and silver (Le Geros R.Z., 2002).

The first bone graft used to repair the craniofacial skeleton was an allograft in 1632 where the cranium of a soldier was constructed with the dog calvarium (Rosdan S., 2000). The first autogenous bone graft procedure was performed in 1881 (McAuliffe. 2003). Bone grafting has become an accepted technique in the early

part of the 20th century due to work of Fred Albee of New York (1876 – 1945) using cortical grafts (Long P.K. and Ibrahim S., 2005)

In modern times, autografts are the gold standard for bone repair and substitution. Success with the use of allografts also has been reported. However, the use of autografts has serious disadvantages, such as additional expense and trauma to patient, possibility of donor site morbidity, and limited availability. The use of allografts also has complications such as viral transmission and immune reaction (Le Geros R.Z., 2002). Therefore, there was a critical need to develop other materials to substitute the autografts and allografts without the drawbacks of these two. In recent years, commercial and experimental materials for bone substitutes have been used including metals, polymers, corals, calcium phosphate and calcium phosphates composites.

3.4.1 TYPES OF BONE GRAFT

3.4.1.1 AUTOGRAFT

Autograft is the term used when the graft is taken from other parts of body skeleton. This is the most suitable graft as it has all the three properties. However, the amount of graft that can be taken is limited. The graft usually is taken from the iliac crest. There are several types:

- Cortical bone graft
- Cancellous bone graft

- Corticocancellous graft
- Particulate bone

Cortical bone grafts are primarily used at the site where there is great mechanical stress with proper fixation. This form of graft is useful in long bone but not in membranous site such as facial skeleton (Habal M. B., 1992).

Cancellous bone graft is used in majority of cases because of its easy application to achieve fusion and to correct discontinuity. It also can be used in clean contaminated and grossly contaminated wounds. However cancellous bone does not have mechanical strength as cortical bone (Habal M.B., 1992).

In cancellous bone with large open areas, revascularization usually takes very well, more rapid and more complete than cortical grafts. Corticocancellous bone usually produces the best results because it enables good vascularization and also gives good mechanical strength. Particulate bone is the graft which composed of small chips of bone and is usually applied without fixation.

3.4.1.2 ALLOGRAFT

Allograft is a graft taken from another donor from same species. Allograft bone is available in fresh, frozen, or freeze-dried (lyophilized). Fresh allografts are rarely used because of immune response and risk of transmission of disease. The frozen and freeze-dried types are osteoconductive but only weakly osteoinductive. The method of preparation has minimized the viable cells to confer the osteogenic properties. Freezing or freeze-drying also appears to lower the antigenicity of the

allograft (Keating J.F. and McQueen M.M., 2001). Frozen grafts usually are larger graft whereas smaller grafts are freeze dried. Frozen allograft has essentially the same biomechanical properties as native bone. But when it is freeze-dried, lyophilization decreases compressive strength by 30%, bending strength by 40%, and torsional strength by 60% (McAuliffe J.A., 2003). These types of grafts are commonly used in patients who require bone grafting but do not have adequate autograft bone.

3.4.1.3 XENOGRAFT

Xenograft is a graft taken from a different species. The immunologic disparity of untreated xenografts causes rapid resorption , which prohibited its use. However due to limited supply of autografts and allografts, xenografts have been explored for the use as materials for bone replacements. Many methods of preparation of xenografts have been explored, including freeze dried calf bone, decalcified ox bone, deproteinized bone, osporum and anorganic bone. Xenograft act as mechanical spacer to prevent soft tissue ingrowth, which would be detrimental to osteogenesis and healing (Murphey M.D. et al, 1992). Several studies on Kiel bone (deproteinized bone from freshly sacrificed calves) had demonstrated osseous formation within the graft (Murphey M.D. et al, 1992).

3.5 PROPERTIES OF BONE SUBSTITUTES

Bone grafting has a well recognized role in orthopedic surgery. However, when extensive grafting is required, amount of autologous bone may not be adequate, as alternative, other materials have been used. The properties of an autologous bone, which is osteogenic, osteoinductive, osteoconductive and completely biocompatible are the basic concept of developing materials as bone substitutes.

Osteogenic materials are materials that have the capacity to form bone, which implies that they have living cells such as osteoblasts or osteocytes or they have capability to produce these cells.

Osteoinductive materials are materials which able to stimulate cells in the local environment to undergo phenotypic conversion into osteoprogenitor cells types which capable of bone formation. The major phases of osteoinduction are chemotaxis, mitosis and differentiation. Chemotaxis is a directed migration of cells in response to the chemical gradient. The next step is mitosis indicated by proliferations of newly attached mesenchymal cells. The mitogenic action can be quantitated by measuring DNA synthesis, using radioautography and isotope incorporation. This phase is followed by differentiation of cartilage, vascular invasion and bone differentiation.

Osteoconductive materials have neither capability to form bone nor induce their formation. They merely provide an inert biocompatible scaffold, which the cellular

elements can grow into the material and gradually regenerate normal bone (Keating J.F and McQueen M.M., 2001). Osteoconductive substances cannot induce bone formation at extraskeletal sites.

There is no bone substitute which embodies all these qualities. Most of the available materials have tended to be either predominantly osteogenic or osteoinductive, or purely osteoconductive (Keating J.F. and McQueen M.M., 2001). The materials can be divided based on these two headings.

3.5.1 OSTEOGENIC AND OSTEOINDUCTIVE MATERIALS

3.5.1.1 COLLAGEN MINERAL COMPOSITE GRAFTS

The main constituent of the organic matrix of bone is type I collagen. Collagen alone is not effective as an osteoinductive material. It is combined with other materials such as tricalcium phosphate as a composite graft. By addition of marrow constituent gives the material osteogenic and osteinductive properties.

3.5.1.2 DEMINERALISED BONE MATRIX (DBM) AND BONE MORPHOGENETIC PROTEIN (BMP).

Urist in 1965 reported that demineralised bone matrix (DBM) had induced the formation of heterotopic bone. He was able to show that implantation of DBM in rat muscle resulted in formation of bone in the soft tissue (Keating J.F and McQueen M.M., 2001). Since then, it had been shown that the active components of

demineralised bone matrix are a series of glycoproteins which belong to a group referred as transforming growth factor (TGF-Beta) family. Within this group, the compounds of particular interests are bone morphogenetic proteins (BMP), which are BMP-2 to BMP-6 and BMP-7 to BMP-9. These BMP are osteoinductive.

3.5.1 OSTEOCONDUCTIVE MATERIALS

3.5.2.1 CALCIUM SULPHATE

This material is most familiar to orthopedic surgeons as 'plaster of Paris'. It has been used to fill defect bony defect in the last century. It resorbs very rapid exceeding the capacity of surrounding bone to regenerate.

3.5.1.2 CALCIUM PHOSPHATE CEMENT

These materials are growing in popularity and are osteoconductive. Their structure is closer to dahllite (carbonated hydroxyapatite which forms the bulk of mineral phase of bone). They are prepared in combination of monocalcium phosphate, tricalcium phosphate and calcium carbonate in powder form, mixed in sodium phosphate solution. This forms a paste which turns to a hard material within 10 to 15 minutes. After 24 to 48 hours, it has a compressive strength similar to cancellous bone (Keating J.F and McQueen M.M., 2001).

Animal studies had shown that this material is biocompatible. However, in man, complete resorption was very slow. In a study of the use of calcium phosphate cement in distal radius and tibial plateau fractures, the material was still present even after two years of implantation (Keating J.F and McQueen M.M., 2001).

3.5.1.3 CERAMICS

When natural mineral salts subjected to a very high temperature, highly crystalline materials are produced known as ceramics. Some of these materials are biocompatible and osteoconductive. The most popular material is tricalcium phosphate. It is very stable and resorbs very slowly (Keating J.F and McQueen M.M., 2001).

3.5.1.4 CORAL DERIVATIVES

These materials are derived from the calcium carbonate of sea corals. The pore structure produced by certain species is similar to human cancellous bone. These derivatives will be further discussed in details as a bone replacement.

3.6 SEA CORAL GRAFT AS BONE SUBSTITUTES

3.6.1 BIOLOGY OF SEA CORAL GRAFT

Natural organic skeleton in invertebrates, such as spiny starfish, sea urchins, corals and others have a great potential as scaffolds for bone regeneration. They provides structural environment which is similar to bone. It also has high macrosity. This property allows vascularization into the pores; allow them to differentiate and

forming bone trabeculae (Ben-Nissan B et al, 2001). Out of these, coralline is the most useful and frequently used.

Coralline apatites can be derived from natural sea coral. Coral exist in two forms: as soft forms and as hard forms. The coral skeletons are composed of calcium carbonate in the form of aragonite which constitutes about 98-99%. The other percentage is formed by trace elements and amino acids. The coral specimen used for bone grafting includes Porites, Acopora and Fungia species.

As it is natural, it has certain optimal strength and structural characteristics. The pore structure of coralline calcium phosphate produced by certain species is similar to human cortical or cancellous bone, making it a suitable material for bone graft applications. Two species of coral with different pore sizes and interconnecting fenestrations have been established for use of bone substitutes, which are coral from genus Porites and Goniopora. Coral from genus Porites (HA-200) has an architectural similar to cortical bone, whereas coral from genus Goniopora (HA-500) is similar to cancellous bone.

Pore interconnection sizes are of utmost importance when hard and soft tissue ingrowth involved. Pores less than 10 um inhibit cellular ingrowth, while pores between 15 and 50 um help fibrovascular colonization. Pores between 50 and 150 um determine osteiod growth and pores higher than 150 um facilitate internal mineralization (Cerroni L. et al, 2002). It has been shown that implants with average pore sizes of around 260 μm had the most successful in-growth as compared to no implants. It was further reported that the interaction of the primary

osteons between the pores via the interconnections allows propagation of osteoblasts (Heness and Ben-Nissan 2004). Coralline hydroxyapatite has macroporosity with a mean diameter of 230 um and interconnecting pores with a mean diameter of 190 um which make it a suitable matrix for bone growth.

