IN VITRO CYTOTOXICITY EVALUATION OF PROCESSED NATURAL CORAL ON HUMAN FIBROBLAST AND OSTEOBLAST CELL LINES

By

NOR SHAMSURIA OMAR

Thesis submitted in fulfillment of the requirements
for the degree of
Master of Science

UNIVERSITI SAINS MALAYSIA
2010

IN VITRO CYTOTOXICITY EVALUATION OF PROCESSED NATURAL CORAL ON HUMAN FIBROBLAST AND OSTEOBLAST CELL LINES

By

NOR SHAMSURIA OMAR

Thesis submitted in fulfillment of the requirements

for the degree of

Master of Science

AUGUST 2010

IN VITRO CYTOTOXICITY EVALUATION OF PROCESSED NATURAL CORAL ON HUMAN FIBROBLAST AND OSTEOBLAST CELL LINES

By

NOR SHAMSURIA OMAR

Thesis submitted in fulfillment of the requirements

for the degree of

Master of Science in Dentistry

AUGUST 2010

Dedication

To my Beloved family for their support and encouragement

ACKNOWLEDGEMENTS

Praise to Allah, the most merciful, the most compassionate Who gave me strength to complete my post graduate study.

I would like to acknowledge the Universiti Sains Malaysia (USM) for awarding the research grant, 304/PPSG/6131202 which supported and made this research project possible. I would like to extend my deepest gratitude to the Vice Chancellor of USM Professor Tan Sri Dato' Dzulkifli Abdul Razak for giving me the opportunity to do my part time post graduate study.

My deepest regard to my supervisor Prof. Dr. Hj. Abdul Rashid Hj. Ismail, Dean and Prof. Dr. Zulkifli Ahmad, Deputy Dean of School of Dental Sciences for making this thesis possible and for all their support and guidance. My heartfelt thanks to my co-supervisor, Dr. T.P. Kannan, for his guidance and advice.

I would also like to thank my co-supervisor Professor Datuk Dr. Haji Abdul Rani Samsudin and Associate Professor Dr. Suzina Sheikh Abdul Hamid, for their support and encouragement and for making this thesis become a reality. My special thank goes to all my colleagues at Craniofacial Science Laboratory, especially Mrs. Fadilah, Mrs. Asiah, Mr. Marzuki, Mr. Ezani, Mrs. Eda, Ms. Khadijah, Mrs. Noor Baizura, Ms. Haswati, Mr. Sehly, Mr. Shahrul and Mr. Rosmadi, for their assistance and support.

Last but not least, I would like to thank to my family for all their support and understanding during the most challenging period especially my beloved husband Muhamad, my lovely daughters Nurnabilah, Nurnasuha and son Muhamad Nabil, my father Omar, my mother Shamsiah, my younger sisters Nor Kamilah, Nor Umairah, and my lovely nephews Aiman, Anis and Amir Shabab to whom I dedicate this thesis.

TABLE OF CONTENTS

CHAPTER FOUR RESULTS

CHAPTER FIVE DISCUSSION

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

ATCC	American type culture collection
CSL	Craniofacial science laboratory
DMEM	Dulbecco's modified eagle's medium
DMSO	Dimethyl sulfoxide
ELISA	Enzyme linked immunosorbent assay
FBS	Fetal bovine serum
HOS	Human osteoblast
ISO	International standard organization
MRC-5	human lung fibroblast
MTT	(3-(4,5-dimethylthiazol-2-yl)-2-5-dipheyl tetrazolium bromide)
NR	Neural red
PNC	Processed natural coral
PBS	Phosphate buffered saline
PNC	Processed natural coral
SEM	Scanning electron microscope

PENILAIAN SITOTOKSISITI TERHADAP BATU KARANG YANG TELAH DIPROSES KEATAS TITISAN SEL OSTEOBLAS DAN FIBROBLAS MANUSIA SECARA *IN VITRO*

ABSTRAK

Tujuan kajian ini dijalankan adalah untuk membuat penilaian ke atas bahan batu karang (spesis *Porites*) yang telah diproses (Kampus Kesihatan, USM) dengan mengunakan kaedah proliferasi, kajian apoptosis dan perlekatan sel dengan menggunakan titisan sel osteoblas (HOS) dan fibroblas (MRC-5)(ATCC, USA). Penilaian sitotoksisiti batu karang yang telah diproses ini adalah mengunakan ujian ekstrak dan sentuhan lansung mengikut format ISO 10993-5. Untuk ujian proliferasi, HOS sel telah didedahkan kepada dua partikel saiz PNC yang berbeza iaitu PNC granul (0.5-1mm) dan PNC serbuk (1-50μm) dan dianalisa mengunakan kaedah MTT dan NR assai. Sesuatu bahan yang diuji adalah dikatakan toksik sekiranya kadar proliferasi adalah kurang daripada 50%. Untuk kajian *apoptosis*, sel HOS telah didedahkan kepada medium ekstrak daripada serbuk PNC pada kepekatan 200mg/ml selama 48 dan 72 jam. Selepas itu sel pelet (1x 10^6 sel) dicampurkan ke dalam larutan Annexin-V-FLOUS dan dianalisa mengunakan *Flow Cytometry*. Kajian proliferasi secara sentuhan langsung ke atas ceper PNC, adalah menggunakan titisan sel HOS dan MRC-5. Sel-sel berkenaan telah didedahkan kepada bahan PNC pada 1, 24, 72 dan 168 jam dan dianalisa mengunakan NR assai. Medium pengkulturan adalah tidak ditukar selama tempoh pengeraman. Sesuatu bahan yang diuji adalah dikatakan toksik sekiranya kadar proliferasi adalah kurang daripada 50%. Untuk kajian perlekatan sel pula, sel MRC-5