The incorporation of host bone into the coral relies on serial sequence of events. These include vascular ingrowth into the coral, differentiation of osteogeniter cells, osseous ingrowth into the porous coralline microstructure with woven and lamellar bone formation followed by bone remodeling (Walsh et al, 2003). These processes occur together with graft resorption by slow simple dissolution and osteoclastic activity. To be an ideal bone substitute, the graft should resorpt fully. The resorption process also allows additional space for new bone formation and it also decreases the load-sharing environment. Complete implant resorption was noted at eight eight weeks in the cortical bone and six weeks in cancellous bone. One of the major disadvantage with coral is that their initial mechanical weakness. But after the osseous ingrowth, their mechanical property will improve as the new bone is overlaid (Walsh et al, 2003).

3.6.2 TYPES OF CORAGRAFT

Coral exists in either true form (coral apatite) or coverted form (coralline hydroxyapatite). The converted form is achieved via hydrothermal conversion or microwave conversion. Coral and converted coralline hydroxyapatite have been used as bone grafts and orbital implants since the 1980s (Heness G and Ben-Nissan B., 2004)

3.6.2.1 Hydrothermal Conversion of Coralline Apatite to Hydroxyapatite

The hydrothermal method was first used in 1974, for hydroxyapatite formation directly from corals by Roy and Linnehan. It was reported that complete replacement of aragonite ($CaCO_3$) by phosphatic material was achieved less than 533 K and 10^3 MPa by using the hydrothermal process (Heness G and Ben-Nissan B., 2004). In 1996, hydroxyapatite derived from Indian coral using hydrothermal process was reported. However, the resultant material was in the form of a powder and required further forming and sintering (Sivakumar, Kumar et al. 1996). During the hydrothermal treatment, hydroxyapatite replaces the aragonite whilst preserving the porous structure. The following exchange takes place:

$$10CaCO_3 + 6(NH_4)_2HPO_4 + 2H_2O \rightarrow Ca_{10}(PO_4)_6(OH)_2 + 6(NH_4)_2CO_3 + 4H_3CO_3$$

The resulting material is known as coralline hydroxyapatite, whether in the porous coralline structure or in powdered form.

3.6.2.2 Microwave Conversion of Coralline Apatite to Carbonated Hydroxyapatite

The aragonite conversion to carbonate hydroxyapatite was achieved by using microwave processing technique. Higher extents of conversion were reported by using this technique. Conversion of Australian coral to monophasic hydroxyapatite by using a two-stage process in which the hydrothermal method was done first, followed by patenting hydroxyapatite sol-gel coating based on alkoxide chemistry.

This had caused 120% increase in the biaxial strength of the double-treated coral in comparison to coralline hydroxyapatite (Heness G and Ben-Nissan B., 2004).

An advantage of coral hydroxyapatite is the beneficial biocompatible properties. It is rapidly integrated into the human body, while at the same time the body is not activated to the invasion by a foreign body. Perhaps this is its most interesting property is that promote bond to bone forming an indistinguishable unions.

Disadvantage of coral hydroxyapatite is its poor mechanical properties (in particular fatigue properties) means that it cannot be used in bulk form for load bearing applications such as use of metallic implants in orthopedics. That is why in certain study, it has been used to coat the metallic implant.

Coating of coral hydroxyapatite have good potential as they can exploit the biocompatible and bone bonding properties of the ceramic, while utilizing the mechanical properties of substrates such as Ti6Al4V (titanium alloy) and other biocompatible alloys. When the metallic materials are required for the strength of mechanical properties, they can be coated with hydroxyapatite which will provides an osteophilic surface for bone to bond to, anchoring the implant to the host bone.

3.7 RESEARCHES ON BONE SUBSTITUTES

The approval for investigation of porous coralline for traumatic defect was achieved in 1982 and it has been experimented for last twenty years. From the studies, they had shown promising results. The studies have been conducted in animals and human. Excellent results as bone substitute were reported particularly in craniofacial, orthognathic, opthalmologic, orthopedic and spine applications (Thalgott J.S. et al, 2002). Along with coral, the other materials have also been studied as bone replacement. In certain studies, they have tried to incorporate the osteogenic and osteoinductive materials to enhance the bone ingrowth into the implants.

Two tests were done on corals regarding its adverse effects: The Gel Clot Test Method and The Ames test. The gel clot test method showed that prepared coral has endotoxin level of 0.3 EU/ml which is less than expected for Food and Drug Administrative Guideline, 1987. The Ames test results also had demonstrated that the prepared coral did not exhibit any mutagenic activity, thus can be considered non-genotoxic (Suzina A.H. et al, 2005).

In vivo study of coral material on the calvarial of Sprague-Dawley rats showed that the implants do not have any adverse effects to the general health of the rats. Histological analysis showed fibrovascular growth, presence of abundant osteoblasts and osteoid seam and very minimal inflammation response with minimal giant cells. This study further confirmed the osteoconductivity of corals.

Wang (1996) had studied the Basic Fibroblast Growth Factor (bFGF) in promoting bone growth using bone conduction chambers. The coralline hydroxyapatite were used as the osteoconductive materials. The study showed that pretreatment of grafts with Basic Fibroblast Growth Factor improves bone incorporation. Basic Fibroblast Growth factor has been shown to stimulate endothelial cell mitosis in vitro and angiogenesis in vivo. Another study conducted by Schnettler et al using bFGF loaded ceramic in pig model revealed that it was superior to allografts in terms of bone ingrowth and did not differ from bone autografts.

A study done by Kim et al (2005) on the periodontal healing in bony defects in dogs comparing between autogenous bone and coral derived material. The study revealed that there was no different between those two groups. The magnitude of new bone formation was similar and can be safely implanted without significant healing aberrations.

Walsh et al (2003) had studied the difference of resorption of coralline hydroxyapatite with different HA layer thickness in rabbit at 14% and 28%. From the study, by altering the thickness of hydroxyapatite layer did not alter the implant resorption in the core of implant, but the surface showed increase in time of resorption rendering it more stable to guide the osteoconduction. There was flowed bone growth from periphery to the centre of implant. Progressive resorption of both implants was evident at six weeks and haversian remodeling was observed by 24

weeks. The new bone formation within the defect supported the osteoconductive properties of coralline hydroxyapatite.

Stubbs D, et al also evaluated the biocompatibility of hydroxyapatite in rabbit tibia. In his study, he had used porous hydroxyapatite (Pro Osteon 200 R) in combination with two other implants; with calcium sulphate slurry as the first group and with calcium sulphate pellets as the second group. Histological examination of specimen revealed early woven bone in both groups, however there was no bone found in the centre of calcium sulphate pellets. There was no significant immunological response noted in both groups.

Several studies have been done on coralline hydroxyapatite in craniofacial applications (Ayers, Simske et al. 1998; Cottrell and Wolford 1998; A., I.T. et al. 2001; Moreira-Gonzalez., I.T. et al. 2001). Ayers R.A (1998) had done a study on evaluation of the long term ingrowth and residual microhardness of porous hydroxyalatite in 17 patients who had undergone maxillary hydroxyapatite implant (four to 138 months of implantation). It had shown that the implant was incorporated into the existing bone. Tissue ossification was present in all implants with significant growth. The amount and maturity of bone growth was proportional to the duration of implantation. Even in the shortest implantation (four months), bone growth within the the pores was normal with presence of a layer of osteoblasts forming an osteoid seam. Over the time, the soft tissue gave way to woven bone followed by lamellar bone. From lamellar stage, it underwent remodeling with presence of Haversian canals. The type of bone was of woven

type with early lamellar stage. The residual microhardness of the bone within the implant was equivalent to the surrounding bone.

Cottrell D.A et al (1998) had evaluated long term clinical and radiographic results of porous block hydroxyapatite as bone graft in orthognathic surgery and craniofacial augmentation. It had shown that use of porous block hydroxyapatite gave high percentage of success and efficacy. However, the use of materials in alveolar cleft grafting resulted in 100 % failure rate.

An interesting study conducted by Moreira-Gonzalez A. (2001) which was a retrospective study reviewing the use of porous coral derived hydroxyapatite in craniomaxillofacial augmentation for cosmetic and reconstructive purposes and long term results was evaluated. The operation was done by placing the porous coral subperiosteally in the bony pocket at the site where to augment. From the study, it was concluded that hydroxyapatite granules can be placed on any bony area of the craniofacial skeleton for cosmetic or reconstructive purposes. The anterior wall of the maxilla, including the paranasal area and pyriform aperture, were augmented primarily for cosmetic reasons. A prominent and well-delineated malar area is considered to be a youthful appearance in both men and women.

Placing the granules in the paranasal area provided fullness and lifting of the nasal tip and partially reduced the depth of the nasolabial fold. It also improved the thickness of the upper lip. The maxilla was the most difficult area to place the granules because they tended to migrate inferiorly out of the "pocket" into the soft

tissue along the path of dissection resulting in loss of projection and need for further augmentation or correction at this site. There has been increasing interest in mandible angle and chin augmentation among male patients with intention to give more prominence to this area. This was easy to do with porous coral-derived hydroxyapatite granules, and a better angle shape frequently produced.

Several studies were conducted on coral hydroxyapatite on spinal fusion (Thalgott J.S. et al 2001, 2002; McConnel J.R. 2003; Minamide, M. et al. 2005). Thalgott J.S, (2001,2002) had done two studies on coralline hydroxyapatite (HA). In the first study, he used HA with or without demineralised bone matrix as an adjunctive to autologous bone in augmented anterolumbar and posterolumbar fusion. He concluded that coralline hydroxyapatite with or without demineralised bone matrix as an adjunct to autologous bone performs similarly to autogenous bone alone.

In his second study, he compared the osteoconduction of coralline hydroxyapatites in combination with others substances in posterolateral lumbar fusion. There were three groups: coralline with 1.5 ml bone marrow; coralline with 1.5 ml autogenous iliac bone; coralline with 500ug bovine-derived osteoinductive bone protein extract. From the results, the coralline combined with bone marrow produced no solid fusion, when combined with autogenous iliac crest bone resulted in solid fusions in 50%. The third group, combination with growth factor resulted in 100% solid fusion.

However in study done by McConnel, which the material was used in cervical fusion. It was found that coralline hydroxyapatite did not maintain adequate

structural integrity and cannot be considered as a reliable alternative in cervical body fusion.

Ninamide M. et al (2005) had done a study on posterolateral intertransverse fusion of L4 and L5. He had compared four materials as implants with seven animals in each group: (1) autologous bone; (2) porous hydroxyapatite (HA) with type I collagen sheet with 100ug BMP; (3) HA with low bone marrow cells – 1 X 10^6 cells/ml; and HA with high marrow cells – 1 X 10^6 cells/ml. The result revealed that cultured bone marrow in type I collagen mixed with porous hydroxyapatite gave good result. All the animals in that group demonstrated bony fusion, followed by group 4 and 1. There was no fusion in group 3 (HA with low marrow cells).

Coral hydroxyapatite has been used as bone substitute in foot and ankle surgery in an eight years study which it has given an excellent result. It also has been used for restoration of osteochondral defects. It was found that during the follow-up, there was some resorption of the material together with new bone formation and fusion of the defects (Shah M, 2003).

Coral hydroxyapatite is a calcium carbonate derivative which has high affinity for proteins. This property makes it an ideal carrier for bioactive peptides, bone growth factors or mesenchymal stem cells. By adding these osteoinductive agents enhance bone growth.

From previous studies that have been done on coral, we can concluded that coral graft is compatible for bone material replacement. In certain circumstances, it can be used in the weight bearing area, such as spine but it has to be augmented with other materials, for example plating (Thalgott J.S. et al. 2002). The use of porous coral hydroxyapatite also shows a good result in craniofacial augmentation (Moreira-Gonzalez., I.T. et al. 2001). The osteoconductive property of the coragraft can be enhanced by mixing with an osteoconductive agents, such as demineralised bone matrix, or marrow cells (Minamide, M. et al. 2005).

CHAPTER 4: METHODOLOGY

4.1 ANIMAL STUDY

The study was an experimental study conducted on 24 New Zealand white rabbits. These rabbits were laboratory bred in the animal house.

The inclusion criteria include:

1. Rabbits more than 3 months old
2. Healthy rabbits

The exclusion criteria include:

1. Rabbits less than 3 month of age
2. Unhealthy rabbits, which was characterized by unkempt appearance, being inactive and presence of discharge from nose or genitalia.
3. Pregnant rabbits

The rabbits were grouped into 4 groups; 6 rabbits in each group. The group were namely as group A, B, C and D.

1. Group A – due for graft harvesting at 4 months post implantation
2. Group B – due for graft harvesting at 3 months post implantation
3. Group C – due for graft harvesting at 2 months post implantation
4. Group D – due for graft harvesting at 1 months post implantation

These rabbits were tagged based on their group and numbers (using the study proforma):

1. Group A - A1, A2, A3, A4, A5, A6
2. Group B – B1, B2, B3, B4, B5, B6
3. Group C – C1, C2, C3, C4, C5, C6
4. Group D – D1, D2, D3, D4, D5, D6

4.2 IMPLANTATION MATERIALS

Material used for grafting was the processed corals from National tissue bank, School of Medical Sciences, Universiti Sains Malysia. It was cut into 4 X 4 X 2 mm in dimension under sterile technique (figure 4.7).

4.2.1 SITE OF IMPLANTATION

The coral graft was implanted into right mandible.

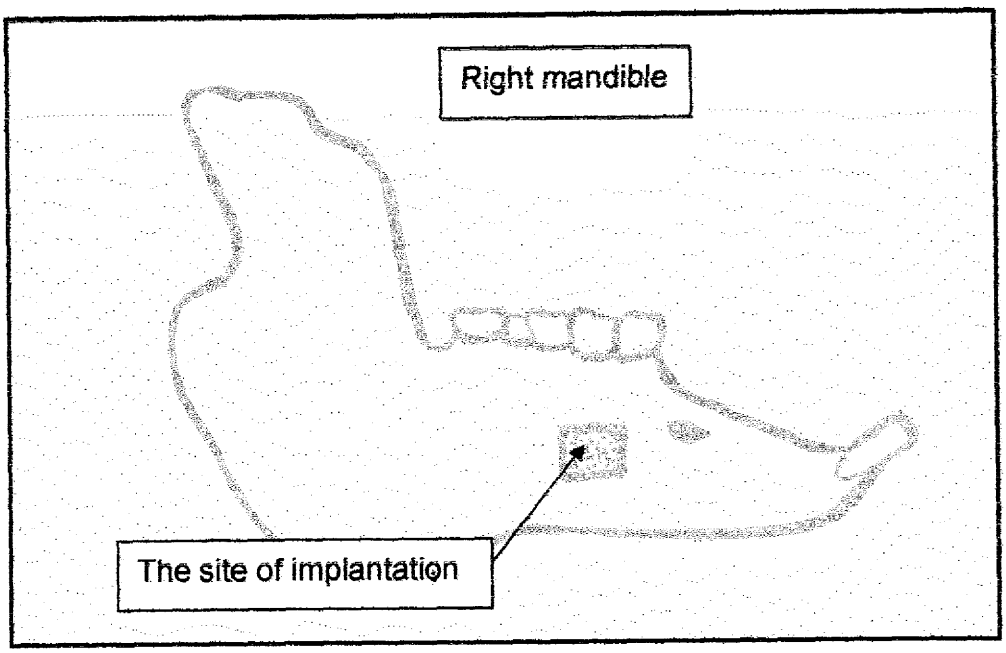

Figure 4.1: The site of coral graft implantation into the right mandible

4.3 EXPERIMENTAL SURGERY

4.3.1 PREPARATION OF ANIMAL

The selected rabbit was weighted and prepared in the scrubing room. Appropriate dosage of Ketamine and Xylazine were calculated and administered, based on per kilogram body weight (Ketamine : 35 mg/kg and Xylazine 5 mg/kg).

4.3.2 SEDATION OF ANIMAL

Intramuscular Ketamine was administered at anterolateral aspect of thigh. After the rabbit was partly sedated, the ear was then prepared for administration of Intravenous xylazine. The rabbit's ear was shaved and large vessel identified. The ear was then swabbed with chlorhexidine. The rabbit was restrained adequately with restrainer for intravenous Xylazine to be given. This resulted in a short and abrupt reaction in which the rabbit seem to resist the injection. After the rabbit was fully sedated, its right mandible was shaved in preparation for implantation procedure. The rabbit then transferred to operation theatre.

4.3.3 IMPLANTATION PROCEDURE

In the operation theatre, the rabbit was placed on left lateral position with head partially extended and supported with a sandbag beneath the neck. The mandible was painted with povidine iodine and sterilely draped. Intramuscular Ampicillin 0.2 ml was given intraoperatively before the procedure started (figure 4.6).

The right mandible examined and the area planned for grafting was infiltrated with 0.5 cc 1% xylocaine and 1:100000 adrenaline for local anesthesia and to reduce the blood loss (figure 4.11). A two centimeter horizontal incision parallel to the

lower border of mandible was done up to fibrous layer. Then the underneath tissue was opened using blunt dissection technique until the mandible exposed (figure 4.12 – 4.13). An area, which is located posterior to the mental nerve was chosen as the site of implantation. The bone was drilled for about 4 X 4 mm in dimension. The coral graft was implanted without fixation (figure 4.15). The periosteum was then opposed using 4.0 catgut to keep the coral graft in position. The Muscles was sutured with 4.0 catgut. Homeostasis secured and skin was sutured using 4.0 silk (figure 4.16). The operative time and blood loss were noted.

4.3.4 POST OPERATIVE CARE

The animal was kept in their cage postoperatively. Two animal per cage provided that they were done on the same day.

4.3.5 ANIMAL MAINTAINENCE

The rabbits were kept in different cages according to date of implantation. Generally, they were monitored for their activity, color of their coats, presence of nasal secretion or any foul genitalia. Local wound was observed for any inflammation or discharge.

4.3.6 ANIMAL EUTHANASIA

The rabbits were sacrificed at 1, 2, 3 and 4 months post implantation. They were euthanized with an overdose of pentobarbital. The head decapitated. The mandible was separated from the head. The right mandible was taken for preparation of specimen.

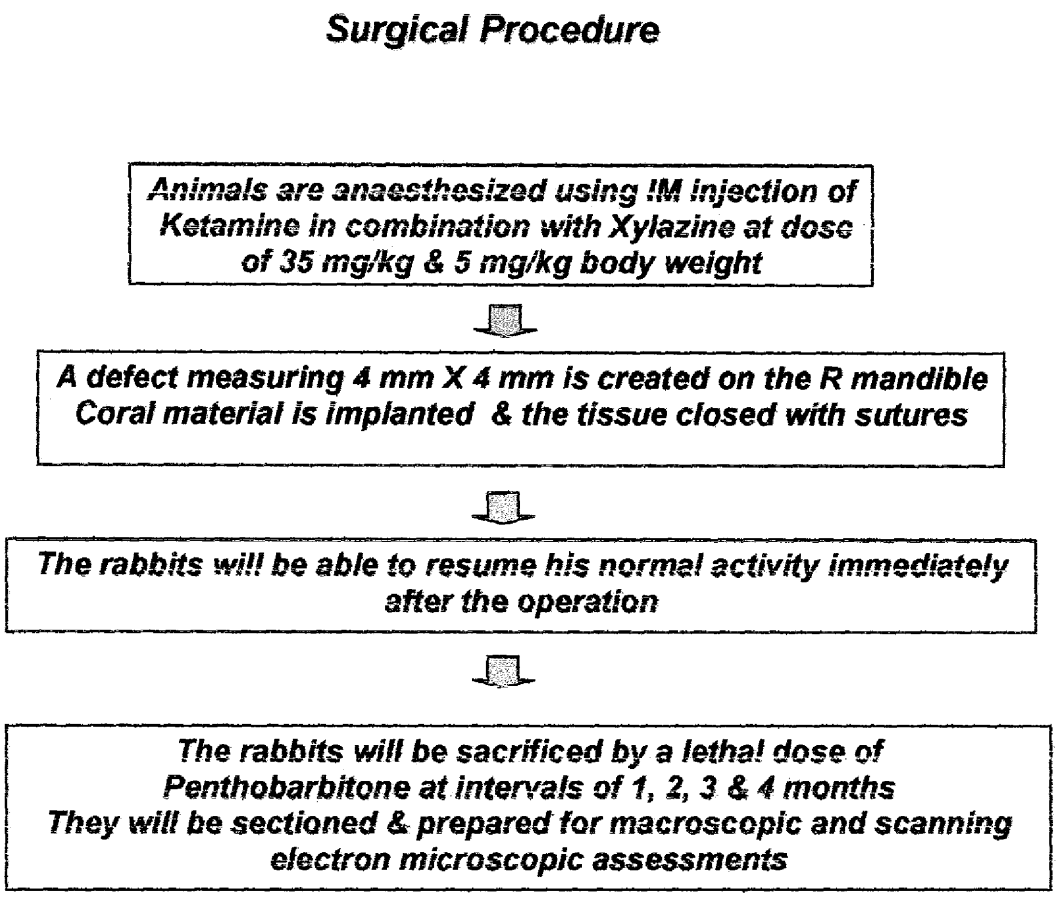

Figure 4.2: The flow chart of surgical procedures done on rabbits

4.4 SAMPLE ANALYSIS

4.4.1 PREPARATION OF GROSS SPECIMEN

Right mandible was carefully dissected from the surrounding tissue and area of the implantation was examined for the smoothness, the edge between the graft and the bone and presence of fibrous tissue (figure 4.17 – 4.18). The specimens were assessed inclusive of macroscopic evaluation and scanning electron microscopy examination.