dan HOS telah dikulturkan ke atas ceper PNC dan getah latex (kontrol positif) dan pemerhatian adalah dibuat di bawah *inverted microscope* selepas dieram selama 72 jam. Untuk analisis melalui *scanning electron microscope* (SEM) HOS sel telah dikulturkan di atas ceper PNC dan plastik *thermanox* (kontrol negatif) dan pemerhatian telah dibuat selepas pengeraman selama 72 jam. Kajian proliferasi sel HOS terhadap granul dan serbuk PNC menunjukkan kedua-dua partikel berkenaan tidak sitotoksik. Kedua-dua partikel granul dan serbuk PNC tidak merangsang sebarang sitotoksisiti dan telah dibuktikan daripada kadar proliferasi yang mana melebihi 50%. Analisa *flow cytometry* telah menunjukkan peratusan sel HOS yang hidup adalah tinggi dan peratusan sel *apoptotic* adalah rendah membuktikan bahan PNC tidak menyebabkan kerosakan kepada sel. Keputusan menunjukkan kadar proliferasi sel HOS dan MRC-5 apabila didedahkan kepada bahan PNC pada masa pengeraman yang berbeza mengunakan NR assai telah menunjukkan tiada ketoksidan terhadap sel sehingga 72 jam masa pengeraman. Kajian perlekatan sel, telah menunjukkan sel MRC-5 dan HOS telah melekat pada pinggir ceper PNC dan berkembang ke dalam liang-liang ceper PNC. Sebagai kesimpulan, keputusan menunjukkan bahan PNC yang telah dihasilkan oleh Kampus Kesihatan, USM adalah tidak sitotoksik dan mengalakkan pertumbuhan sel HOS dan MRC-5.

IN VITRO CYTOTOXICITY EVALUATION OF PROCESSED NATURAL CORAL ON HUMAN FIBROBLAST AND OSTEOBLAST CELL LINES

ABSTRACT

The aim of this study was to evaluate the *in vitro* cytotoxicity of the locally produced processed natural coral (Health Campus, Universiti Sains Malaysia) from the *Porites* species in terms of proliferation, apoptosis study and cell attachment by using human osteoblast (HOS) and fibroblast (MRC-5) cell lines (ATCC, USA). The *in vitro* cytotoxicity of the processed natural coral (PNC) was evaluated using test on extract and direct contact as per ISO 10993-5 (1999). HOS cells were used to study the magnitude of proliferation when exposed to the extraction medium of two different particle sizes of PNC, granules (0.5-1mm) and powder (1-50μm). The proliferation of HOS cells was analyzed using MTT and NR assays. The material is considered toxic if the proliferation rate was less than 50%. For the apoptosis study, HOS cells were exposed to the extraction medium of PNC powder at a concentration of 200mg/ml for 48 and 72 hours. Then the cell pellet (1x 10^6 cells) was resuspended in Annexin-V-FLOUS labelling solution and subjected to flow cytometric (FCM) analysis. Proliferation study via direct contact of PNC disc was carried out using HOS and MRC-5 cell lines. Those cells were exposed to the PNC material at 1, 24, 72 and 168 hours and analyzed using NR assay and the medium was never changed through out the incubation periods. The material is considered toxic if the proliferation rate was less than 50%. For the cell attachment study, MRC-5 cells and HOS cells were cultured on the PNC discs and rubber latex (positive control) and observed under inverted microscope after 72 hours of incubation period. For the Scanning Electron Microscopic (SEM) analysis, HOS cells were cultured

on the PNC disc and thermanox plastic (negative control) and observed after 72 hours of incubation period. Proliferation study of HOS cells on the extraction of PNC granules and powder showed that both particles were not cytotoxic. Also, both PNC granules and powder did not induce any cytotoxicity as was evident from their proliferation rate, which was above 50%. The flow cytometry analysis showed that the viable cell percentage for the HOS cells was high and the apoptotic cell percentage was low, indicating that PNC did not cause a remarkable damage to the cells. The results of the magnitude of proliferation of HOS and MRC-5 cells when exposed to the PNC material at different incubation periods using NR assay indicated that there was no cytotoxicity until an incubation period of 72 hours. The cell attachment study showed that both MRC-5 and HOS cells were attached on the edge of the PNC disc, which later grew into the pores of the PNC disc. All the above results show that the locally produced PNC material by Health Campus, Universiti Sains Malaysia is non cytotoxic and favours the growth of HOS and MRC-5 cells.

CHAPTER ONE

INTRODUCTION

1.1 Background of the study

The development of bone-graft substitutes has evolved from the understanding of how autografts and allografts are used intraoperatively and how they are remodelled by the body after transplantation. Bone formation requires a physical structure to which osteoblasts can adhere. Therefore, the concept of using porous devices composed of biocompatible materials was conceived. The earliest work used inert metals, such as cobalt chrome and titanium alloy. These materials can provide passage ways for bone integration, but bone cell does not directly bond to proliferate along their surfaces (Shors, 1999).