4.5 ASSESSMENT OF SAMPLES

After dissection of the specimens, each specimen was examined by evaluating the margin between the coral and the host bone and the incorporation of soft tissue to the implant surface.

For scanning electron microscopy evaluation, the specimens were examined using scanning electron microscopy which was done in USM Penang. The freeze dried specimens were placed in sputter coater for coating process. After completed coating process, they were analyzed with the electron microscopy (figure 4.19 – 4.20).

Figure 4.3: The setting of operation theatre in the animal house

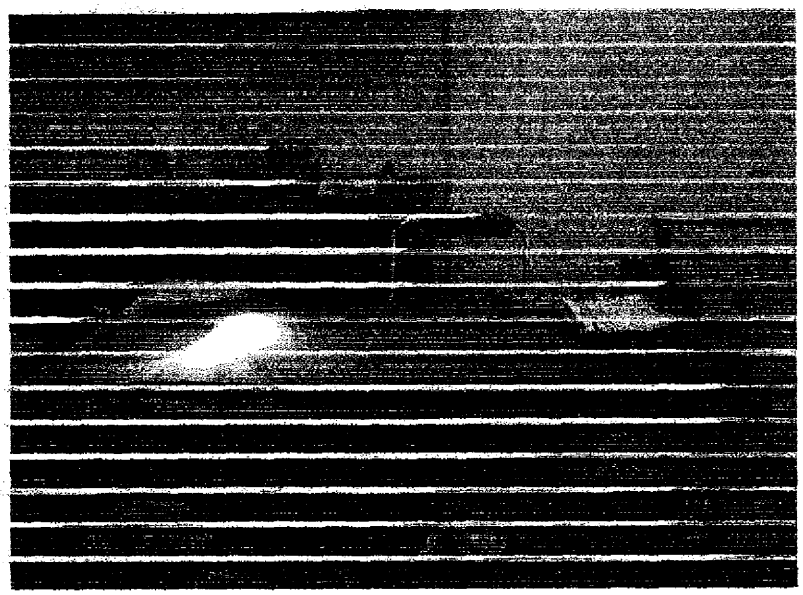

Figure 4.5: The instruments used during the operation

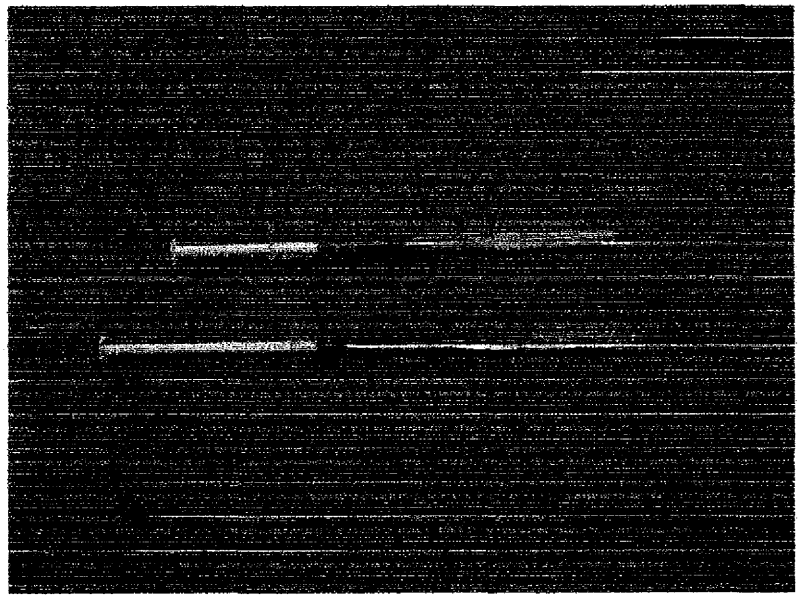

Figure 4.6: The intramuscular Ampicillin (above) and the local anesthesia (below)

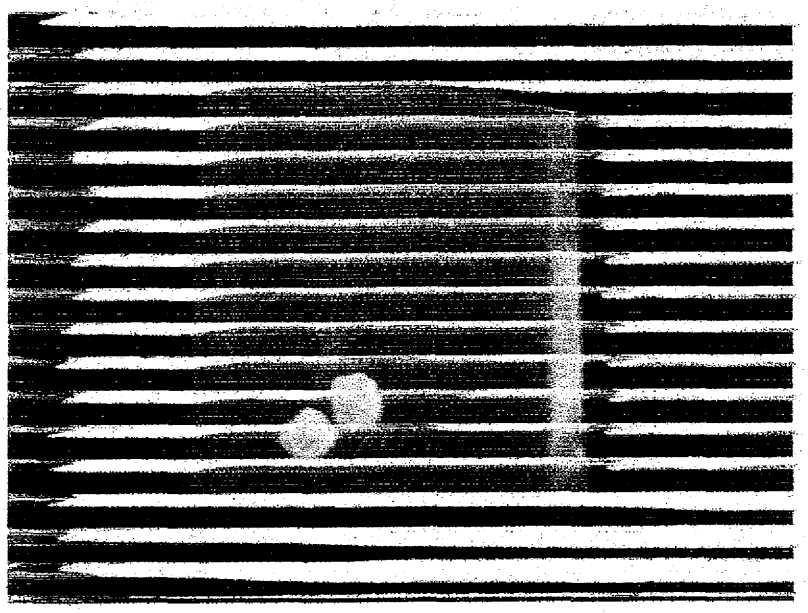

Figure 4.7: The coral graft used for implantation

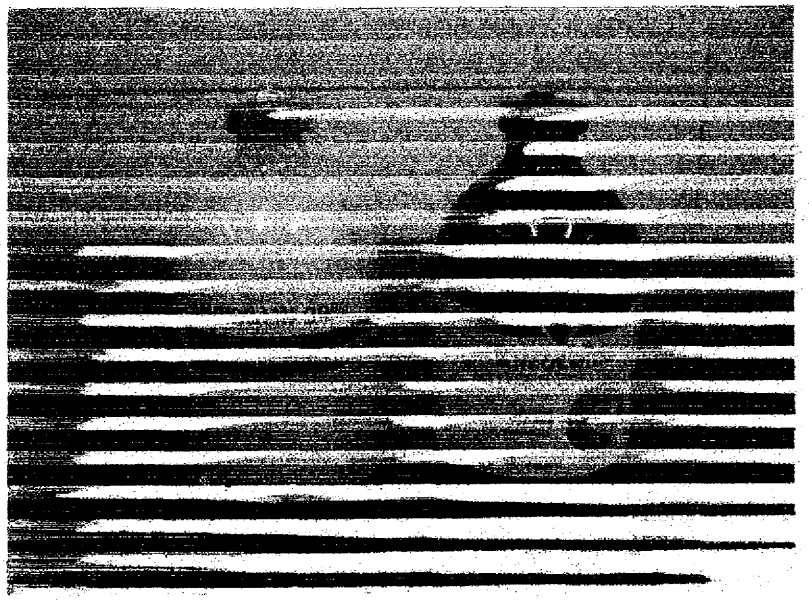

Figure 4.9: The rabbit is placed on the operation table

Figure 4.10: The right mandible is painted with povidone iodine

Figure 4.11: Administeration of local anesthesia

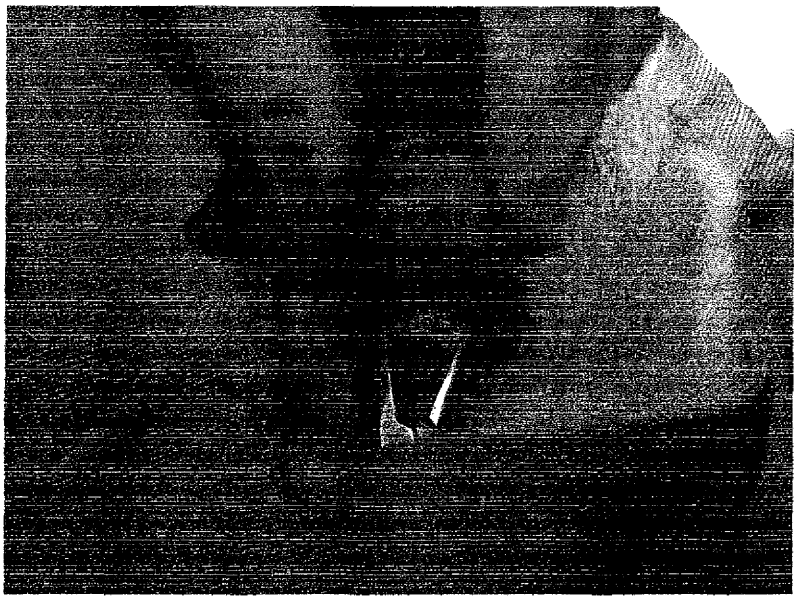

Figure 4.12: Blunt dissection of soft tissue

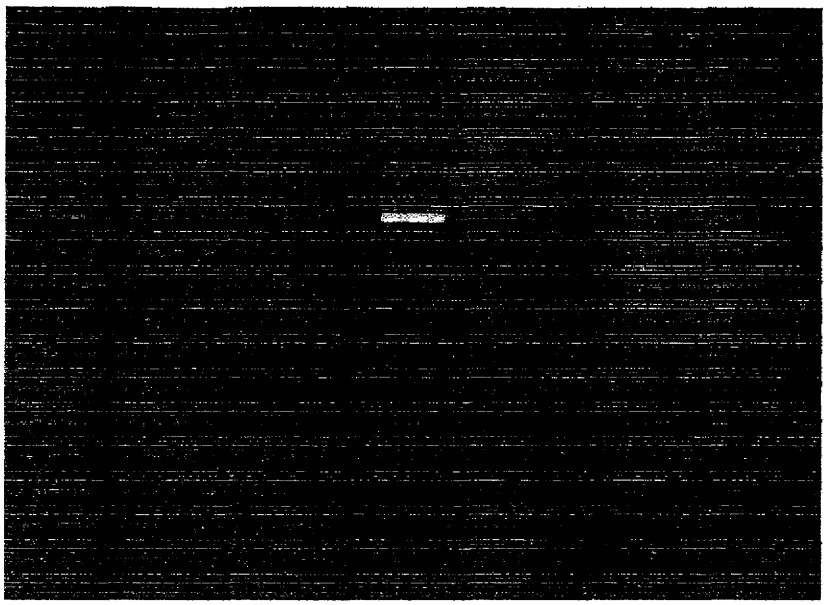

Figure 4.13: The exposed mandible before creating the defect

Figure 4.14: The prepared bony defect for the implantation process

Figure 4.15: The implanted coral graft in the bony defect

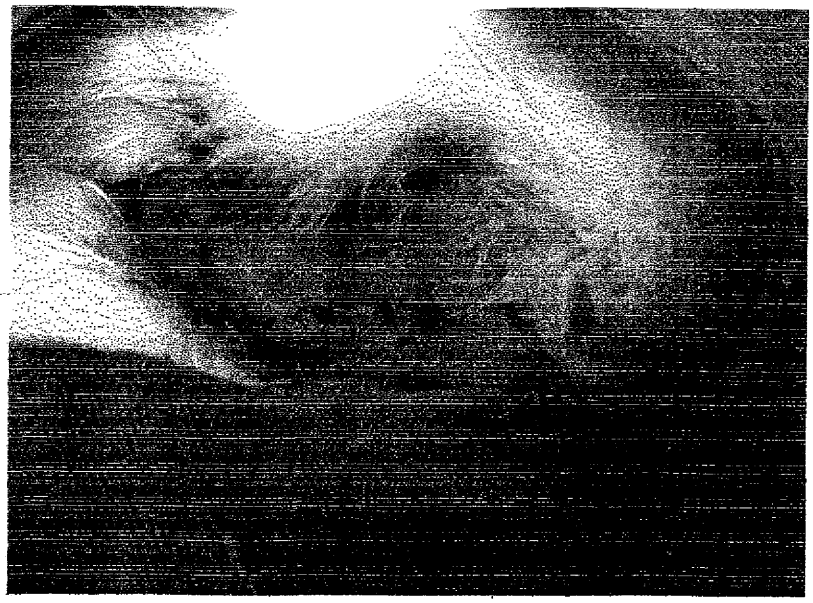

Figure 4.16: The wound closed with silk suture

Figure 4.17: Preparation of gross specimen

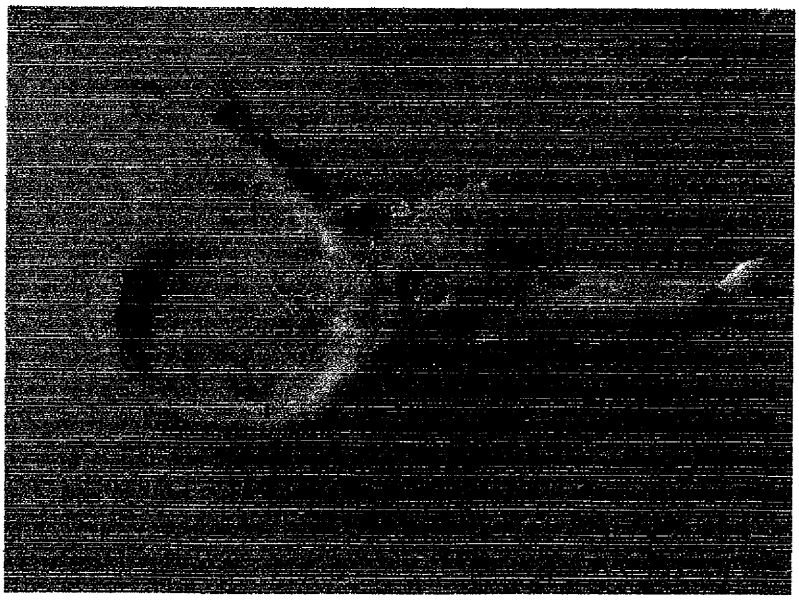

Figure 4.18: The right mandible post dissection, for macroscopic evaluation

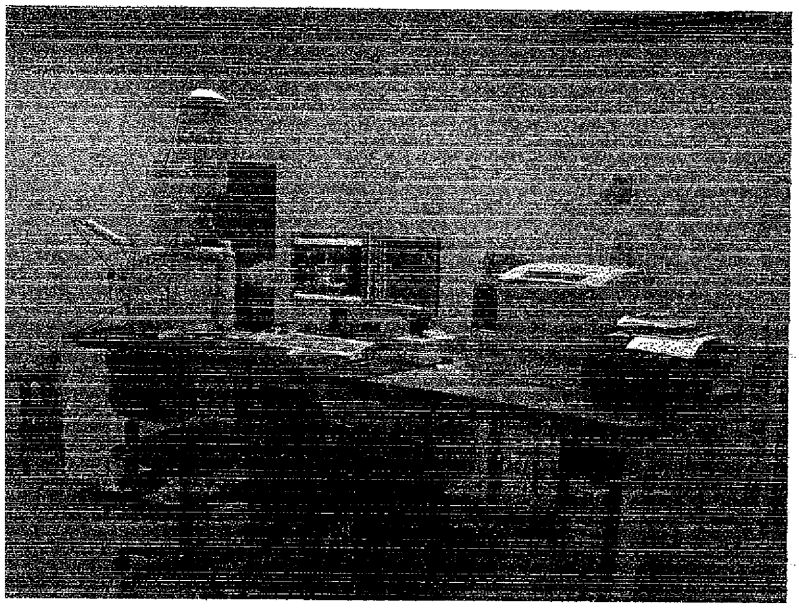

Figure 4.19: The setting for electron microscopy examination

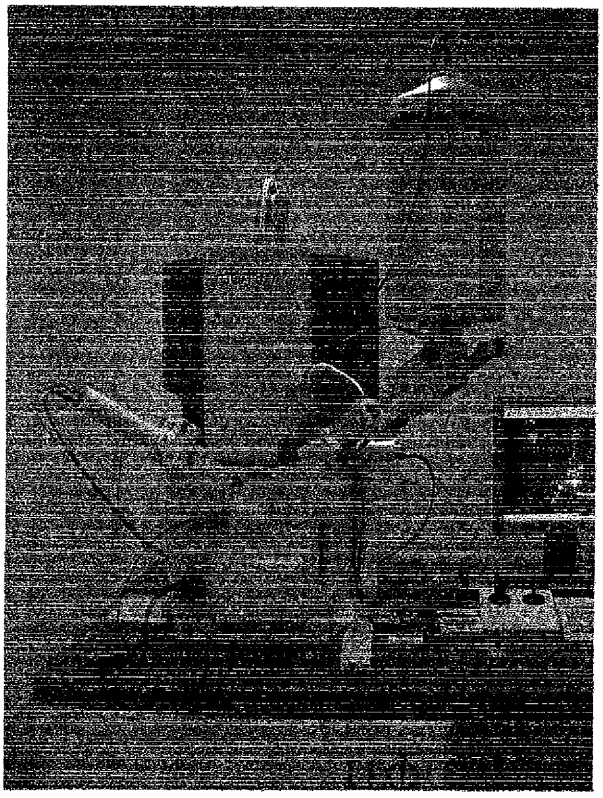

Figure 4.20: The scanning electron microscopy machine

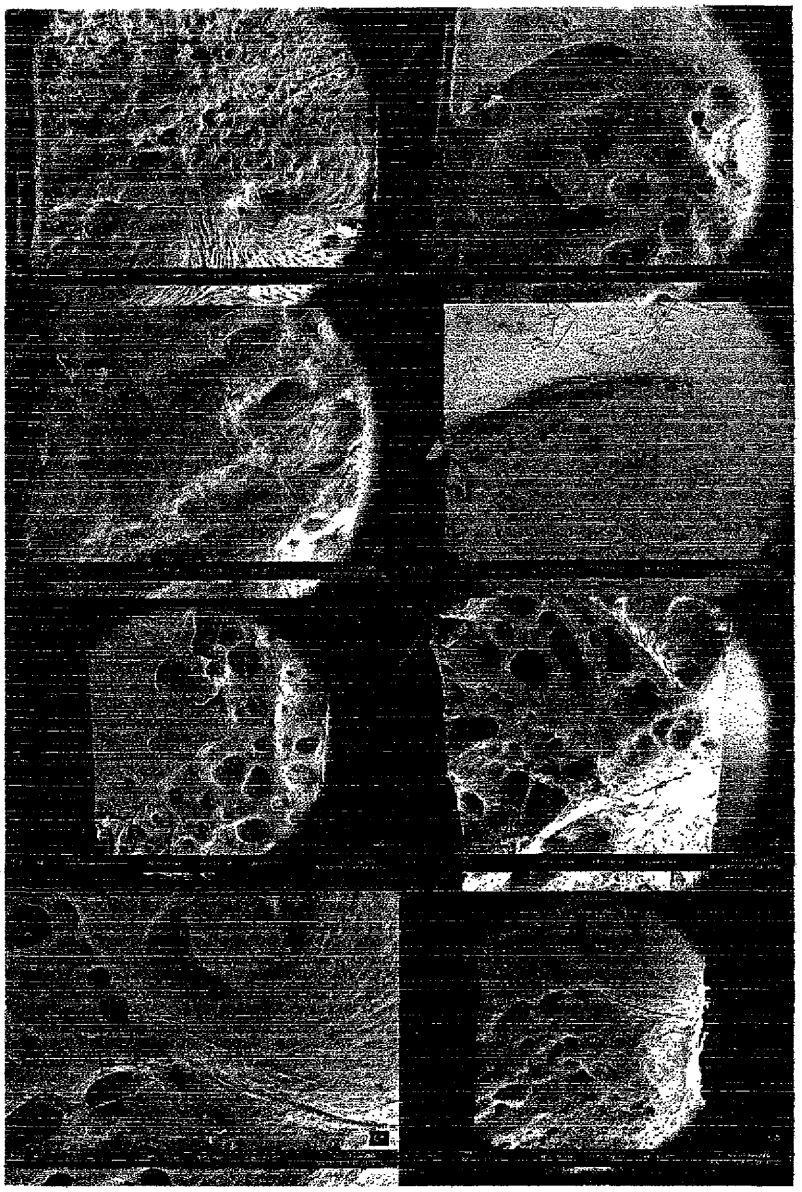

Figure 4.21: The SEM pictures of the specimens

CHAPTER 5: RESULTS

5.1 GENERAL CONDITION OF THE ANIMALS

A total of 24 New Zealand White rabbits were involved in the study, all the rabbits survived until the day of euthanasia. All the rabbits were healthy postoperatively and they resumed normal activity immediately after recovering from the effects of anesthetic drugs. They had demonstrated good activity with no sign of infection or any discharge at the wound site. There was no nasal discharge or any discharge from the genitalia. They were physically active throughout the postoperative period until the day of sacrifice. All the rabbits gained weight by the day of sacrifice.

A rabbit was noted had developed an ear infection secondary to head lice, which was tagged as A1. But this infection occurred at the 14^{th} weeks after the surgery performed. However it did not affect the rabbit's activity.

The duration of operation taken from starting of incision till closing of wound ranged from 15 to 20 minutes.

5.2 MACROSCOPIC ASSESSMENT

5.2.1 MACROSCOPIC ASSESSMENT OF GROUP A

Macroscopic inspection of the site of implantation revealed bone formation at the margin between the implant and host bone. In most of the specimens, it was difficult to delineate the margin between the host bone and the implant. Some bony continuity between the host bone and the implant was also noted. The surface of implant was irregular and adhered to fibrous tissue but still can be scrapped out. There was minimal bleeding during the dissection of fibrous tissue from the implant surface.

5.2.2 MACROSCOPIC ASSESSMENT OF GROUP B

The edge of coral was blunt and was partly covered by host bone. In some part, there was some difficulty to delineate the margin of the implant and the host bone. The implant surface was irregular and rough with several bands of fibrous tissue attached to the surface of implant.

5.2.3 MACROSCOPIC ASSESSMENT OF GROUP C