Osteogenesis, osteoinduction and osteoconduction are the essential elements of bone regeneration along with the final bonding between host bones and grafting material which is called osteointegration. The term osteoconduction means that bone grows on a surface. An osteoconductive surface is one that permits bone growth on its surface or down into pores, channels. The term osteoinduction means that primitive, undifferentiated and pluripotent cells are somehow stimulated to develop into the bone-forming cell lineage (Giannoudis *et al*., 2005). The different characteristics of bone graft substitutes are shown in table 1.1.

Biomaterials or scaffolds for osteogenesis should mimic bone morphology, structure and function in order to optimize integration into surrounding tissue. Bone is a structure of hydroxyapatite ($Ca_{10}(PO_4)_6(OH)_2$) crystals deposited within an organic matrix in which 95% is type 1 collagen. The morphology is composed of trabecular

bone, which creates a porous environment with 50 – 90% porosity (Karageorgiou and Kaplan, 2005).

Table 1.1 Characteristics and bone graft substitutes

Characteristics	Grafts
Osteoconduction	Calcium sulphate Calcium phosphate cements Ceramics Collagen Synthetic polymerss
Osteoinduction	DBM BMPs Growth factors Genetic therapy
Osteogenesis combined	Bone marrow aspirate (BMA) Composite graft

The necessity for porosity in bone regeneration has been shown by Kuboki *et al.* (1998) using rat ectopic model, whereby solid and porous particles of HA were used for BMP-2 delivery. They found that no new bone was formed on the solid particles, while in the porous scaffolds, direct action of osteogenesis occurred.

Scaffolds for bone regeneration should meet certain criteria to serve the skeletal functions including mechanical properties similar to those bones at the repair site,

biocompatibility and biodegradability appropriate with remodelling (Karageorgiou and Kaplan, 2005). Scaffolds serve primarily as osteoconductive moieties, since new bone is deposited by creeping substitution from adjacent living bone. In addition to osteoconductivity, scaffold can serve as a delivery vehicle for cytokines such as bone morphogenetic proteins (BMPs), insulin-like growth factors (IGFs) and transforming growth factors (TGFs) that transform recruited precursor cells from the host into bone matrix producing cell, thus providing osteoinduction (Groeneveld *et al*., 1999).

The success of implanted device is affected by the ability of cells to interact with the exposed device material because properties such as surface topology are stable features of the surface, compared to chemical modifications device which may be degraded over time. There has been immense interest in directing cell behavior by controlling the topology of materials. Cells have been found to respond differently to smooth surfaces compared to materials with micro or nanoscale roughness in a cell type dependent manner (Jiyeon *et al*., 2008).

The research on natural coral as a bone substitute has been reported in many experimental studies. It has been proven to be biocompatible, biodegradable and has not been found to cause any inflammatory responses (Tuominen *et al*., 2000). Coral is made of calcium carbonate (98-99%) in the form of aragonite with the trace elements and amino acid (Louisia *et al*., 1999). It has been used as a biomaterial for bone replacement because of several reasons such as, the material simplifies the surgical procedure, harvesting of autologous bone is no longer necessary and transmission of infections such as AIDs, and hepatitis can be avoided with certainty. Furthermore, coral has porous architecture, high compression breaking stress and resorbability. It has been reported

that the porosity and three-dimensional structure of coral implant encourage bony ingrowth (Marchac *et al*., 1994).

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 distinguishes it from non-resorbable materials extensively used in these surgeries. An optimal clinical utilization of coral requires a 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 (Guillemin *et al*., 1989).

Coral mineral has had considerable success considering its porous structure (which ranges from 150 to 500μm), its similarity to cancellous bone and also because it is one of a limited number of materials that will form chemical bonds with bone and soft tissue *in vivo*. Studies indicate that a favorable pore size and micro-structural composition are important factors facilitating in-growth of fibrovascular tissue or bone from the host. Pore interconnection sizes are utmost importance when hard and soft tissue in-growth is involved (Ben-Nissan, 2003).

The structure of the commonly used natural coral porites species is similar to that of cancellous bone and its initial mechanical properties resemble those of bone. It is also an osteoconductive material (Shahgaldi, 1998). Bone grafting mediated via tissue engineering of stem cells for repairing defects represents a new direction towards bone regeneration in this millennium. Natural coral has found considerable interest as scaffold materials (Parfitt, 2000).

All newly developed biomaterials must fulfilled stringent criteria laid out by government authorities and international agencies, such as the United States Food and

Drug Administration (FDA) and International Organisation for Standardization (ISO), before receiving approval for clinical application (Gallagher *et al*., 2006).

ISO is a worldwide federation of national standard bodies (ISO member bodies). The work of preparing International Standards is normally carried out through ISO technical committee. The International standards ISO 10993-part 5 "Biological Testing of Medical devices-Part 5: Test for *in vitro* cytotoxicity" was prepared by technical committee ISO/TC 194, Biological Evaluation of Medical Devices. The crucial parameters for cytotoxicity testing are addressed but not specified in ISO, 10993-5 (1999).

In practice, standard cell-based toxicity assays are performed *in vitro* and high-risk materials are removed at this early stage. This method has the advantages of simplicity, good sensitivity and reproducibility and is widely used in the initial evaluation of biocompatibility of biomaterials (Gallagher *et al*., 2006).

1.2 Problem statement

Virtually, every operative day, orthopedists, neurosurgeons, maxillo-craniofacial surgeons and periodontists need to fill defects in bone or augment deficient bone. When the defect is small, an autologous bone graft is the best solution but in larger defects, the addition of homologous bone graft and biomaterials are a necessity. Recent advances in orthopaedic and maxillo-craniofacial surgery can be attributed to the revolution in biomaterials. During the last decade, a large number of biomaterials have been proposed as artificial bone filler for repairing bone defect (Shors, 1999)

Bone graft is the second most common transplantation tissue, next to blood. More than 500,000 bone grafting prosedures are happening annually in the United States

and 2.2 million worldwide in order to repair bone defects in orthopaedic, neurosurgery and dentistry (Giannoudis *et al.*, 2005).

The coral of *Porites* species possess anatomical structure, physical, chemical and mechanical characteristics that stimulate the human bone. The material is acceptable by people from all races, ethnicity, religious and belief. The material is economical and affordable to patients and this will help to reduce the cost and increase the quality of health care in the country (Hamid *et al.*, 2005).

1.3 Justification of the study

This study was carried out using a dead coral identified by Universiti Sains Malaysia (USM), Health Campus, isolated from *Porites* species. Dead coral of *Porites* species was processed at the Tissue Bank Unit of USM. The specimen was collected from Pulau Perhentian, Terengganu and the species was identified by marine biologist Prior approval was obtained from Department of Fisheries, Malaysia (Appendix).

In this present study, The coral of *Porites* species was chosen because the interconnectivity between its pores was quite similar to that of the human cancellous bone. An *in vitro* cytotoxicity test was carried out on this processed natural coral (PNC) on human osteoblast (HOS; CRL-1543) and human embryonic lung fibroblasts (MRC-5; CCL-171) cell lines (ATCC, USA) by using extraction/indirect method and direct methods. Two different types of cell lines were used in this study; HOS cell line which corresponds to hard tissue and MRC-5 cell line which corresponds to soft tissue.

1.4 Objectives of the study

1.4.1 General objective

The objective of this study is to evaluate the *in vitro* cytotoxicity of processed natural coral as a bone substitute.

1.4.2 Specific objectives

1. To determine the cytotoxicity effects of different particle sizes of processed natural coral on the proliferation of human osteoblast cell line
2. To assess the apoptosis of human osteoblast cell line on the extract of processed natural coral material
3. To determine the cytotoxicity effects of processed natural coral on the proliferation of human lung fibroblast and human osteoblast cell lines based on different incubation periods.
4. To study the attachment of the human osteoblast and human lung fibroblast cell lines on the processed natural coral material

CHAPTER TWO

LITERATURE REVIEW

2.1 Definition of Biomaterials

The definition of biomaterial endorsed by consensus of experts in this field is that "a biomaterial is a nonviable material used in a medical device, intended to interact with biological systems" (Ratner, 1996). The first reported use of synthetic biomaterial in facial plastic and reconstructive surgery occurred around 1600 when Fallopius implanted a gold plate to repair a calvarial defect (Costantino *et al*., 1993).

Biomaterials have been used extensively in orthopaedic surgery, craniofacial and maxillofacial surgery, biomedical engineering and more recently, the delivery of therapeutic agents. Many definitions have been proposed for the term biomaterial. According to Dorland (2000), biomaterials are substances (other than drugs), synthetic or natural, that can be used as a system or part of a system that treat, augment or replace any tissue, organ or function of the body.

2.2 Historical development of biomaterials

The ancient Chinese and the Aztec used gold in dentistry for more than 2000 years ago. Perhaps the most widely used class of material is metals for implants**.** For instance, some of the most common orthopedic surgeries involve the implantation of metallic implants. These range from simple wires and screws to fracture-fixation plates and total joint prostheses. Aluminium, platinum, and nikel-plated devices were used as screws. By the early 20^{th} century, high-carbon steel was used for these purposes. Cobalt-chromium alloys were introduced in the 1920s and titanium came into vogue in the late

1940s. Currently, the most commonly used metals for orthopaedic implants are low-carbon stainless steel and cobalt-chromium alloy (Agrawal, 1998).

Stainless steel is used extensively for fracture fixation devices. Compared to the other metals used in orthopedic, stainless steel exhibit a moderate to high elastic modulus and tensile strength. Stainless steel is fairly biocompatible although they never appear to fully integrate with bone tissue. Cobalt-chromium alloys are highly corrosion resistant. Compared to stainless steel, they exhibit higher elastic modulus, strength and hardness, but they have relatively low ductility and are difficult to machine. Titanium is used in two forms; commercially pure titanium and Ti-6A1-4V. Pure titanium is relatively weak (Agrawal, 1998).

At the turn of this century, synthetic plastic became available. Their ease of fabrication led to many biomaterial experiments. Most of these, were doomed to failure due to the light of our contemporary understanding of biomaterials toxicology (Ratner, 1996). The popularity of biodegradable polymers as biomaterials has been steadily increasing in the past two decades. The beauty of these materials is that they can be designed as temporary implants that stay intact until the healing process in the body is complete, whereupon they degrade by hydrolytic or enzymatic action and excreted from the body as waste products. The most popular biodegradable polymers are polylactic acid (PLA) and polyglycolic acid (PGA) (Agrawal, 1998).