The edge of coral graft was blunt and partly covered by host bone. In small part, it was difficult to delineate the margin of the implant and the host bone. The surface of implants was irregular. There was presence of fibrous tissue over the surface of implant.

5.2.4 MACROSCOPIC ASSESSMENT OF GROUP D

The edge of coral graft was blunt. The margin between the implant and host bone was visible and easily delineated. The surface of implants was irregular. There was presence of fibrous tissue over the surface of implant.

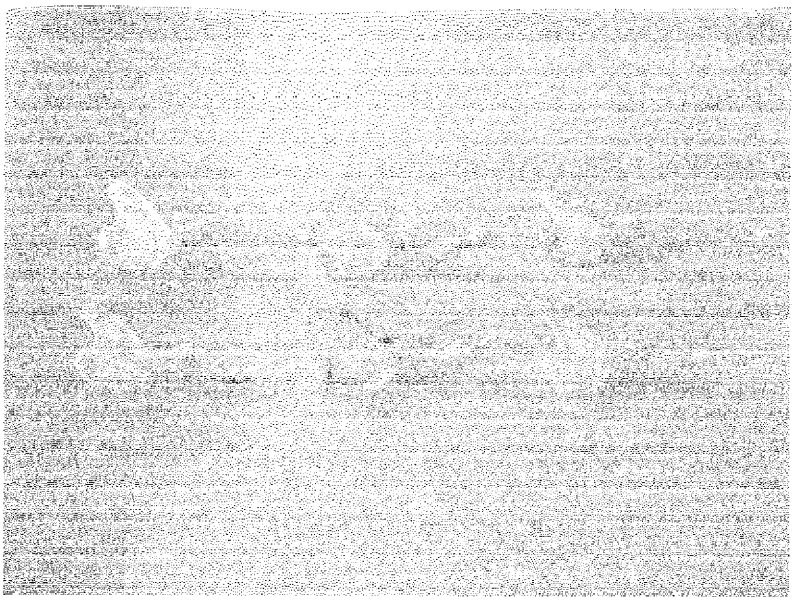

Figure 5.1: The macroscopic appearance of the specimens – one month duration

Figure 5.2: The macroscopic appearance of a specimen – one month duration (close view)

5.3 SCANNING ELECTRON MICROSCOPY (SEM) ASSESSMENT

5.3.1 SEM ASSESSMENT OF GROUP A

Scanning electron microscopy examination showed a good integration between the host bone and the coral graft (figure 5.3). The coral still maintain their porous microstructure with some pores diminished in size. There was cellular invasion within the pores especially at the periphery of implant. Several pores were completely filled with cellular tissue (figure 5.4). There was presence of extracellular matrix extended into the coral pores. There was granularity on the coral surface signifying an active cellular growth. The overlying periosteum also noted intermingled with the coral graft.

5.3.2 SEM ASSESSMENT OF GROUP B

Scanning electron microscopy examination showed a good integration between the host bone and the coral graft (figure 5.5). The coral still maintain their porous microstructure with some pores diminished in size. There was cellular invasion within the pores (figure 5.6). Several pores were completely filled with cellular tissue. There was presence of extracellular matrix extended into the coral graft. There was granularity on the coral surface signifying an active cellular growth.

5.3.3 SEM ASSESSMENT OF GROUP C

Scanning electron microscopy examination revealed a good integration between the host bone and the coral graft (figure 5.7 and 5.8). The coral still maintain their porous microstructure with some pores diminished in size. There was cellular

invasion within the pores. Several pores were filled with cellular tissue. There was presence of extracellular matrix extended into the coral pores. There was granularity on the surface signifying the cellular growth. The results were not much different if compared to B5 and B6 (three months implantation). However one of the specimens tagged C6 showed a good incorporation irrespective of implantation duration (figure 5.9).

5.3.4 SEM ASSESSMENT OF GROUP D

Scanning electron microscopy showed good integration between the graft and the host bone. There was cellular tissue response noted within the pores (figure 5.10 and 5.11). There was presence of fibrous tissue. There was presence of granularity on the coral surface signifying an active cellular growth.

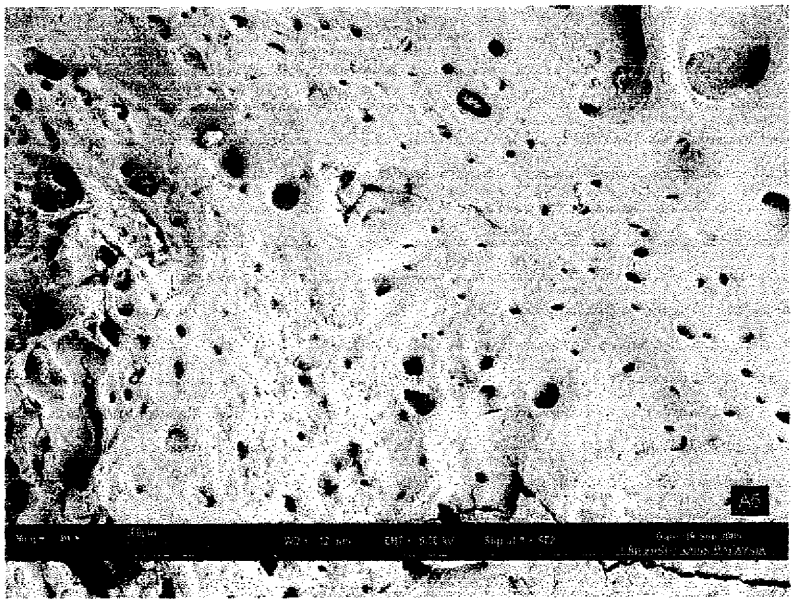

Figure 5.3: The scanning electron microscopy (SEM) picture showed the margin between coral and host bone in four months specimen

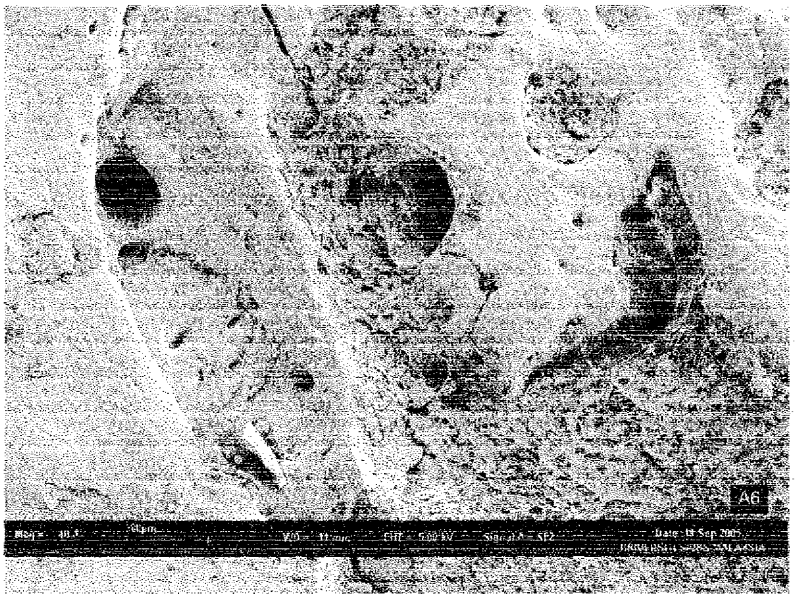

Figure 5.4 : The SEM picture showed soft tissue infiltration into the pores in four months specimen.