A variety of polymers are used in medicine as biomaterials. Their applications range from facial prostheses to tracheal tubes, from kidney and liver parts to heart components, and from dentures to hip and knee joints. Polymethyl methacrylate (PMMA) was introduced in dentistry in 1937. During the World War II, debris of PMMA from shattered gunnery turrets, accidentally implanted in the eyes of aviators

suggested that some of these materials evoked only mild foreign body reaction (Ratner, 1996). In this century, PMMA was used extensively as bone cement which is primarily used to support the stems of total joint prostheses in the medullary cavity of bone (Agrawal, 1998).

Gruninger *et al*. (1984) introduced the term "calcium phosphate cement" which can be prepared by mixing a calcium phosphate salt with water or an aqueous solution to form a paste that reacts at room temperature. The cement is applicable in grafting and reconstruction of damaged parts of the body system. Yu *et al*. (1992) found that calcium phosphate cements can be used as drug delivery systems for a variety of remedies and act as a vehicle for antibiotics, anti-tumor and anti-inflammatory drugs. Despite the wide range of possible clinical applications of calcium phosphate cements, there are very few literature reports on its *in vitro* biocompatibility.

Diamond-like carbon films (DLC) have been studied extensively for military applications as a single-layer antireflection coating for infrared vision system (Lettington and Smith, 1992). DLC film has attracted much attention in recent years because of its hardness, wear resistance, chemical inertness and low coefficient of friction. Thomson *et al.* (1991) were the first few authors to study the biological effects on DLC films. Du *et al.* (1998) studied the morphological behavior of osteoblasts cells on DLC coatings *in vitro* and they found that after a period of time the cells attached, spread and proliferated on the DLC coated film.

Several materials derived from the animals or plant is also being considered for use as biomaterials. One of the advantages of using natural materials for implants is that they are anatomically similar to materials familiar to the body. Natural materials do not usually offer the problem of toxicity often faced by synthetic materials. They may also

carry specific protein binding sites and other biochemical signals that may assist in tissue healing or integration. However, natural materials can be subjected to problems of immunogenicity. Collagen has been studied extensively for use as a biomaterial. It has shown good promise as a scaffold for new tissue growth and is commercially available as a product healing. Other natural materials under consideration include chitin, keratin, cellulose and natural coral (Amarjit *et al.*, 2003).

2.3 Characteristic of an ideal biomaterial as a bone substitute

Screening for an ideal biomaterial for bone substitute is still a challenge for researchers. An ideal biomaterial should be osteoconductive, osteoinductive and porous for cellular infiltration. Furthermore, it should be biocompatible, mechanically stable with respect to the native bone and be biodegradable to prevent having foreign material in the body for prolonged period of time. Other considerations include ease of sterilization without loss of properties (Tortora and Grabowski, 2000).

Bone is not completely solid but has many small spaces between its hard components. Some spaces provide channels for blood vessels that supply bone cells with nutrients. Other spaces are storage areas for red bone marrow. Depending on the size and distribution of the spaces, the regions of a bone may be categorized as compact or spongy. Overall, about 80% of the skeleton is compact bone and 20% is spongy bone (Tortora and Grabowski, 2000).

Bone matrix contains abundant of inorganic mineral salts, primarily hydroxyapatite (HA) and some calcium carbonate. In addition, bone matrix includes small amounts of magnesium hydroxide, fluoride and sulfate. As these minerals salt are deposited in the framework formed by the collagen fibers of the matrix, they crystallize

and the tissue hardens. This process of calcification or mineralization is initiated by osteoblasts (Tortora and Grabowski, 2000).

The organic components including cell, collagen, and various macromolecules have the capacity to initiate immune responses. The inorganic component, however, is biocompatible and nonimmunogenic. These calcium salts, primarily calcium phosphate, constitute approximately 70% of bone by weight. The major constituent is in the form of a poorly crystalline calcium phosphate compound, known as HA. Calcium salt, particularly HA and calcium carbonate are bioactive and osteoconductive (White and Shors, 1986).

Porosity allows soft tissue and bone to regenerate within the pore space. Porosity alone however is not adequate for bone ingrowth. Porosity with interconnectivity is the most essential prerequisite (White and Shors, 1986). Pore size is important in determining cellular ingrowth, factor release and vasculirization. White and Shors (1986) reported that the requisite pore size for bone ingrowth into porous implants is 100 to 500um. Material scientists at the Clemson University in the early 1970s conducted seminar studies. They showed that the diameter of the interconnecting pore dictate the kind of tissue growing into the porosity of implant place next to the bone. To generate mineralized bone, the interconnecting must be larger than 100um. If they are 40 to 100um, osteoid forms and if they are 10 to 40um, fibrovascular tissue forms (White and Shors, 1986)

There has been conflicting studies on the size of the pores of biomaterial that will influence the bone formation. Pinade *et al.* (1996) recommended that pore sizes up to 200um will achieve the bone growth while Burg *et al.* (2000) have claimed that an interconnecting pore size of 200-400um is the optimum pore size for bone ingrowth.

Kuhne *et al.* (1994) investigated the new bone formation between two different pore size of coralline hydroxyapatite (HA500 and HA200) by using rabbit model. They found that the new bone formation in coralline hydroxyapatite (HA 500) with pore size 500um is faster than in coralline HA (HA200) with the pore size 200um. They concluded that, 500um pore size hydroxyapatite may be suitable as bone substitute in metaphyseal defects.

Gao *et al.* (1997) investigated the effect of tricalcium phosphate (TCP) and Biocoral®(natural coral) cylinder on bone regeneration during the healing of segmental defects of sheep in terms of radiomorphometry, histology and biomechanics. They compared the osteointegration and mechanical strength of the two different kinds of bioceramics and found that biocoral had better osteointegration and biomechanical performance than TCP. The three-dimensional structure of pores and interconnecting fenestrations in Biocoral as compared to TCP might be more favorable for new bone ingrowth.

2.4 Coral for medical application

Marine reefs are primarily composed of corals and exist in two forms: as a soft form without significant inorganic structure and as a hard form which is called stony corals or scleractina. These scleractina corals are colonies of many individual animals called polyps, which is derived originally from a single animal. The polyp, an invertebrate grow most aggressively in warm, shallow along the equator. The polyps deposit an interconnecting, porous skeleton composed primarily of calcium carbonate form of aragonite. As the polyps grow, they vacate their skeleton leaving behind a

network of interconnecting porosity, which has the correct pore size for bone ingrowth (Shors, 1999)

The species of marine invertebrates exploited in medical applications are presented in Table 2.1. *Porites* and *Goniopora* species, belonging to the Poratidae family, are widely used as a coral grafts and also for developing coralline HA bone substitute implants (Damien and Revell, 2005). The Image of *Porites* and *Goniopora* species are shown in Figures 2.1 and 2.2. (www.reefcorner.com/).

Table 2.1 Classification of corals in biomedical use

	Coral Taxonomy
Kingdom	Animalia
Phylum	Coelenterata
Order	Scleractenia
Family	Paratidae
Genera	Porites species Goniopora species

Figure 2.1 Image of *Porites* species

Figure 2.2 Image of *Goniopora* species

Since, these skeletons are composed primarily of calcium carbonate, material scientists in mid-1970s used these coral skeletons as templates for making bone-graft substitutes. The implant is called coralline and manufactured in two forms. One approach is to use coral directly in the calcium carbonate form and termed as natural

coral. The other form is conversion of calcium carbonate to hydroxyapatite and termed as replamineform (Shors, 1999).

Replamineform ceramics are made from natural coral. Coral is composed of 97% calcium carbonate, but is structurally similar to bone. Two common types of coral, by genus, have structures that emulate cancellous and cortical bone, respectively. *Goniopora* species creates a structure with 500-600um pores and 220-260µm interconnections. This 'trabecular pattern' is similar to cancellous bone, with 20% matrix and the rest 'marrow space' (Kurz *et al*., 1989). Porites on the other hand, is similar to cortical bone with 200-250µm pores and parallel channel connected by 190µm fenestrations. Unlike the random pores structure of sintered ceramics, the unique structural geometry of coralline promotes rapid resorption and reossification. One form of replamiform ceramic employs hydrothermal exchange to replace calcium carbonate with calcium phosphate (Lebwohl *et al.*, 1994). This material, marketed as Pro-Osteon (Interpore, Irvive, CA), is essentially coralline HA. Both forms are extremely biocompatible. An incompletely converted, calcium phosphate and calcium carbonate material, termed Pro-Osteon 500R, is being investigated for a potentially more predictable profile (Truumees and Herkowitz, 1999).

A large number of biomaterials have been used as artificial bone substitutes for repairing bone defects such as hydroxyapatite, tricalcium phosphate, polymer and bioactive glasses. Roux *et al*. (1988) reported that coral was frequently used in maxillofacial surgery during 1980s but its use became rarer during the last few years. This is perhaps due to difficulty of using this material, as it is brittle and rough, but more probably because in clinical use the variations in the porosity values have not been taken into account according to the species. All coral species secrete calcium carbonate

in the form of aragonite, but each species constructs an original architecture which results in a unique porosity (Roudier *et al*., 1995).

According to Guillemin *et al.* (1987) and Shors *et al.* (1989), certain coral species are rapidly resorbed after implantation. Experiments *in vitro* and *in vivo* to characterize the behavior of resorption of bone implanted with three different coral materials was conducted by Roudier *et al*. (1995) and they found that the porosity plays the important role to influence the speed of resorption. Biocompatibility for living cells was studied *in vitro* by seeding the developing differentiated bone marrow cells and they observed that osteoblastic cells grew and maintained their *in vitro* differentiation on the material for more than a month.

2.5 Applications of tissue engineering

Despite the many advances in bone graft substitutes, new materials and approaches to bone healing continue to be investigated. One exciting area is tissue engineering, which can be defined as the application of biological, chemical, and engineering principles to the repair, restoration, or regeneration of living tissues by using biomaterials, cells, and biological factors alone or in combination. Tissue engineering is a new development in biomedicine, involving a series of strategies and the key element is the use of biologically based mechanisms in order to repair and heal damaged and diseased tissue. The key elements include a specific living cell type (or several cell types), a biomaterial as a scaffold that form a supportive structure for culturing cells *in vitro* and surgical delivery *in vivo* to the patient. For the majority of mammalian cell type, a growth stimulus is also required to control the differentiation of cells into the appropriate cell linage and to promote their proliferation (Di-Silvio *et al.*, 2003).