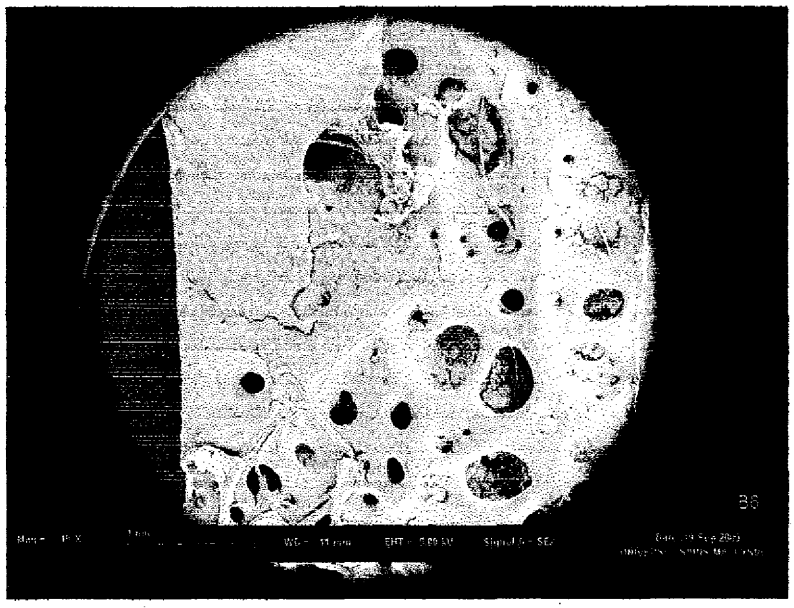

Figure 5. 5: The SEM picture showed the margin between the graft and host bone in three months specimen

Figure 5.6 : The SEM showed the cellular ingrowth into the pores in three months specimen.

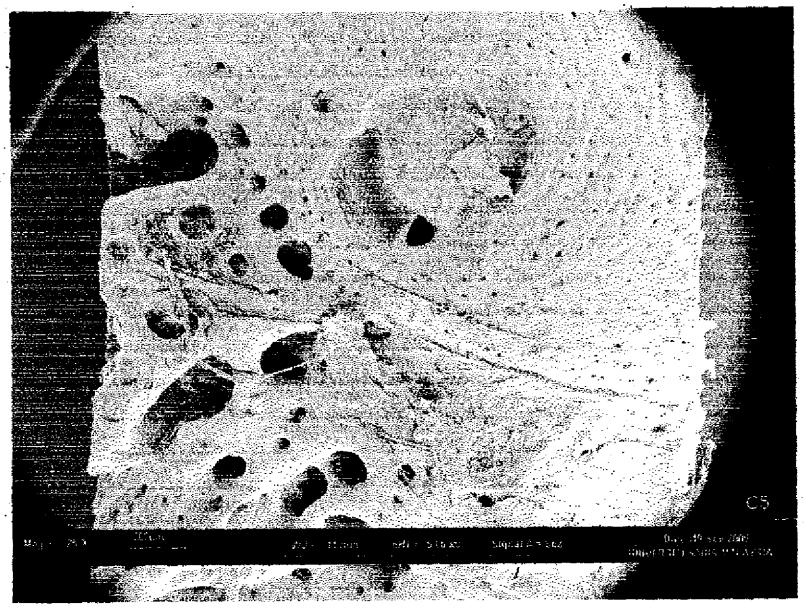

Figure 5.7 : The SEM picture showed the margin between the graft and the host bone in three months specimen

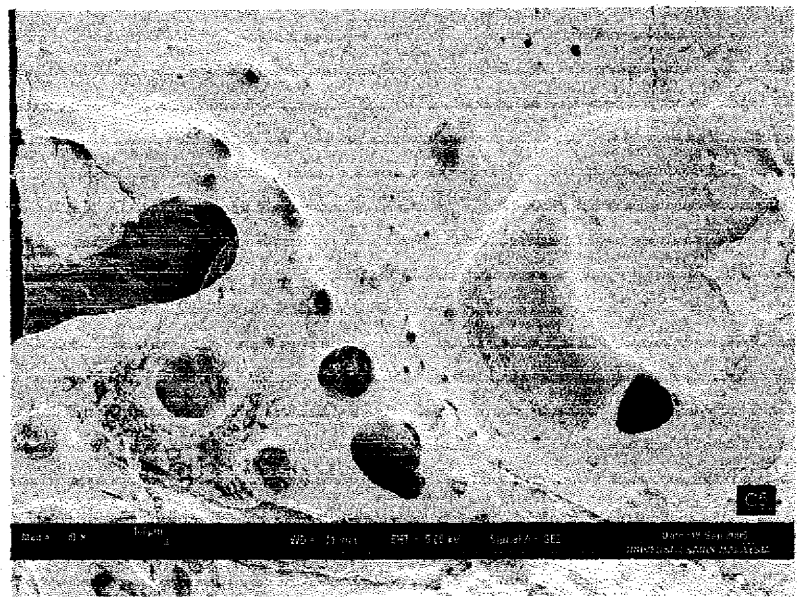

Figure 5.8 : The SEM picture showed the cellular ingrowth into the pores in three months specimen

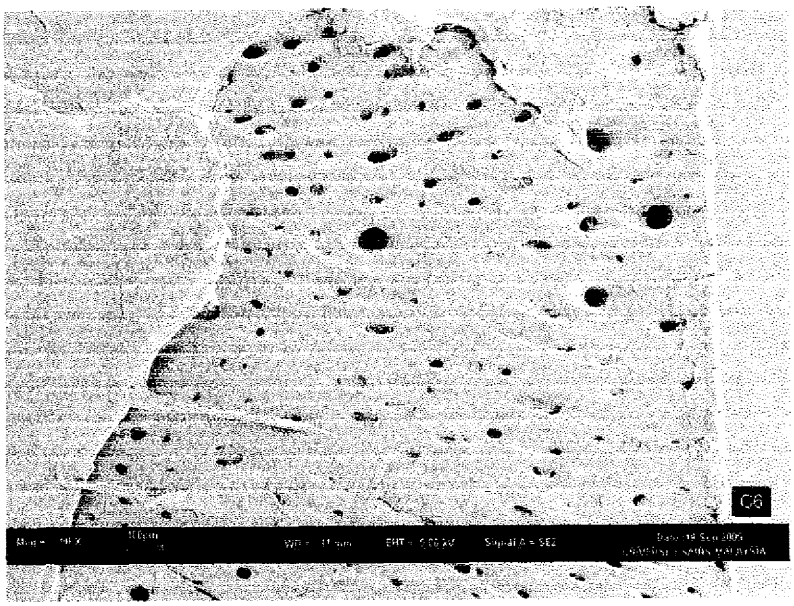

Figure 5.9 : The SEM picture showed the reduced pores size in a three months specimen (C6) which exhibited a remarkable result irrespective of the duration of implantation.

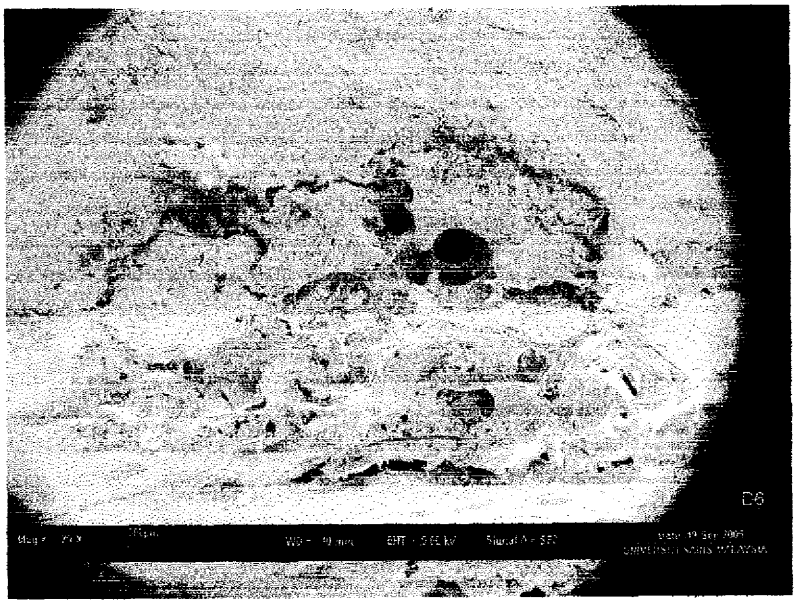

Figure 5.10 : The SEM picture showed the visible margin between the graft and the host bone in one month specimen.

Figure 5.11 : The SEM picture showed the cellular ingrowth into the pores in one month specimen.

CHAPTER 6: DISCUSSION

Processed coral has been used as bone substitute for surgical management of skeletal defects. It has a matched porosity similar to cancellous bone. It is gradually resorbs to be further replaced by new bone when implanted into bony defects. It has been reported to be biocompatible and osteoconductive (Nather A. et al, 2005). Its potential use as a bone substitute in the management of alveolar and periodontal defects has been reported (Kim et al, 2005). With the success of using coral as a bone replacement in the previous study elsewhere has triggered this study to further assess its biocompatibility as the preliminary study done by Rosdan et al (2004) had shown a promising result.

The main objective of this study is to assess the biocompatibility of processed coral as a bone substitute, focusing on the mandible as a part of craniofacial area. The assessments were inclusive of postoperative behavioral observation, macroscopic appearance of the specimen and scanning electron microscopic evaluation. In this study, no controls were used as the main objective is to assess its biocompatibility as bone replacement materials.

From the behavioral observation, the rabbits gained load bearing immediately after recovering from anesthesia. None of the rabbits developed any signs of significant inflammatory reaction: the rabbits were healthy, no sign of fever, no nasal discharge or discharge from genitalia. Clinical wounds healing were uneventful. All the wounds healed without signs of inflammation or infection. All the rabbits gained

weight. However one of the rabbit developed ear tick infection at 14 weeks post implantation which was considered not related to the implantation procedure.