Osteoblasts, chondrocytes and mesenchymal stem cells obtained from patient's hard and soft tissues can be expanded in culture and seeded onto scaffold that will slowly degrade and resorb as the tissue cultures grow *in vitro* and/or *in vivo* (Langer and Vacanti, 1993). The scaffold or three-dimentional (3-D) construct provides the necessary support for cells to proliferate and maintain their differentiated function and its architecture defines the ultimate shape of the new bone and cartilage. Several scaffold materials have been investigated for tissue engineering of bone and cartilage including hydroxyapatite, poly(α-hydroxyesters) and natural polymer such as collagen and chitin (Di-Silvio *et al.,* 2003).

2.6 Nanotechnology

After years of evolutionary research, nanoscience and nanotechnology can address one of the greatest challenges in the post-genomic era of the 21st century. Nanotechnology in the life science is omnipressent. Several biological systems operate at the nanoscale with remarkable precision and regulation. A fine example is that of biomolecular motor proteins, designed by nature to carry out critical functions in the cell. Most of the properties of these proteins are nanoscale. A similar situation is encountered with biomaterials, another area with tremendous application potential where novel materials are being created by material designer based on inspiration from complex nanobiological systems (NSTI, 2005).

The ability to mimic the dimensions of constituent components of natural tissues, like proteins, nanophase materials may be an exciting successful alternative. Nanophase materials are defined as materials with constituent dimension less than 100nm in at least one direction. Materials investigated to date include nanophase ceramics, metals,

polymers and composites. Nanophase materials may be optimal materials for tissue engineering applications which is not only due to their ability to stimulate dimensions of proteins that comprise tissues, but also because of their higher reactivity for interactions of proteins that control cell adhesion and, thus, the ability to regenerate tissue (NSTI, 2005).

2.7 Medical devices

A medical device is defined as any instrument, apparatus, of other article that is used to prevent, diagnose, mitigate or treat a disease or to affect the structure of function of the body with the exception of drug (SMDA, 1990). It has been agreed that biomaterials are also considered as a medical device. Thus, medical device has extremely large item and biomaterial is classified either as class II or class III depending on the regulation in the particular activity. The classification according to Food and Drug Act (FDA) is as follows:

a) Class I:

Wheel chairs, patient electrode, Scalpels, dental drills, wound management systems, hearing aid tester.

b) Class IIa:

All patient monitoring equipments, syringes, needles, ultra sound devices, external ECGs, diagnosis devices.

c) Class IIb:

Laser devices for applications, internal ECGs, non-energized implants, treatment devices.

d) Class III:

Energized implants, all Intracardiac applications, heart valves, catheters, non-energized implants, all devices in contact with the central nervous system (CALISO, 2006).

2.8 Biosafety of Medical devices

2.8.1 Biocompatibility of biomaterials

Biocompatibility is generally defined as the ability of a biomaterial or medical device to perform with an appropriate host response in a specific application (Anderson, 2008). Bioresponse or biocompatibility assessment (i.e. evaluation of biological responses) is considered to be a measure of the magnitude and duration of the adverse alterations in homeostatic mechanisms that determine the host response. From a practical view, the evaluation of biological responses to a medical device is carried out to determine that the medical device performs as intended and presents no significant harm to the patient (Anderson, 2008).

Kirkpatrick *et al.* (1997) reported that biocompatibility involved two principle areas. The first is the principle of 'biosafety' which involves the exclusion of severe deleterious effect of the biomaterial on the organism. This encompasses both cytotoxicity and the field of mutagenesis and carcinogenesis. The second area is the 'biofunctionality' which deals with the ability to perform with the appropriate host response in a specific application.

2.8.2 Cytotoxicity testing *in vitro*

Definition of cytotoxicity means to cause toxic effects such as cell death, alteration in cellular membrane permeability, enzymatic inhibition at the cellular level. A toxic material is defined as a material that releases a chemical in sufficient quantities

through inhibition of key metabolic pathway. The number of cells that are affected is an indication of the dose and potency of chemical (Northup, 1993).

Cytototoxicity tests are recommended for all medical devices because

(i) they allow for rapid evaluation,

(ii) standardized protocols are employed,

(iii) quantitative and comparable data are produced and

(iv) due to their sensitivity, they allow for discarding toxic materials prior to animal testing.

The categories of test are listed as an extract test, direct-contact test and indirect-contact test. Experimental studies have demonstrated that good correlation between *in vitro* and *in vivo* tests, thus confirming the usefulness of *in vitro* tests as systems to select the materials (Freshney, 2000).

Many experiments carried out *in vitro* are for the sole purpose of determining the potential cytotoxicity of the compounds being studied, either because the compounds are being used as pharmaceuticals or cosmetics and must be shown to be nontoxic or because they are designed as anticancer agents and cytotoxicity may be crucial for their action (Freshney, 2000).

2.8.2.1 Nature of cytotoxicity assay

The choice of assay will depend on the agent under study, the nature of the response, and the particular target cell. Assays can be divided into five major classes:

a)Viability

Viability assays are used to measure the proportion of viable cells following a potentially traumatic procedure, such as primary disaggregation, cell separation, or

freezing and thawing. Most viability tests rely on a breakdown in membrane integrity that is determined by the uptake of a dye to which cell is normally impermeable (e.g., trypan blue, erythrosin, naphthalene black) or the release of a dye normally taken up and retained by viable cells (e.g., diacetyl fluorescein or neutral red). However, this effect is immediate and does not always predict ultimate survival (Freshney, 2000).

b) Survival

Short term tests are convenient and usually are quick and easy to perform. They reveal only cells that are dead (i.e., permeable) at the time of the assay. Frequently, however, cells that have been subjected to toxic influences (e.g., irradiation, antineoplastic drugs) show an effect several hours, or even days, later. The nature of the tests required to measure viability in these cases is necessarily different, since by the time measurement is made, the dead cells may have disappeared. Therefore, long term tests are used to demonstrate survival rather than short term toxicity, which may be reversible. Survival implies the retention of regenerative capacity and is usually measured by plating efficiency (Freshney, 2000).

c) Metabolic

Metabolic assay is usually microtitration based, of intermediate duration that can either measure a metabolic response (e.g., dehydrogenase activity, DNA, RNA or protein synthesis) at the time of, or shortly after, exposure, or measure the same parameter two or three population doublings after, exposure, when it is more likely to reflect cell growth potential and/or survival (Freshney, 2000).

d) Transformation

Transformation is associated with genetic instability and three major classes of phenotypic change, one or all of which may be expressed in one cell strain:

i) Immortalization, the acquisition of an infinite life span

ii) Aberrant growth control, the loss of contact inhibition and anchorage dependence

iii) Malignancy, as evidence by the tumorigenic potential of the cells

The characteristic of a cell line do not always remain stable. Normal, human finite cell lines are usually genetically stable but cell lines from other species, particularly the mouse are genetically unstable and transform quite readily (Freshney, 2000).

e) Irritancy

A response analogous to inflammation, allergy or irritation *in vivo*; as yet difficult to model *in-vitro*, but may be possible to assay by monitoring cytokine release in organotypic culture (Freshney, 2000).

2.8.3 Biocompatibility testing

2.8.3.1 MTT assay

Several indirect methods are commonly used for the measurement of cell viability, cytotoxicity and cell proliferation. These assays are simple, rapid and well suited for the analysis of large number of sample in 96-well microtitre plates. These methods are usually based on the measurement of an enzymatic activity which reflects the general metabolic status of cell. One of the best known is the 3-[4,5-dimethylthiazol-2y]-2-5-diphenyltetrazolium bromide (MTT) assay. The MTT assay is quantitative calorimetric assay based on the cleavage of the yellow water-soluble tetrazolium salt, MTT, to form water-insoluble, dark-blue fomazan crystals. MTT cleavage occurs only in living cells by the mitochondrial enzymes succinate dehydrogenase. The formazan crystals are solubilised using suitable organic solvent,

usually isopropanol, and the optical density of the resulting solution is measured using spectrophotometer. The absorbance is directly proportional to the concentration of the blue formazan solution, which is in turn proportional to the number of metabolically active cells (Mosmann, 1983).

2.8.3.2 Alkaline phosphate assay

An alternative indirect method for measurement of cell number is the alkaline phosphate assay which is based on conversion of non-fluorogenic substrate 4-methyl umbelliferyl phosphate to the fluorescent product 4-methyl umbelliferylone by the widely distributed enzyme alkaline phosphatase (Freshney, 2000).

2.8.3.3 Neutral red assay

Neural red uptake assay, initially described by Finter, (1969) is being used commonly in biochemical and immunological studies and it has also been adopted as a recommended method for qualitative assessment of biomaterial safety by some national agencies for normalization of testing (AFNOR, 1988). The neural red assay quantifies the number of viable cell after exposure to toxicants and based on the cellular uptake of the dye which passes through intact membranes of viable cells and is concentrated in the lysosomes.

However, neutral red is not retained by nonviable cells. Uptake of neutral red is quantified by fixing the cells in formaldehyde and solubilizing the stain in acetic ethanol, allowing the plate to be read on an ELIZA plate reader at 570nm. Neutral red tends to precipitate, so the medium with stain is usually incubated overnight and centrifuged before use. This assay does not measure the total number of cells, but it does show a reduction in the absorbance related to loss of viable cells and is readily automated (Fotakis and Timbrell, 2006).

2.8.3.4 Direct measurement of mitogenetic activity in cell culture

a) [^{3}H] thymidine into acid-insoluble materials

This is a direct method which is used for measuring the mitogenic activity of a growth factor in any adherent or non-adherent cell type, which can be made to arrest in G0 or G1 phase of the cell cycle after suitable period of cell culture or serum deprivation (Freshney, 2000).

b) Autoradiography of labelled nuclei

This method measures the incorporation of [^{3}H]thymidine into nuclei autoradiographically by exposing the labeled cell into film. As the sensitivity of this method is less than that of scintillation counting of [^{3}H]thymidine incorporated into acid-insoluble material, a higher specific activity of radiolabelled precursor is used, usually 5µCi ml^{-1}[^{3}H] thymidine. The exposure to such cells to a high level of radioactivity causes DNA damage resulting in arrest of cells after S phase (Freshney, 2000).

c) BrdU incorporation and staining

This method measures incorporation of the thymidine analogue BrdU into DNA. BrdU is detected by immunocytochemistry using anti-BrdU antibody, or commonly using fluorescent DNA-binding dyes such as Hoechst 33258, whose fluorescence is quenched by BrdU (Freshney, 2000).

2.8.4 Genotoxicity testing

The identification of substances capable of inducing mutations has become an important procedure in safety assessment. Chemicals that can induce mutations can