From these observations, it had demonstrated that all the rabbits tolerated the operative procedure and the graft implantation. There was no presence of fever or any unhealthy appearance that might signify a significant immunological reaction between the host and the grafts. In a normal condition, when a foreign body is introduced into any part of the body, there will be an inflammatory reaction which can lead to graft rejection. That is why in any graft implantation procedure, steroid cover is given to prevent this immunological reaction. But the phenomenon was different in this study even though the coral graft is a xenograft. It was observed to be well accepted by the rabbit thus is considered biocompatible.

Corals are grouped under calcium carbonate, which has been stated to be a biocompatible and an osteoconductive material. The coral graft, even though is a xenograft and biological in origin, the material is bioinert and does not evoke any inflammatory reaction which can lead to graft rejection (Suzina A.H et al, 2005). The processed corals that were used in this were thoroughly cleaned from the debris and washed with distilled water. They were cut into smaller pieces and chemically treated followed by freeze-drying process. The materials were then sterilized at Malaysian Institute for Nuclear Technology Research (MINT) using gamma radiation.

This processed corals had undergone several studies before being validated as a safe biomaterial. There was no microorganism isolated from the processed

radiosterilised coral samples which could evoke an inflammatory reaction (Suzina A.H et al, 2005). The Gel Clot Test Method showed that the prepared coral has endotoxin level of 0.3 EU/ml, less than accepted by FDA. The Ames test results also demonstrated that it did not exhibit any mutagenic activity. In vitro study to assess its cytotoxicity was done, it was found that the coral is biocompatible and non cytotoxic to fibroblast (MRC-5) and osteoblast of human cell-lines (Suzina A.H et al, 2005).

A study done by Stubbs et al (2004), which he has evaluated the biocompatibility of coral in rabbit tibia. He had used coral derived material, Pro Osteon 200 R in combination with two other implants that was calcium sulphate slurry as the first group and calcium sulphate pellets as the second group. There was no significant immunological response in these groups. The finding is similar with previous studies (Kim et al, 2005; Walsh et al, 2003). All the animals tolerated the operation without complications and were loads bearing on the day after surgery as in study by Walsh et al (2003). With all these results in combination with other previous studies, the coral graft is concluded as a bioinert material, it is non cytotoxic, does not evoke intense inflammatory reaction and is biocompatible.

Macroscopic evaluations of the implanted corals of all the specimens had demonstrated that the coral was well incorporated within the bony defect. It was difficult to delineate the edge of corals despite of duration of implantation. However minimal differences were noted between each specimen. When comparing the

specimens in relation to duration of implantation, the longer the duration, the harder the delineation of the margin. The four months post implanted specimens showed the best incorporation and were the most difficult specimen to delineate the margin. The three and two months specimens showed no obvious difference macroscopically. The presence of fibrous tissue anchored to the surface of coral signified that it was well incorporated by soft tissue.

From these findings, coral grafts were considered biocompatible and were well incorporated into the mandibular defects. In an experimental dog model by Shors, found that bone grew into coralline hydroxyapatite within first month by 14% and averaged 56% by twelve months. This finding supported that that the bone incorporation into the graft depended on duration of implantation. He also concluded that coralline hydroxyapatite implants are biocompatible, osteoconductive and promotes healing within clinically relevant time frames (Nather A. et al, 2005). . Coral grafts have been demonstrated to support bone regeneration in a wide variety of procedures (Kim et al, 2005). It is good in alveolar augmentation and periodontal regeneration as use in the mandibular area. In this study, it showed good incorporation with rabbit's mandible. The edge of coral graft were also noted to be blunt signified that some resorption process had occurred, but these findings better recognized by the scanning electron microscopy examination.

Scanning electron microscopy evaluation of the implanted corals demonstrated cellular ingrowth on the surface of coral and had extended within the pores. There was extracellular matrix formation within the pores. The calcium carbonate components of corals were noted to resorb with time. The coral's pores also reduced in size and reduced in depth indicated that the cellular ingrowth has occurred within the pores. New bone ingrowth within the pores supported the osteoconductive nature of coral. However in one of specimen tagged C6 showed a remarkable incorporation. It showed good incorporation between the implanted coral and the host bone despite of only two months of implantation. The reason that can explain this finding is that the response of animals towards any implanted graft can differ. The capacity for bone formation varies between different rabbits even from the same breeding stock. Perhaps in this particular rabbit, it had exhibited a good reaction towards the graft. There was a study conducted by Aspenberg et all (1996) gave the same result, the difference was that it was conducted in rats.

From the scanning electron microscopy evaluation showed the coral acts as a good bone conductor. It posses the correct pores size to invite capillaries into it. When discussing the porosity, it is one of essential criteria for bone and soft tissue ingrowth. The recommended pore size is 100 um to 500 um to allow bone ingrowth. The interconnections must be larger than 100 um for mineralized bone to regenerate. Pore connections of 100 um to 200 um is necessary for the development of Haversian systems and vessels anastomosis. If it is between 40 to 100 um, the osteoid will form and if less than 40 um, the fibrovascular will form

(Nather A. et al, 2005). The processed corals by National Tissue Bank, School of Medical Sciences, Universiti Sains Malaysia with pores size ranging from 60 um – 800 um with mean size is 280 um have fulfilled the criteria, thus rendering it as a promising future bone substitute.

The scanning electron microscopy examination also showed some resorption of coral surface had occurred. Resorption is one of essential process to allow the bone ingrowth. Resorption process occurs together with new bone formation.It provides additional space for new bone formation. The rate of resorption of an implant is influenced by implant porosity, chemical composition, crystallinity, size of coral graft, site of implantation and the animal species. In general, there are two mechanisms of implant resorption; dissolution and resorption (Nather A. et al, 2005).

Dissolution is a simple physical chemistry process of coral dissolution in the surrounding body fluid. It is influenced by the surface area to volume ratio, solubility of coral, fluid convection, local acidity and temperature. Resorption is a biological process of degradation mediated by cells mainly osteoclasts. The resorptive process by osteoclasts is mediated through the action of the carbonic anhydrase enzyme, which converts carbonate to carbon dioxide and hydrogen ions (Nather 2005). The replacement of implant with host owns tissue needs to be titrated with the rate of new bone ingrowth (Walsh et al, 2003).

From all the findings, the coral graft used for the study showed its property as a biocompatible bone graft. It is inert and several tests have confirmed this; Gel Clot Test and the Ames test. The processed coral graft also biocompatible and served as good osteoconductive material. Several studies that have been done have demonstrated the biocompatibility and the osteoconductivity of processed corals.

A study on implanted coral into the calvarial of Sprague-Dawley rats was done by Suzina A.H. et al (2005), histological assessment of the implanted coral showed fibrovascular ingrowth with very minimal inflammatory response and presence of occasional giant cells. Abundant osteoblasts and formation of osteoid seam with mature bone bone formation were noted. Another study on histological assessment of implanted coral block in the created mandibular defect also showed bony ingrowth into the pores. The bone growth and incorporation into the coral was noted similar to a study done by Walsh et al (2003) where the coral derived material was implanted in the rabbit's proximal tibial defect.

A preliminary clinical trial in dental patients where it was implanted in the tooth socket showed no complaint from the patients. The absence of symptoms and signs demonstrate the biocompatibility of the material in the human system. Further trials on human should be further explored as the coral graft has a great tendency to be commercialized as a future bone substitute for our national use.

The osteoconductive property of coral can be enhanced by adding the osteogenic or osteoinductive components to the material. Several studies had supported this idea (Wang and Aspenberg 1996; Boden, Martin et al. 1999). Several materials have been used for the purpose; the transforming growth factor, demineralised bone matrix, autolous bone marrow component or autogenous bone. Among all these, the superior result was obtained when the material combined with transforming growth factor.

Transforming Growth Factor is a family of growth factor known as Transforming Growth Factor Beta (TGF-B). The important growth factor in this family is Bone Morphogenetic Protein (BMP). BMP exists in the animal of any species. It was found to be non-species specific meaning that BMP from the animal can induce bone formation in human. The BMP hypothesis assumed that it is released from a supramolecular aggregate of noncollagenous proteins(NCPs) in the normal bone turnover process or as a response to injury or transplantation (Rosdan S., 2000).

By addition of a certain concentration of these osteoinductive agents will increase the bone formation within the implanted materials. This has been confirmed by several studies which have demonstrated formation of heterotopic bone when placed in non bony sites such as soft tissue.

CHAPTER 7: CONCLUSION

This study confirmed that the processed coral is biocompatible as well as being a safe and osteoconductive material. The implantation of the corals into the rabbit's mandible showed no reactive inflammatory reaction, no significant infection as well as being a safe graft. The macroscopic and the scanning electron microscopy evaluation revealed bony and good cellular incorporation into the graft further supported its osteoconductive property.

From the observation of the rabbits and the evaluations that had been done, it can be concluded that the processed coral is biocompatible and can be further develop as future bone material replacement in human.

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STUDY PROFORMA

ANIMAL EXPERIMENTAL STUDY

PARAMETERS

1. Date :
2. Animal :

New Zealand White Rabbit	
Sprat Dolly Rat	
Sheep	

3. Tag. No :
4. Age :
5. Weight :
6. Sex :

GENERAL CONDITION

1. Activity — Active/not active
2. Coat — Healthy/Not healthy
3. Nasal/oral secretion — Absent/Present
4. Genitalia — Odorless/Foul smelling

IMPLANT

1. Biomaterial

		Dimension (height/length/width)
Dense Hydroxyapatite Block		
Dense Hydroxyapatite Granules		
Porous Hydroxyapatite Block		
Coral Block		
Coral granules		
Bone allograft		
Other:_____		

2. Implantation date :

3. Procedure :

    a. Anesthetic drugs :

    b. Antibiotic :

    c. Operative time : Surgery commenced : _____

    Surgery finished : _____

4. Site of implantation :

5. Sacrifice date :

6. Post implantation duration :

7. Preparation

Tag no :

Sacrifice date :

ASSESSMENT

1. Behavioral assessment

2. Macroscopic assessment

3. Scanning electron microscopy

COMMENTS: