ABSTRACT

Ceramic for bone replacement is one of the active areas of biomaterial research today. One of the active ceramic biomaterial research is hydroxyapatite (HA). The HA has been used clinically for the last 20 years because of its excellent biocompatibility, osseoconduction and osseointegration. The only problem with pure HA is that it is brittle. Because of this, many modifications have been done by scientist to develop a stronger biocompatible ceramic material. Our local scientists have developed a value added HA that is stronger and less brittle than pure HA.

The aim of this study is to perform an *in vitro* biocompatibility test for a value added HA based biomaterial.This biocompatibility study will look at the cytotoxic charateristic of the value added test material compared with pure HA standard material (SM) and known positive control (CM). The experiment is designed to detect biofunctionality (proliferation) of the human lung embryonic fibroblast cell line (MRC-5) in response to the material tested.Thus the cell count at the end of the specific test period will be the principal determinant of the biocompatibility result for the material tested. The test material was developed by a group of scientists at our local university. The standard material is (pure HA) $Ca_{10}(PO_4)_6OH_2$ and the positive control used is latex rubber (standard used in *in vitro* testing)

A preliminary study of the relationship between the different weight (concentration) of the value added HA (TM) and pure HA (SM) against the proliferation of cell lines was carried out. The test was done by chosing 72 hours as the incubation time. The SM and TM were introduced to the cells at different

concentrations of 1 mg/ml, 1.5 mg/ml, 2 mg/ml, 2.5 mg/ml, 3 mg/ml, 4 mg/ml and 5 mg/ml. At the end of the 72 hours incubaion period, the cells were stained with neutral red and the optic density (OD) was measured using a spectrophotometer. The percentage killing of the cells was then calculated. The Percentage killling of the cells after treatment with TM and SM was compared. The results showed that value added HA had significant increased percentage killing on the cells when tested with concentrations from 1 mg/ml to 5 mg/ml but the pure HA was not cytotoxic to MRC-5. For different concentration percentage killing will increased 10.541% every 1 mg/ml increased.

From this preliminary test the optimum concentration/weight of test material effective on proliferation of cells was determined. 2 mg/ml of test material was chosen to be the suitable concentration. For the definitive tests, the SM, TM and CM at 2 mg/ml each were introduced to the cells and the final cell count was done after incubation periods of 1 hour, 3 hours, 24 hours, 3 days and 7 days. The percentage killing of the cells were then compared for TM, SM and CM. At different time the value added HA had killing effect starting at day 3. The pure HA also had kiling effect (20.9 %) but in day 7. Percentage of killing of TM at day 7 were 46.27 %. For control material killing effect started after the first hour and at day 7 percentage killing were 93.66 %. Statistically show that for TM, percentage killing in 1 hour were 0.279 %/hour, compare to SM 0.122 %/hour and for CM were 0.494 %/hour.

The present *in vitro* evaluation indicated that the value added HA was more toxic than pure HA but the toxicity of value added HA was low compared to the positive control. In this screening, we can conclude that there is toxicity effect by both value

added HA and pure HA but the value added HA was more toxic. The toxicity effect was more on value added HA when the concentration was increased by 1 mg/ml compared to pure HA, which has no effect. Statistical analysis showed a strong correlation between the effect of TM concentration on the percentage of killing. In this test, the conclusion can be made that the value added HA has less biocompatibility than the commercialized pure HA.

ACKNOWLEDGEMENTS

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

I would like to thank the following people who have helped me throughout the course of my study:

First and foremost, I am deeply grateful to The Lecturer of Oral Maxillofacial Surgery, Faculty of Dentistry, University Malaya, Professor Dato' Dr. Lian Chin Boon and Associate Professor Datin Dr. Norhanom Abdul Wahab, Deputy Director of The Centre for the Foundation Studies in Science, University Malaya for their advice, and guidance, under whose supervision the work was carried out, in spite their extremely busy schedule and to Associate Professor Radzali of USM for providing me with the biomaterial.Also to Dr. Fauziah and her staff for provided me the MRC-5 cells.

I wish to thank R. Sujatha from The BioMolecular Laboratory, Institute of Postgraduate Studies and Research (IPSP), University Malaya who painstakingly and patiently helped me with the tissue culture technique.

I am grateful to my sponsor, University Sains Malaysia for granting me leave and financial and also to Associate Professor Dr. Rani, The Dean of Faculty Dentistry University Sains Malaysia, who really help in successful of my research and his staff for their continued support.

I would like to thank to Head of Oral Maxillofacial Surgery Department, Associate Professor Dr. Ong Siew Tin for her understanding, help and encouragement throughout my postgraduate programme.

I want to thank Associate Professor Dr Zainal for understanding and encouragement. Also to all Lecturers in Deparment of Oral Maxillofacial Surgery for help and guidance.

Miss Lily,Sister, Staff Nurses and All DSAs for their kind help and assistance.

For Dr Ayu , Dr Mastura, Mrs.Suria and Mr.Hakim I really appreciate your help and guidance.

I would also thank Puan Adida (Head of Dental Library, Faculty of Dentistry) and her staff for their help and assistance.

Also thank to Dr. Syed Hatim (Statistician, USM, Kubang Kerian) for guidance in statistic analysis.

Thank you to my best friends, Dr. Raja Azman, Dr. Iqbal, Dr. Shaiful, Dr. Akram, Dr. Rahmi, Dr. Norhayati, Dr. Rosrahimi, Dr.Shim, Dr. George, Dr. Ajeet and Dr. Ravi, without whose participation and help, this study would not have been possible.

Thank you to my fellow colleagues in PPSG USM who constantly gave me advice and support while doing my project.

Finally, I would like to thank my deareast, beloved Maziana Hussin, and my lovely children, Muhamad Irham and Nurul Batrisyia for their love, understanding, moral support and encouragement during my absence.

Last but not least, my parents and mother-in law for their understanding and their Prayer.

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mg	: microgramme
HA	: hydroxyapatite
CO_2	: carbon dioxide
FBS	: foetal bovine serum
TM	: test material
SM	: standard material
CM	: control material
ml	: mililitre
µm	: micrometer
ISO	: International Standardization Organization
DMEM	: Dulbecco's Modified Eagle's Medium
$NaHCO_3$	: sodium bicarbonate
Nacl	: sodium chloride
NR	: neutral red
°C	: degree centigrade
PBS	: phosphate buffered saline
rpm	: revolution per minute
SDS	: sodium dodecyl sulphate
sq. cm	: square centimeter
Na_2HPO_4	: sodium phosphate anhydrous
KH2PO4	: potassium dihydrogen orthophosphate
OD	: optic density

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CHAPTER 1

INTRODUCTION

1.1 Biomaterial

An alloplastic material is an inert foreign substance implanted within living tissue. Phenomenal expansion in the use of implant materials has occurred since 1945 as industrial improvements and developments in metal alloys and developments in polymer chemistry have been adapted for biomedical application. Over 50 different inorganic materials are in current use in an estimated 2 million patients annually because: (a) they are available when autologous tissue is not (e.g. hydrocepholus shunt or pacemaker units); (b) there is no donor site morbidity or scaring; (c) nonbiodegradable alloplastic material do not undergo resorption, as do bone or cartilage graft; (d) biodegradable implant systems are being developed for uses such as controlled-release drug delivery system (Mc Grath, 1989).

1.1.1 Fundamental Properties of All Implants

The tissue response to different implants varies with the chemical composition and micro- and macrostructure of the synthetic material, and these differences are used clinically. For example, vigorous tissue ingrowth with Marlex mesh hernia repairs provides a strong and lasting support, while the fibrous encapsulation of a

silicone tendon prosthesis ensures free gliding of a tendon graft. However, certain properties and concerns are common to all implants (Mc Grath, 1989). The properties are discussed below.

Noncarcinogenic

The implant must not be carcinogenic. Foreign body induced sarcomas, predominantly fibrosarcoma, can be observed in animals with metal, polymer and glass implant. This appears to be species-specific, occuring in rats, mice, and hamsters but not in dogs or primates. It is also time-dependent, with a latent period in the rat that corresponds to 10-15 years of human life. The incidence of rodent tumour is related to the size and the configuration of the implant; there is a higher frequency of tumours with larger implants and with smooth-surfaced implants that evoke a long axis-oriented collagenous shell than with powdered, perforated or porous forms of the same material (Mc Grath, 1989).

Nontoxic

Toxicological evaluation of a material includes tissue culture and *in vivo* testing of cell growth, attachment, and movement parameters as well as histochemical analysis of enzyme activity and solute concentration in the boundary layer at the implant-tissue interface (Mc Grath, 1989).

Nonallergic

The highest incidence of hypersensitivity reactions to inorganic implants is reported with metallic devices.On skin testing, as many as 6% of patients with metallic prostheses are found to be sensitized to constituent nickel, colbalt, or chromium, (Elves *et al.,* 1975; Deutman *et al.,* 1977).

Nonimmunogenic

Despite the absence of organic molecules in non biological materials, inorganic implants may not be immunologically inert. Theoretically, either a humoral or a cell-mediated immune response could be induced by leaching out of nonproteinous substances stimulating immunoglobulin release and an autoimmune reaction, or by absorption of endogenous proteins into implants, which would alter their conformation in such a way that new antigenic complexes are created (Mc Grath, 1989). While standard *in vitro* immunogenicity testing is required for all implants, new interest in implant immunopathology (Bagnall, 1983) has been stimulated by recent reports of autoimmune connective tissue disease developing after the injection of paraffin or liquid silicone (Mc Grath, 1989).

In termed human adjuvant disease, the clinical manifestations are similar to those of scleroderma, rheumatoid arthritis, polymyositis and other (Mc Grath, 1989). Some clinical discrepancies exist, the association with implants has not been proven, and immunological mechanisms of the diseases induction are uncertain.

Mechanical Reliability

In spite of the physical and mechanical functions, biomaterials must show enviromental stability. All of the properties designed into an implant, such as tensile strength, elastic modulus, impact resistance, conductivity, crystallinity and cross-linking, can be tested during the fabrication process. However, the implant must function in a dynamic biochemical enviroment where tissue enzymes, free radical, bacteria and drugs are present. Screening for hydrolytic resistance, oxidative degradation, extractability, electrical stability and numerous other factors can make the testing of biomaterials a complex process (Mc Grath, 1989).

Biocompatibility

All implanted materials initially give rise to an inflammation reaction with migration of macrophages and fibroblast into the zone around the implant and vascularization to form typical granulation tissue. The course thereafter depends on the chemical composition of the implant, the physical nature of the implant (size, shape, porosity,surface finish) and the tissue in which the prothesis is implanted (Mc Grath, 1989).

Absorbable material

The degradation of an absorbable material can occur by dissolution, hydrolysis or enzymatic degradation (Mc Grath, 1989). Macrophage phagocytosis and intracellular digestion clears the residue.

As the material is absorbed, granulation tissue replaces it with the eventual formation of a stable scar. The rate of degradation is accelerated by increasing hydrophilic properties, surface charge, or surface energy and or by adding amino acids (Mc Grath, 1989) : these all increase material reactivity.

Nonabsorbable Material

The cellular response around a nonabsorbable substance varies with its chemical composition, reflected in the water content, charge distribution, ion exchange and protein deposition at its surface. Shape affects the in vivo reactivity for unknown reasons. Roughness of the surface profoundly affects the adjacent macrophage behavior and lysosomal enzyme production. The absolute size of the implant has less effect than its contiguous distances or pore size.

These differences in inflammatory cell population and behavior are accompanied by differences in fibroblast activity and collagen deposition. The relatively inert, smooth surface implant becomes encapsulated in a dense collagenous connective tissue sheath. The reactive, rough, woven or knitted implant is incorporated or firmly fixed by the fibrous tissue.

1.2 Objective

The aim of this study is to perform an *in vitro* biocompatibility test for a value added HA based biomaterial. This biocompatibility study will look at the cytotoxic charateristic of the material tested compare with pure HA standard material (SM), the value added test material (TM) and known positive control (CM).

The experiment is designed to detect biofunctionality of the cell lines in response to the material tested and in this respect the proliferation of the fibroblast will be the principal criteria of the bioassay.

Thus the cell count at the end of the specific test period will be the principal determinant of the biocompatibility result for the material tested.

The test material was developed by a group of scientists at the School of Material Science and Engineering, Malaysia Science University (USM). The standard material (pure HA) Ca_{10} ($PO_4)_6$ OH_2 is readly available in the market. The positive control used is latex rubber (urine cateter).

1.2.1 General objective

The general aims of the present study are :

(i) To develop a biocompatibility biomaterial that can be use as a bone replacement substitute.

(ii) To develop a value added biomaterial which will maintain its biocompatibility similar to the standard biomaterial that is already used in clinical practice.

1.2.2 Specific objective

The study is aimed at testing a value added hydroxyapatite base material for its cytotoxicity properties in an *in vitro* study using a human embryonic lung fibroblast cell line (MRC-5).

CHAPTER 2

LITERATURE REVIEW

2.1 Synthetic biomaterial

The first reported use of synthetic biomaterial in facial and reconstructive surgery occurred around 1600 when Fallopius implanted a gold plate to repair a calvarial defect (Costantino *et al.*, 1993). Since that time, contour reconstruction of the non-stress-bearing, craniofacial skeleton has continued to be a technical and materials problem. Metals are difficult to shape and are hampered by problems such as infection and corrosion (Gordon & Blair, 1974).

Although we have had several hundred years of experience with synthetic biomaterial, very few implants have proven to be ideal. The difficulty in developing biocompatible materials underscores the complex interaction between the body and any implant.

Materials that initially seem to be appropriate and successful, frequently fail to remain integrated with the surrounding tissue or are degraded by the body over time. A recent example of this, is the silicone gel breast implant.

Biomaterial actually can be natural, semi synthetic or synthetically produced. Numerous natural and semi synthetic materials are available in the form of xenograft

(from species), allografts (from the same species) or auto graft (from the same organism), and these function well in facial plastic and reconstructive surgery. Any synthetic biomaterial can also be referred to as an alloplast. There is a wide variety of alloplast, most of which are specifically designed for either soft tissue or skeletal application .

The success or failure of any alloplast hinges on many factors, such as its chemical composition, biostability, physical form, mechanical properties, site of implantation and presence of impurities. Of those alloplastic materials used to augment and replace the facial skeletal, the most promising and well tolerated are calcium phosphate-based compound (Kent, 1986a).

Although it would seem that the chemical composition of the implant would be of primary importance, its physical form is equally critical in determining biocompatibility (Holmes & Hagler, 1988) .In fact, an implant with excellent biocompatibility on a chemical level can fail just because its physical form is not appropriate.

Focusing on chemical composition, physical form, and appropriate application are good starting points when evaluating any new biomaterial for potential use, since most have not yet demonstrated long-term biocompatibility in humans beings (Costantino *et al.*, 1993).

Ceramic for bone replacement is one of the active areas of biomaterial research today. At least 40 to 50 different ceramic materials are under evaluation as implant materials. These are divided into three categories: (a) inert, (b) reactive or bonding, (c) resorbable. Like an autologous cancellous bone graft or a coral template,

the resorbable ceramic prosthesis serves as a scaffold for new bone growth and eventually is replaced by living tissue. The advantage of the resorbable material is that there will be no long term stability or compatibility problems. The disadvantages are strength degradation during the remodelling process, which may cause mechanical implant failure (Bajpai PK *et al.*, 1976; Ferraro J.W., 1979) .

One of the active resorbable ceramic biomaterial research was calcium phosphate which was mainly composed of hydroxyapatite. Virtually all current calcium phosphate biomaterial can be classified as polycrystalline ceramics since their material structure is derived from individual crystals of highly oxidized substances that have been fused together at the crystals grain boundaries by a high temperature process called sintering.

2.1.1 Hydroxyapatite

Hydroxyapatite (HA) forms the principal mineral component of bone and comprises 60 to 70% of the calcified skeleton.

Its chemical composition is $Ca_{10}(PO_4)_6(OH)_2$ and it has been produced synthetically since the early 1970s and used clinically for the last 20 years (Jarcho, 1981; Costantino *et al.*, 1993).

All forms of HA have excellent biocompatibility and when placed in contact with viable bone, results in osseoconduction and osseointegration. If macropores (200

to 300 μm) are fibricated into an HA implant, bone grows into macropores (Grenge et al., 1989). There is no evidence that HA is osteogenic. HA does not cause a chronic inflammatory response, toxic reactions, or a foreign body giant cell reaction (Costantino *et al.*, 1993). There are two types of HA: ceramic and non ceramic. Until recently, all forms of HA in clinical use were ceramic preparations. Ceramic HA is synthesized in a crystal form at a very low pH, then heated to 800° to 1300° to form a solid mass of HA.

This heating process, called sintering, results in a hard, strong, functionally non resorbable biomaterial. Ceramic HA is available in two forms: dense or porous (Costantino *et al.*, 1993). Porous HA were more useful then dense. Porous HA, has interconnecting 200μm pores that allow bone to grow onto the internal surface area of the implant by osseoconduction. The porous pattern of this material is based on marine corals. The calcium carbonate skeleton of the coral is chemically converted to HA while maintaining the porous structure of the coral.

Porous HA is ingrown by fibro-osseous tissue and becomes fixed to the surrounding bone within several weeks (Costantino *et al.*, 1993).

After tissue ingrowth is complete, the implant consists of approximately 17 % bone, 43% soft tissue, and 40% residual HA implant. Although these implants are very slowly resorbed (1 % per year), they can be considered functionally non resorbable. This material comes in block form but is difficult to shape because it fractures easily (Costantino *et al.*, 1993). It cannot be used for any stress-bearing

application and is most appropriate as an onlay graft or spacer material following facial osteotomies (3 year evaluation) (Salyer and Hall, 1989).

Because it is fragile and difficult to contour, the application has been largely limited to dentistry. Experience with porous HA for alveolar ridge augmentation has shown that this material is resistant to infection after ingrowths by fibro-osseous tissue, but can become exposed if the overlying soft tissue is not adequate.

In summary, porous ceramic HA is a very biocompatible alloplast with some significant structural limitations. It should be considered as a bone graft substitute only for selected applications in facial plastic and reconstructive surgery (Costantino et al., 1993).

The principal limitation of the calcium phosphate implant material is their mechanical properties. Like most ceramics, these materials are quite brittle, have low impact resistance and relatively low tensile strengths. A comparison of the ranges of mechanical properties observed with calcium phosphate ceramics versus those of currently used metallic materials, as detailed in table 2.1, would appear to preclude their use as hard tissue prosthetic material (Jarcho, 1981). So we should find the HA base material with very biocompatible, osseoconductive, stronger and easier to contour without breaking.

Table 2.1: The Mechanical Properties Of Bone and Metallic And Calcium Phosphate Implant Materials

Material	Compressive Strength (10^3 psi)	Tensile strength (10^3 psi)	Modulus (10^3 psi)
Bone			
Cortical bone	20	10.0	2
Cancellous bone	6-9	0.5	_
Metals			
316 L Stainless	_	80-145	30-40
Cor-Cr alloy	_	97	30
Titanium	_	50	16
Calcium Phosphates			
Porous	1-10	0.36	_
Dense	30-130	10-28	5-15

As a result, implantation of this material is limited to stress regions of the body (Ogiso *et al.*, 1996). This is because of the low fracture toughness (0.8 to 1.2 Mpam $^{1/2}$) and the low flexural strength (< 130 Mpa) of the material (Wang and Chaki, 1993).

A possible explanation of the basic biologic profile of calcium phosphate implant material lies in the chemical nature of these materials; all are composed

mainly of calcium and phosphate ions, the same ions, which make up the bulk of natural bone minerals. Hence, these materials, when implanted in bone, are capable of participating in calcium phosphate solid solution equilibrium at their surface .The requisite calcium and phosphate ions needed to establish this equilibrium may be derived from the implant or surrounding bone, or both (Jarcho, 1981).

Takeshita F. *et al.* in 1997 did a study of bone formation around dense HA implants using light microscopy, image processing and confocal scanning microscopy .He found that the percentage of bone contact of HA is superior to titanium throughout the experimental period (5, 7, 14, 28,84 and 168 days). HA coating has a great attraction because HA has high biocompatibility and different form of HA can stimulate bone regeneration and increase the bone–HA contact ratio (Takeshita *et al.*, 1997).

Yu-Liang *et al.* conducted a study in 1999 about osteoblastic cell attachment to hydroxyapatite coated implant surfaces *in vitro*. The results show that HA coatings on titanium were altered by interaction with culture medium. Cellular attachment to HA surfaces was slightly higher than titanium surfaces, thus confirming the bone-enhancing properties of HA. The level of HA crystallinity seems to have no influence over initial cell adherence in either the untreated or pretreated conditions. However, the higher osteoblastic attachment on pretreated HA surfaces observed in this study implies that the active interaction of HA with living tissue plays an important role in the initial cellular response to this material (Yu-Liang, 1999).

2.1.2 Zicornia

Zirconia or zirconium oxide is derived by heating zirconium hydroxide or zirconium carbonate (Hawley G.G., 1981). Zirconia is a new class of ceramic known as engineering, special or technical ceramics (Anderson J.C., 1990). This zirconia can be developed to retard the crack propagation by a kind of crack-closing mechanism, this will increase the toughness of the ceramics (Smith W.F., 1996; Anderson J.C., 1990). Yttrium oxide partially stabilized zirconia has been advocated as an alternative to alumina. This class of ceramic has a higher toughness than alumina, since it can be transformation toughened (Cahn R.W. 1992).

Kutty M.G. and Ramesh Singh undertook a study on the effects of zirconia additions on the sintering behaviour and phase stability of hydroxyapatite ceramics. They found that the presence of zirconia phase in hydroxyapatite matrix played an important role in the stability of the HA phase (Kutty M.G. & Ramesh Singh, 1999).

2.2 Biocompatibility Testing

Biocompatibility has undergone a change of emphasis in the past years and is now generally accepted as involving two principal areas. The first is the principle of 'biosafety', which involves the exclusion of severe deleterious effects of the biomaterial on the organism. This encompasses both cytotoxicity and the complicated field of mutagenesis and carcinogenesis. The second area is concerned with 'biofunctionality', an aspect which deals with the 'ability to perform with an appropriate host response in a specific application'. The pressure in industrialized societies to reduce experiments on animals, as well as the considerable advances in *in vitro* methodology, have paved the way for a variety of tests which are suitable for testing regimes and, in particular, for the International Standardization Organization (ISO) standard. The need to apply modern methods of cell and molecular biology to biocompatibility testing and risk assessment, as well as the requirement for quantification are emphasized (Kirkpatrick, 1998).

2.2.1 Biosafety

(A) Cytotoxycity testing *in vitro*

Definition of cytotoxicity - means to cause toxic effects (death, alteration in cellular membrane permeability, enzymatic inhibition, etc) at the cellular levels. A toxic material is defined as a material that releases a chemical in sufficient quantities

through inhibition of key metabolic pathways. The number of cells that are affected is an indication of the dose and potency of the chemical (Northup, 1996).

This component of biological evaluation of medical devices is central to the International Standard (ISO 10993-5), which sensibly does not specify a single test, but rather attempts to present guidelines for the choice of suitable tests and define important principles of these tests. The latter includes positive and negative control materials, extraction conditions, choice of cell lines and cell media, as well as important aspects of the test procedures, including test on extracts, and test by direct and indirect contact. The standard also allows for qualitative or quantitative evaluation. The present authors have been concerned about critically assessing these methods from a scientific viewpoint in order to achieve a maximum standard of evaluation. Theoretical and practical elements of performing such *in vitro* cytotoxicity test have been published elsewhere (Kirkpatrick & Mittermayer, 1990).

The ISO standard categories of evaluation are as follows:

(a) assessments of cell damage by morphological means;

(b) measurements of cell damage;

(c) measurements of cell growth;

(d) measurements of specific aspects of cellular metabolism.

(i) MTT test

This quantifiable test is a measure of cell metabolic function, dependent on the intact activity of a mitochondrial enzyme, succinate dehydrogenase, which is impaired after exposure of cells to toxic surroundings. In brief, the test involves the conversion of the tetrazolium salt, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), to an insoluble formazan product, which can be quantitated by spectrophotometry (Kirkpatrick *et al.*, 1998).

(ii) DNA synthesis

Cells *in vitro* in a non-confluent state in monolayer undergo mitosis, prior to which deoxyribonucleic acid (DNA) synthesis occurs (in the S-phase of the cell cycle). Culture which are entering the log growth phase can be pulse labeled with a nucleotide building block of DNA, which has been tagged, for example, with radioactive atom to enable detection and assisted by the case of tritiated thymidine. Some people prefer the use of the non-radioactive precursor, bromodeoxyuridine (BrdU), which after uptake can be detected by a fluorescent dye-conjugated monoclonal antibody against BrdU (Kirkpatrick *et al* , 1998).

(iii) Membrane integrity test

Toxic effects on cells involve alterations in membrane functions, of which the structural and functional integrity of the plasma membrane separating the cell from its

surroundings plays a vital role. This test using a combination of fluorescein diacetate (FGDA) and ethidium bromide (EB).

(B) Mutagenesis/ carcinogenesis

This extremely complicated and vexed question of risk assessment of biomaterials has unfortunately failed to be influenced by the enormous progress which has been made in understanding of pathomechanism in conventional oncology.

In vitro assays using human cells, an approach will involve specialized techniques to investigate nucleic acids (DNA, RNA), whether at macromolecular level or, in the case of DNA, as an integrated element of a chromosome. It is thus possible to study cell regulatory mechanism at two principal levels- the level of *gene transcription* and the levels of *gene translation.* The nucleic acid level of gene activity can be relevantly studied using techniques such as Northern blotting (NB), *in situ* hybridization (ISH) and the polymerase chain reaction (PCR). The level of gene translation involves the gene product, usually in the form of a protein, which could be a structural protein, a receptor or an enzyme concerned with (every conceivable) cell function (Kirkpatrick *et al.*, 1997).

2.2.2 Biofunctionality

Cytotoxicity testing *in vitro* usually involves permanent cell lines, such as HeLa cells (derived from a human cervical carcinoma) or L-929 cells (a mouse fibroblast cell line). However, the above-mentioned tests can equally be carried out on primary isolated human cells. As biofunctionality concerns the specific function of

ı medical device, it is evident that non-transformed human cells relevant to the ;unction of the device should be used. Thus, endothelial cells (EC) would be the ogical choice for biofunctional testing of vascular prostheses. Platelets, monocytes and granulocytes for all blood-contacting devices, and fibroblasts, chondrocytes and osteoblast are all ideal for studying cell interaction with orthopaedic or dental implants.

The essential philosophy of studying biofunctionality *in vitro* is that one aims at establishing reproducible and quantifiable assays which center on cells involved in and biological parameters relevant to the medical device application. It is clear that these parameters will include some of the functions studied under the heading of cytotoxicity, for example, cell proliferation. A vascular prosthesis designed to permit EC pre-seeding must be shown to promote an acceptable rate of EC growth on its inner surface.

This is, however, not the only parameter. Others include those in the following subsections (Kirkpatrick *et al.*, 1998).

(A) Cell adhesion

If biomaterial cytotoxicity can be excluded, cell adhesion is probably the single most important aspect of cell interaction with a biomaterial and may or may not be desirable. A medical device designed to be integrated into a host tissue, e.g. a bone or joint prosthesis, must in the first analysis promote cell adhesion, whereas cell adhesion to a blood-contacting surface can elicit serious medical complications, as in catheters and blood dialysis devices. Quantification can be carried out using image

analysis system at both light microscopically and ultra structural level in assays designed to give response curves at various times after beginning cell-substratum interaction from an initial suspension of cells applied to the biomaterial surface. An alternative to computer assisted image analysis of a microscopic image of the cell-bio-material interaction is a spectrophotometric method using an elisa-reader for 96-well microtitre plates, coated with the material to be studied or in the presence of a material extract. The latter procedure has been described previously (Kirkpatrick *et al.*, 1998)

(B) Cell spreading

This phenomenon, which involves complex cytoskeletal reorganization, is an essential function of a cell which has become adherent to a surface and precedes the function of cell proliferation to give a cell-covered surface.

Cell spreading is thus a parameter of vital importance in studying biomaterials designed to be fully integrated into the host tissues. This parameter can be assessed, for example, by quantitative scanning electron microscopy and has been used to evaluate the suitability of surface modified poly(carbonate urethane)s for vascular prostheses (Kirkpatrick *et al*, 1998).

(C) Cell proliferation

Already presented as an important parameter concerning risk assessment, cell proliferation determination is a central component of studying materials designed to

be integrated into host tissue, as in case of osteointergrated into host tissues, as in the case of osteointegration of a joint prosthesis or dental implant. The requirement to use the relevant cell type in *in vitro* assays has already been stressed. Thus, human osteoblasts have been used to study how ceramic coating affect cell growth (Labat *et al.*, 1995). Bone cements in dental practice have also been tested with respect to their ability to influence the cell cycle of osteoblast (Granchi *et al.*, 1995).

The formation of a confluent monolayer of endothelial cell (EC) on vascular prostheses is a prerequisite for pre-seeding technologies and can be readily tested *in vitro* studies on how fibroblasts can proliferate on, for example, poly L-lactic acid (Kirkpatrick *et al.*, 1998). This method will be used in this study.

(D) Cell biosynthetic function

This element of biofunctionality testing cannot be emphasized enough. *In vitro* techniques offer an almost inexhaustible store of possibilities.

The relevance for biocompatibility is that the evaluation of proper function of a medical device must go far beyond the exclusion of cytotoxicity. It is thus possible that a material which does not alter the ability of a relevant cell to proliferate may reduce an important biosynthetic function (Kirkpatrick et al., 1998).

2.2.3 Future Perspectives in Biofunctionality Testing *in vitro*

There are many exciting fields of endeavour in current biomedical research, which as yet have only tangentially affected biomaterial research but which are highly relevant to this field and also promise to facilitate progress.

Examples are as follows:

(A) Fluid shear systems.

(B) New aspects of cell functionality.

(i) Cell adhesion molecules (CAM);

(ii) Production of cytokines and growth factors;

(C) Application of modern cell and molecular biological techniques.

(D) Encapsulated cell technology.

(E) Haemocompatibility testing.

New applications will make *in vitro* testing of biocompatibility more relevant, both to those developing and testing new biomaterials, and to those employing them in the clinical situation (Kirkpatrick et al., 1998). In the present study the biofunctionality (cell proliferation) testing method was chosen.

2.3 Biocompatibility Testing of Hydroxyapatite (HA) Against Human Bone and Soft Tissue Cell Lines

Jui- Sheng Sun et al in 1997 did a study to test the biological effects of various sized HA particles on myoblast and fibroblast. Both the myoblast and fibroblast were mixed in *in vitro* culture 0.1% (1 $mgml^{-1}$) of various sized HA particles (0.5 – 3.0, 37-63,177-250,420-841 μm) for 1 h, 3h, 1 days, 3 days and 7 days to test their effects on the cell count significantly. The transforming growth factor-β1 (TGF - β1) concentration in the culture medium decreased significantly on the addition of HA particles.

When calculated as a ratio to the cell number, the TGF-β1 titre increased most significantly in the groups of medium-sized particles. The prostaglanding E_2 (PGE_2) concentrations in the medium increased significantly. The changes in TGF- β1 and PGE_2 concentration with the smallest particle were most significant and persisted longer.

The inhibitory effects of the HA particles on the cell culture were mediated by the increased synthesis of PGE_2. Caution should be exercised before considering the use of an HA product which could easily break down into a fine powder (Jui- Sheng Sun *et al.*, 1997a)

Jui- sheng Sun also in same year did another study on the effects of calcium phosphate particles on the growth of osteoblast. The focus of this work is to elucidate the biocompatibility of the particulates of various calcium phosphate cytotoxicities.

Four different kinds of calcium phosphate powder, including β- tricalcium phosphate (β-TCP), hydroxyapatite (HA), β-dicalcium pyrophosphate (β-DCP), and sintered β-dicalcium pyrophosphate (SDCP), were tested by osteoblast cell culture. The results were analyzed by cell count, concentration of transforming growth factor-β1 (TGF-β1), alkaline phosphatase (ALP), and prostaglandin E_2 (PGE_2) in culture media.

The changes were most significant when osteoblast were cultured with β-TCP and HA bioceramics. The changes in the cell population of the β-TCP and HA were quite low in the first 3 days, then increased gradually towards the seventh day. The changes in TGF-β1 concentration in culture medium was inversely related to the changes in the cell population. The ALP titer in the culture media of the β-TCP and HA were quite high in the first 3 days then decreased rapidly between the third and seventh day. The concentrations of PGE_2 in the culture media tested were quite high on the first day, decreased rapidly to the third day, and then gradually until the seventh day.

There were changes in the β-TCP but these were much less significant. We conclude that HA and β-TCP have an inhibitory effects on the growth of osteoblast. The inhibiting effects of the HA and β-TCP powders on the osteoblast cell cultures possibly are mediated by the increased synthesis of PGE_2 (Sun *et al.*, 1997c).

The effects of implants on bony tissue have been investigated.

The effect upon adjacent skeletal muscle have not been determined. So Jui-Sheng Sun *et al.*, in 1997 did focus their work to elucidate the biological effects of various calcium phosphate biomaterial including β-tricalcium phosphate (β-TCP), hydroxyapatite (HA), β-dicalcium pyrophosphate (β-DCP) and sintered β-dicalcium pyrophosphate (SDCP), which were tested by myoblast cell cultures. The results were analyzed by cell count, cell morphology and concentration of transforming growth factor-β1 (TGF-β1) in the culture medium. The cell population and TGF-β1 concentration of the control sample increased persistently as the time of the culture increased.

The changes in the cell population and TGF-β1 concentration in the culture medium of the β-TCP and HA were quite low in the first 3 days of culture, then increased gradually towards the seventh day. The changes in the cell population and TGF-β1 concentration in culture medium of the silica, β-DCP, and SDCP were quite similar. They were lower during the first day of culture but increased and reached that of the control medium after 7 days' culture. Most cells on the β-TCP and HA diminished in size with radially spread, long pseudopods.

They conclude that HA and β-TCP are thought to have an inhibitory effect on the growth of the myoblasts. The HA and βTCP may interfere with the repair and regeneration of injured skeletal muscle after orthopaedic surgery (Sun *et al.*, 1997b).

J. Li *et al* had also studied on the evaluation of the biocompatibility of various ceramic powders with human fibroblast *in vitro*. Cell reaction to powders of ceramics was studied *in vitro*. Cultured human fibroblast were exposed to different types of ceramic powders: zirconia (ZP), alumina (A), tricalcium phosphate (TCP) and hydroxyapatite (HA), at various concentrations.

The cell viability at the different exposure times was measured by the colony formation (expressed as colony forming efficiency (CFE)), Neutral red uptake (NR) and colorimetric tetrazolium (MTT) reduction. Alumina and hydroxyapatite showed no cytotoxic effects at studied doses (1-500 μg/ml) while zirconia and tricalcium phophate inhibited cell viability, with 50% of CFE reduction at the concentration of about 50 μg/ml.

In order to study the cytotoxic mechanism of zirconia powder, two further experiments were included, viz. the cellular response to the sintered zirconia ceramic powders (CZP) which were obtained by crushing the sintered ceramic material and the measurement of the degradation of zirconia ceramic plate in the different solutions, i.e. either in saline or in 0.2 lactic acid (pH 2.72). Similar cell reactions were obtained for the CZP and ZP by using MTT and NR assay. Slow releases of ions from zirconia ceramic plate, yttrium in both solution and zirconium and yttrium in lactic acid, were detected (Li *et al.*, 1993).

Wilke *et al.*, in 1998 conducted a study on the biocompatibility analysis of different biomaterials in human bone marrow cell cultures.

The human bone marrow cells have been chosen because these cells are in direct contact with the biomaterial after implantation *in situ*. The sensitivity of this method is evaluated for materials which are already being used as implants in humans and animal, e.g., hydroxyapatite (HA) ceramic, pure titanium, and ultra-high-molecular-weight polyethylene (UHMWPE). As indicative parameters of biocompatibility primary cell adherence, cell proliferation, production of extra cellular matrix, cell vitality and cell differentiation are described. After 2 weeks in culture, obvious differences between the biomaterials with respect to the indicative parameter could be observed. Cell numbers were greatest on the HA specimens.

In the case of titanium alloys, they observed a decreased number of cells. The production of extra cellular matrix was high for the HA ceramics but reduced for titanium specimens. The polymers allowed only a few adherent cells and showed no signs of extra cellular matrix production. The results can be correlated astonishingly well to animal experiments and clinical experiences. Therefore, they concluded that this cell culture system seems to be a useful tool for biocompatibility testing of bone implantation materials.

It also helps reduce animal experiments. With the help of flow cytophotometry, they analyzed the influence of biomaterials on large number of cells with respect to differentiation.

There were similar populations of T cells and monocytes on all specimens tested. Extended B-cell and granulocyte populations, however, were observed with titanium and UHMPE. Most osteicalcin-containing cells adhered to the HA ceramics (Wilke *et al.*, 1998).

2.4 Cell lines

Although attempts at maintaining tissue explants *in vitro* have been reported since 1907, the beginning of the modern era of cell culture was marked by the establishment of the first cell lines, the mouse L-cell fibroblast line and the human HeLa cervical adenocarcinoma line. However, it was not until the 1960s that functional differentiated mammalian cell lines were first established (Sato *et al.*, 1994). Cell lines (cells of a known origin with an unlimited lifespan in culture) offer an important tool for research (Gilchrist *et al.*, 2000).

Cell and tissue models make it possible for us to study the biological responses of cells to a wide range of stimuli (biological, chemical, mechanical and so on) in vitro right down to molecular level. Such model has a number of advantages:

i) The cells' physicochemical environment (pH, temperature, osmotic pressure and tension of gases such as oxygen and carbon dioxide) can be controlled and monitored.

ii) Cell population are more homogeneous than those in tissues; culture conditions can be designed to ensure that cells required grow more vigorously than other cells in the population.

iii) Agents under test can be used at much lower concentrations if cells exposed to them directly in culture rather than by injection in vivo.

iv) The need to use animals in preliminary characterization studies is greatly reduced.

There are disadvantages in the used of culture methods:

i) Dissociation of cells from their normal three-dimensional structure can lead to the loss of specific cell interactions.

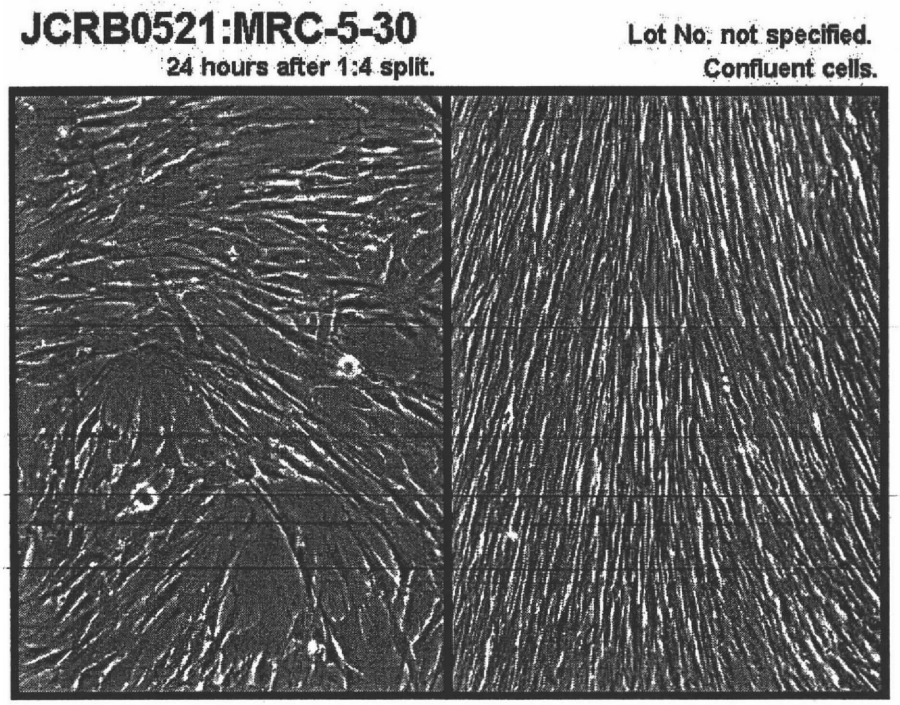
ii) The mechanisms involved in homeostatic regulation of the cells are lost, mainly the endocrine and nervous system (Silvio, 1999).

Cell lines can be divided into two general types: adherent (monolayer cells) or non-adherent (suspension cells). Adherent cells attach to the plastic substratum of a flask or plate and therefore need to be detached from this surface before they can be used (Morgan and Darling, 1993). Example of adherent cells were lung embryonic fibroblast cell (MRC-5).

2.4.1 MRC-5 cell line

MRC-5 are human embryonic lung normal diploid fibroblast cell. This cell establish by Jacob. The cell derived from foetal lung tissue, taken from a 14-week foetus with a genetically normal family history and no sign of neoplastic disease both at abortion and for at least three years afterward (Jacob *et al.*, 1970). The cells as shown in Figure 2.1.

Figure 2.1: MRC-5 cells, left side 24 hours after 1:4 split and on the right side showed the confluent cells.



CHAPTER 3

MATERIALS AND METHODS

3.1 Biomaterial

The pure hydroxyapatite (HA) and the value added HA base material which were produced by a group of scientists at the School of Engineering Universiti Sains Malaysia were obtained through the courtesy of Associate Professor Radzali of USM.

This value added HA is stronger and easier to contour without breaking (it is not too brittle as compared to pure HA). The test material is HA that has been added with zirconia to make it tougher. For the testing, the pure HA which is the standard material (SM) and the test material (TM) were in the powder form. The powder form was used because it is easier to measure the weight. The pure HA (Ca_{10} $(PO_4)_6$ OH_2) is commercially available in the market. The latex rubber of a urine catheter tube was used as the positive control material (CM). All the test material(TM) and pure HA (SM) were weighed in micro tubes. Their weight ranged from 1 mg to 5 mg each. The micro tubes containing TM and HA were sealed and placed in sealed autoclavable plastic bags before being autoclaved.

The positive Control material (CM) was placed in an autoclavable plastic bag and sealed before being autoclaved. All the materials were kept in the drawer in the lab before testing.

3.2 Cell Line

Fibroblast cells were used in this study. Fibroblast cells are primary cells from the human embryonic lung tissue (MRC-5). The Fibroblast cells were obtained by courtesy of Dr Fauziah of the Department of Microbiology, Universiti Sains Malaysia, Kubang Kerian, Kelantan. Fibroblast are sticking cells. Figure 3.1 shows a photograph of fibroblast cells used in the study.

Figure. 3.1 MRC-5 cell line (magnification 200X)

3.3 General Preparation and Sterilization Methods

3.3.1 Washing of glassware

Stringent washing of all glassware was followed to achieve cleanliness. All glassware were soaked overnight in 7X detergent (Flow Laboratories), which was followed by tap water. The glassware was then soaked in distilled water overnight and dried at 60°C in a drying oven.

3.3.2 Sterilization by dry heat

Pipettes were sterilized using dry heat. Pipettes were plugged with cotton wool. Commercial pipette canisters were used to enclose pipettes and exposed to180°C for 2 hours in a hot air oven.

3.3.3 Sterilization by autoclaving

Glassware was sterilized using the autoclave. Bottles with plastic caps were loosely screwed and beakers were covered with aluminium foil. Pipette tips were enclosed in plastic containers and wrapped with aluminium foil before autoclaving. The items were autoclaved for 20 minutes at 1.1 kg/cm^2 (15 lb) pressure.

.4 Tissue Culture Techniques

3.4.1 Precaution for tissue culture

All procedures pertaining to cell culture were carried out in a sterile environment using a Biohazard cabinet Class II (Flow Laboratories). Alcohol (70% v/v in water) was used to wipe the area in the hood and all apparatus.

Sterile glassware, plastic ware and micropipet tips were used during tissue culture work. All solutions were prewarmed in a 37°C water bath before they were used in the tissue culture medium.

3.4.2 Biohazard precautions

Gloves were worn during all experiments. Before the disposal of flasks and other containers used for cell culture, they were autoclaved at 121°C for 30 minutes at 15 psi.

3.4.3 Basic medium preparation

DMEM medium

One bottle (10 gram powder) of DMEM (Dulbecco's Modified Eagles's Medium), Sigma with L-Glutamine and 20mM HEPES was made up to one litre distilled water (Flow Lab, Australia). 3.7 grammes of sodium bicarbonate ($NaHCO_3$,

Analar) was added to the solution. The medium was filter sterilized using a 0.22 μm filter membrane (Millipore, UK) and stored at 4°C for up to 4 months.

3.4.4 10 % Supplemented medium

10 % Supplemented DMEM medium (Maintenance medium/MRC 5)

Hundred millilitres of 10% supplemented medium DMEM was prepared using 96 ml of basic medium, supplemented with 2 ml inactivated Foetal Bovine Serum (FBS), 1 ml (10.000 U/ml) peniciline and 10 mg/ml streptomycin respectively and 1 ml (1.000 U/ml) of nystatin. The media was filter sterilized using a 0.22 μm filter membrane and stored at 4°C for up to 2 weeks. The Fibroblast cells were maintained using this medium. All reagents used were obtained from the Sigma Lab, UK.

3.4.5 20% Supplemented medium

20% Supplemented DMEM medium (DMEM Growth medium)

Hundred milliliter of DMEM growth medium was prepared using 89 ml of DMEM basic medium, supplemented with 10 ml inactivated FBS and 1 ml peniciline streptomycin (10.000U/ml-10mg/ml). The media was filter sterilized using a 0.22 μm filter membrane and stored at 4°C for up to 2 weeks. The medium was used for the purpose of reviving fibroblast cells.

3.4.6 Cryopreservation of Fibroblast Cells

The cryopreserving solution consisted of 4 ml FBS, 5 ml Dimethyl sulfoxide (DMSO). The solution was filter sterilized using 0.45 μm filter membrane and stored at 4°C for not more than 1 month. Exponentially growing fibroblast cells in culture flask were washed with 10 ml PBS and then treated with 3 ml PBS and 1 ml of Trypsin (Amresco, USA) not longer than 10 minutes. The cells were spun down at 1000 rpm for 5 minutes using a bench centrifuge. The cells were resuspended in 2 ml fresh cryopreservation solution and alliquoted into sterile provials in 1 ml volumes. The provials were left to stand in ice and placed in a 1 inch thick polystyrene box. The box was kept in a –70°C freezer overnight. The provials were then transferred into liquid nitrogen (-196°C).

3.4.7 Revival of Cells

The provial of cells was removed from liqiud nitrogen and plunged into a beaker of ice. It was then, transferred to a 37 °C water bath for quick thawing.The cells were transferred into 5 ml of 20% supplemented growth medium DMEM in a polypropylene tube (Falcon, USA) and spun at 1000 rpm for 5 minutes.

The supernatant was discarded and the pellet resuspended in 5 ml of 20% supplemented medium DMEM and incubated in a 25 ml tissue culture flask (Falcon, USA) at 37°C in a 5% CO_2 incubator.

3.4.8 Maintenance of Culture

Fibroblast cell culture was maintained in maintenance medium/MRC 5 medium. The cells were in 25 ml tissue culture flasks. The tissue culture flasks were incubated in the 5% CO_2 incubator at 37°C in a humidified atmosphere. The cells are routinely checked under an inverted microscope (Olympus, Japan) for any bacterial or fungal contamination. Media depletion of nutrition was indicated by a change in the colour of the medium. The cultures were fed twice a week by a replacement of 80 % of the supernatant with fresh 10% supplemented FBS medium. The cultures (Fibroblast) were split or subcultured when the cell have been confluent in a monolayer.

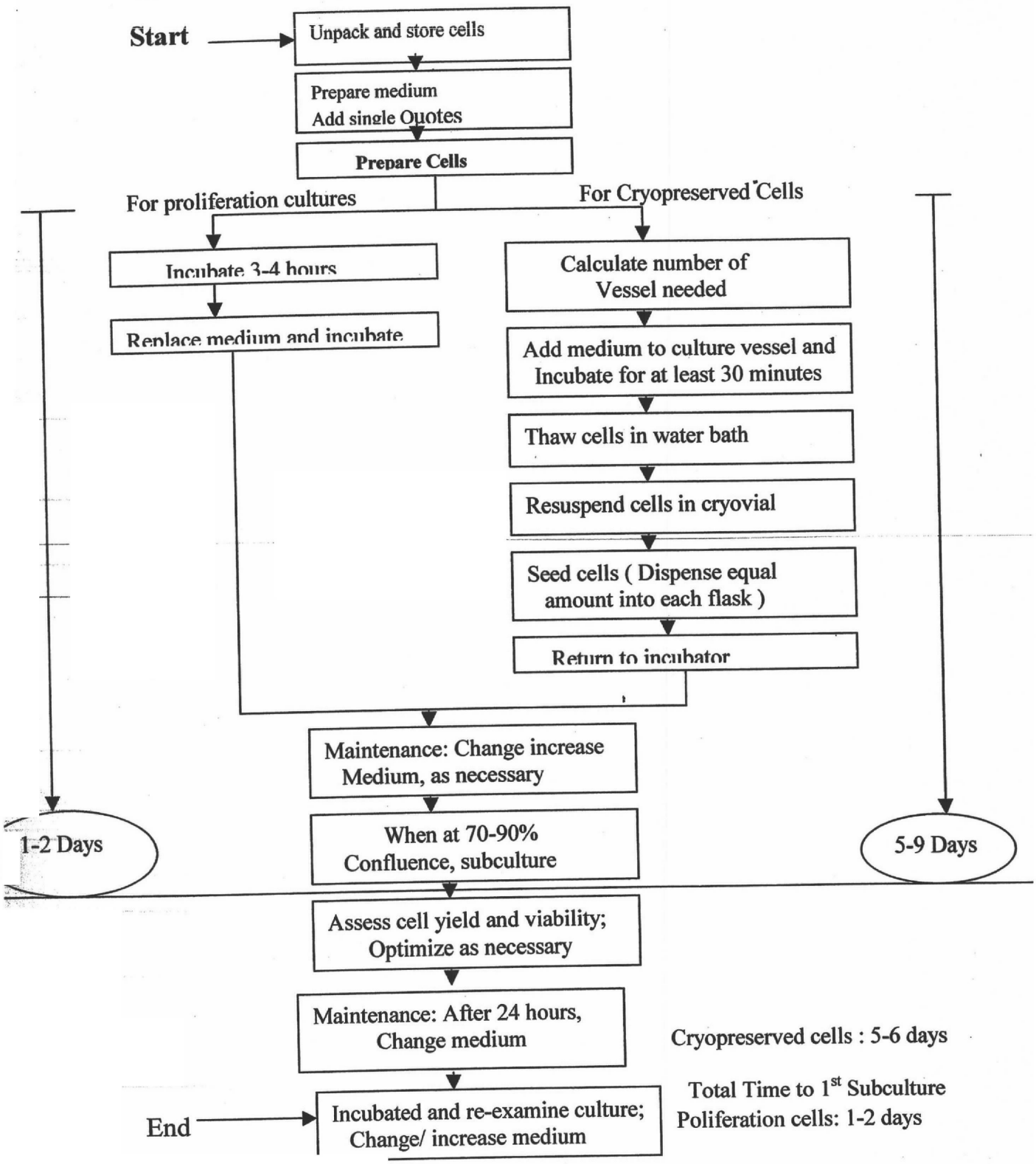
3.4.9 Subculturing of Fibroblast Culture

Fibroblast attached and formed a single layer in a culture flask. Before subculturing, the culture medium was removed and the cells were washed twice with PBS.

The cells were isolated from the flask by incubating such cells in 1 ml of 0.25% trypsin and 3 ml of PBS for 5 minutes at 37°C, and then sharply tapping the flask to detach the cells from the flask. The isolated and floating cells were transferred into a Falcon tube and centrifuged for 5 minutes at 1000 rpm.

The supernatant was removed and 2 ml of 10% supplemented medium was added to the pellet. The cells were split and transferred into different flasks containing 4 ml – 5ml culture media each. The flask were then further incubated at 17°C in a CO_2 incubator. An Overview of fibroblast cell culture process is shown in Figure 3.2.

Fig.3.2 Overview of Fibroblast Cell Culture Process



3.4.10 Cell Enumeration

A drop of the diluted cell suspension (1:10) was placed on haemocytometer (Neubauer, Germany). The dead cells were stained blue while the living cells were not stained. The haemocytometer was placed under a microscope and the unstained living cells were counted under the 20X objective.

The number of living cells in 1 ml of the culture media was calculated using the following formula :

$$\text{Cells per ml} = \frac{\text{No. of cells counted}}{\text{No. of squares}} \times 10^4 \times 10 \times 0.9$$

10^4 : Counting chamber conversion factor

10 : Dilution factor with Trypan Blue

0.9 : Volume of original cells suspension

3.5 Preparation of Buffers/Chemicals/ Enzyme

3.5.1 Preparation of Phosphate Buffered saline (PBS)

The PBS buffer was prepared using 1.52 gm of sodium phosphate anhydrous (Na_2HPO_4, BDH), 0.58 gm of potassium dihydrogen orthophosphate (KH_2PO_4, BDH) and 8.5 gm of sodium chloride (NaCl, Merck, Germany) that were dissolved in distilled water and the volume was made up to 1 litre. The PH of the buffer was adjusted to 7.2 using a PH meter (Hanna Instruments, Singapore). The buffer was then filtered using a 0.22 μm filter membrane and stored at room temperature.

3.5.2 Preparation of Trypsin

Trypsin solution was prepared by dissolving 0.25 g of trypsin (Amresco, USA) in 100 ml of PBS. The solution was sterilized by in filtration using a 0.22 μm filter membrane and stored at 4°C.

3.5.3 Preparation of Trypan Blue

A 0.4 % stock solution of Trypan Blue was prepared using 0.2 gram Trypan Blue powder with 50 ml distilled water and stored at room temperature. For use in the cytotoxicity assay, 10 ml of stock solution was further diluted with 90 ml distilled water.

3.5.4 Preparation of Neutral Red

One percent solution of neutral red was prepared using 1 gram neutral red which was dissolved in 50 ml ethanol and 50 ml distilled water.

It was then filter sterilized using 0.22 µm membrane filter (Millipore,UK), stored as stock solution at room temperature and kept away from sunlight. One mililitre of stock solution was further diluted with 49 ml sterile distilled water to give a final concentration of 0.02% and it was then filter sterilized using 0.22 µm membrane filter (Millipore,UK), and stored at room temperature.

3.5.5 Preparation of 1% Sodium Dodecyl Sulfate (SDS)

One gram of Sodium Dodecyl Sulfate (SDS) (Bio-Rad, USA) was dissolved in distilled water with gentle stirring and the volume was made up to 100 ml. This solution was stored at room temperature.

3.6 Testing for Cytotoxicity of Biomaterial

3.6.1 Cytotoxic assay

The fibroblast cells were maintained in 10 % supplemented culture medium for Fibroblast cells. The medium was changed 24 hours before the assay. Confluent fibroblast cells were washed twice with PBS and treated with 0.25% trypsin (Amresco, USA) for 5-10 minutes to detach the cells from the flask. The detached cells were then centrifuged at 1000 rpm for 5 minutes.

The density of the fibroblast were adjusted to 30,000 cells/ml.One mililitre of the cell suspension was transferred into a 24 well tissue culture flasks (Falcon).

In preliminary test the biomaterials were added at concentration of 1mg/ml, 1.5 mg/ml, 2 mg/ml, 2.5 mg/ml, 3mg /ml, 4 mg/ml and 5 mg/ml in the well of tissue culture flask, immediately after the cell dispersion. The fibroblast cells were incubated for 72 hours in a humidified incubator at 37°C with 5% CO_2 in air. Without change of media, tests were performed in duplicates. The test control consisted of

untreated cells. The material used were test material (TM)- HA value added based material and HA pure (SM). In this testing we wanted to see the cell count relation to the changing of the biomaterial concentration.Time were fix to 72 hours.

For other testing(the definitive test), we want to ascertain the relationship between the cell count to the changing of time. (the concentration or weight was fix).

Similarly the density of fibroblast cell were adjusted to 30,000 cells/well. One mililitre of the cells suspension was transferred into 24 well tissue culture flask (Falco).

We fix the concentration or weight to 2mg/ml or 2mg and then added it to the well immediately after the cells dispersion. All this procedure was done in lamina flow (sterile working place). The time to read the result was 1 hour, 3 hours, 24 hours, 3 days and 7 days. All the 24 well tissue culture flask were different for each hour and day.

The tissue culture flask with cells were then incubated for the time above in humidified incubator at 37°C with 5% CO_2 in air,without changing media and all tests performed in duplicate.The control consisted of untreated cell. Materials used for testing were TM, SM and CM (Positive control material).

3.6.2 Determination of Dye Uptake (Spectrophotometer)

At the end of the 72 hours incubation period, the sticking cells (Fibroblast) were stained with 1 ml 0.02% neutral red. The cells were further incubated for 2 hours to allow maximum uptake of dye by the surviving cells. The culture medium with the dye solution was aspired from the flask and the cells were rinsed with PBS. The dye taken up by cells were extracted by lysing the cells with 1% SDS for 30 minutes by sacking.

The cells were transferred into cuvettes and using a spectrophotometer (Hitachi 20000) with the optic density (OD) at 540 nm was read. Cytotoxicity was observed and confirmed when cell death was observed compared to the mean value of the controls.

The percentage of killed cells were determined by the formula below. The percentage of killed of the TM was then compared with the percentage killed of CM and SM in different concentrations/weight and time.

$$\text{Killed (\%)} = \frac{\text{OD of control (untreated)} - \text{OD of test}}{\text{OD of control}} \times 100\%$$

CHAPTER 4

RESULT

4.1 Cytotoxicity of Standard and Test Material Tested at Different Concentrations

4.1.1 Standard material (SM)

When the standard material was tested on the MRC-5 cell at different concentrations of 1 mg/ml, 1.5 mg/ml, 2 mg/ml, 2.5 mg/ml, 3 mg/ml, 4 mg/ml and 5 mg/ml, it was found that the standard material was not cytotoxic to the cells after 72 hours incubation period. The results are shown in Table 4.1 and Figure 4.1.

Table 4.1: Percentage Killed of MRC-5 Cells treated with Different Concentrations of SM (Standard Material). Incubation Time: 72 Hours.

Mean of Cell Optical Density Without SM = **0.025**

SM (Standard Material) Concentration (mg/ml)	Spectrophotometer Optical Density (OD)			% Killed
	1	2	Average	
1 mg/ml	0.038	0.049	0.0435	0 %
1.5 mg/ml	0.038	0.183	0.1105	0 %
2 mg/ml	0.099	0.139	0.119	0 %
2.5 mg/ml	0.147	0.032	0.0895	0 %
3 mg/ml	0.032	0.051	0.045	0 %
4 mg/ml	0.043	0.049	0.046	0 %
5 mg/ml	0.045	0.054	0.0495	0 %

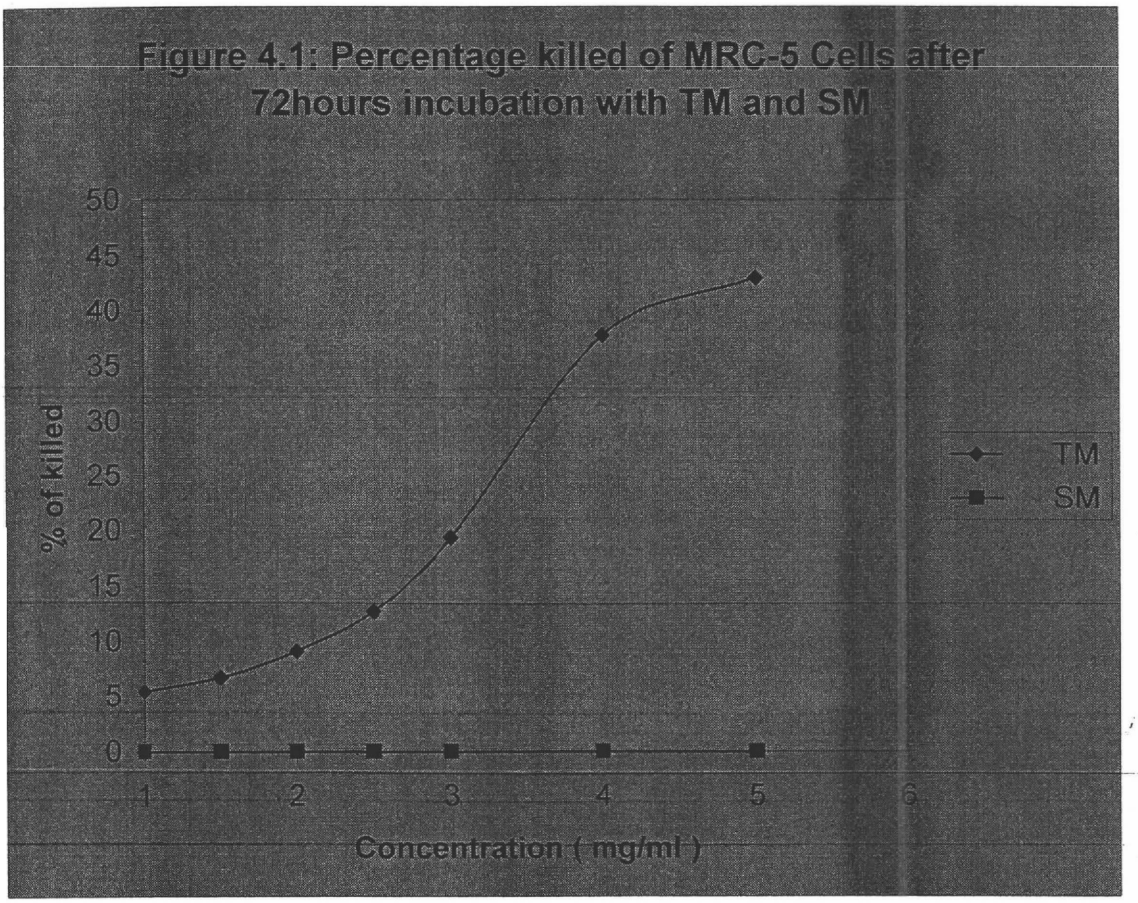
4.1.2 Test material (TM)

When the Test material was tested on the MRC-5 cell at different ɔncentrations of 1 mg/ml, 1.5 mg/ml, 2 mg/ml, 2.5 mg/ml, 3 mg/ml, 4 mg/ml and 5 ıg/ml, it was found that the test material was cytotoxic to the cells even at 1 mg/ml ɔncentration after 72 hours incubation period. At 1 mg/ml concentration, only a low ytotoxic effect of about 5.45 % of MRC-5 cells was observed. When the ™ oncentration was increased to 5 mg/ml the percentage of killed increased to 43.03 %. 'herefore the test material showed an exponential degree of cell death with increase ı concentration. The results are shown in Table 4.2 and Figure 4.1.

Table 4.2: Percentage Killed of MRC-5 Cells treated with Different Concentrations of TM (Test Material). Incubation Time: 72 Hours.

Mean of Cell Optical Density Without TM = **0.0825**

TM (Test Material) Concentration (mg/ml)	Spectrophotometer Optical Density (OD)			% Killed
	1	2	Average	
1 mg/ml	0.082	0.074	0.078	5.45 %
1.5 mg/ml	0.083	0.071	0.077	6.70 %
2 mg/ml	0.087	0.063	0.075	9.09 %
2.5 mg/ml	0.074	0.070	0.072	12.73 %
3 mg/ml	0.068	0.065	0.0665	19.39 %
4 mg/ml	0.049	0.054	0.0515	37.76 %
5 mg/ml	0.048	0.046	0.047	43.03 %



Statistical Analysis

Linear regression statistics were used in this evaluation.

Sig. (1-tailed)	KILLED	.	.000
	TM	.000	.
N	KILLED	7	7
	TM	7	7

Model Summary

Model	R	R Square	Adjusted R Square	Std. Error of the Estimate	Change Statistics				
					R Square Change	F Change	df1	df2	Sig. F Change
1	.972	.946	.935	3.9050	.946	86.925	1	5	.000

a Predictors: (Constant), TM

ANOVA

Model		Sum of Squares	df	Mean Square	F	Sig.
1	Regression	1325.510	1	1325.510	86.925	.000
	Residual	76.245	5	15.249		
	Total	1401.755	6			

a Predictors: (Constant), TM
b Dependent Variable: KILLED

Coefficients

Model		Unstandardized Coefficients		Standardized Coefficients	t	Sig.	95% Confidence Interval for B	
		B	Std. Error	Beta			Lower Bound	Upper Bound
1	(Constant)	-9.448	3.405		-2.774	.039	-18.202	-.694
	TM	10.541	1.131	.972	9.323	.000	7.635	13.448

a Dependent Variable: KILLED

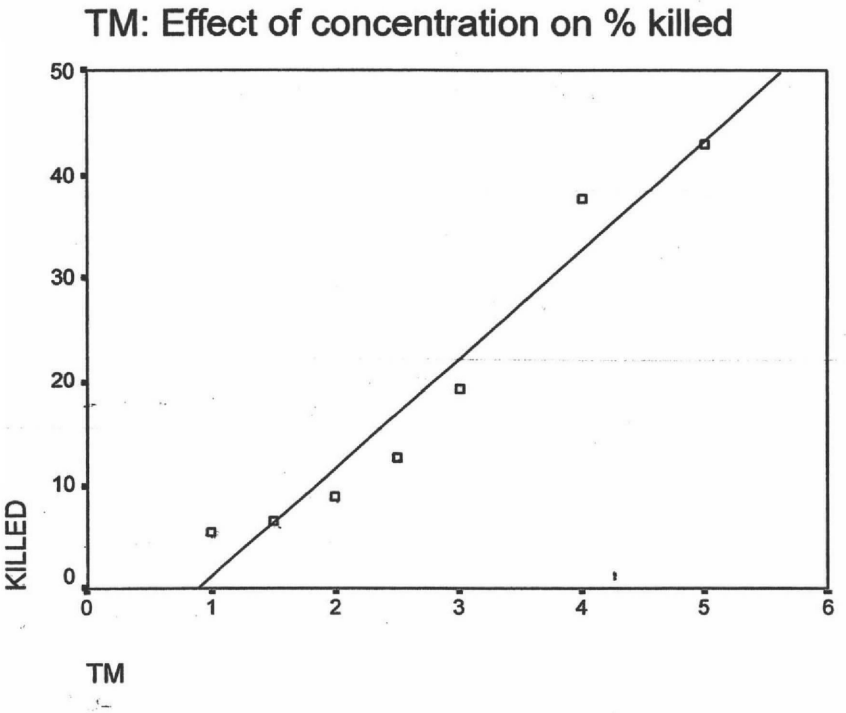
Interpretation from simple linear regression analysis:

Test material (TM)

For the TM at different concentrations the R was 0.972 which means that it is statistically high correlation (Excellent correlation if R= 0.76-1.0). The scatter graph (Fig. 4.3) shows positive correlation. The P value were 0.000, means concentration of TM has a significance effect on the percentage of killed.

Confidence interval was 7.635-13.448, this not include 0. This means it is significant. β was 10.541.

Figure 4.2: Effect of TM Concentration on % of Killed of MRC-5 Cells



Killed = % of killed
TM = Time of incubation(hours)

4.2 Comparison of cytotoxicity of CM, SM and TM against MRC-5 cells.

The CM, SM and TM were tested at 2 mg/ml on the MRC-5 cells in order to compare their cytotoxicity. The incubation periods was fixed at 1 hour, 3 hours, 24 hours, 3 days and 7 days.

1 hour incubation

At this incubation period no killed of cells was observed for both SM and TM but for CM, a low cytotoxicity of 0.99% was observed. See Table 4.3.

Table 4.3: Cytotoxicity of 2 mg/ml SM, CM and TM on MRC-5 cells after incubation period of 1 hour .

No.	Material	Spectrophotometer (Optical Density)		% of Killed
			Average	
1. 2.	Cell	0.114 0.130	0.122	0 %
3. 4.	Pure HA (SM)	0.132 0.127	0.1295	0 %
5. 6.	Positive Control (CM)	0.126 0.116	0.121	0.99 %
6. 7.	Test Material (TM)	0.121 0.123	0.122	0 %

3 hours incubation

At this incubation period no cytotoxicity for SM and TM but for CM the % of killed increase to 16 %. As showed in Table 4.4.

Table 4.4: Cytotoxicity of 2 mg/ml SM, CM and TM on MRC-5 cells after incubation period of 3 hours.

No.	Material	Spectrophotometer (Optical Density)		% of Killed
			Average	
1. 2.	Cell	0.028 0.022	0.025	0 %
3. 4.	Pure HA (SM)	0.035 0.042	0.0385	0 %
5. 6.	Positive Control (CM)	0.028 0.014	0.021	16 %
6. 7.	Test Material (TM)	0.043 0.027	0.035	0 %

24 hours incubation

After this incubation period no cytotoxicity effect by SM and ™ was observed but for the CM the percentage killed increased to 58.53 %. This is shown in Table 4.5.

Table 4.5: Cytotoxicity of 2 mg/ml SM, CM and TM on MRC-5 cells after incubation period of 24 hours.

No.	Material	Spectrophotometer (Optical Density)		% of Killed
			Average	
1. 2.	Cell	0.108 0.097	0.1025	0 %
3. 4.	Pure HA (SM)	0.128 0.101	0.1145	0 %
5. 6.	Positive Control (CM)	0.051 0.042	0.0465	58.53 %
6. 7.	Test Material (TM)	0.097 0.110	0.1035	0 %

3 days incubation

After 3 days incubation periods SM still did not show any cytotoxicity but TM started to show a 9.44 % killed cells. Percentage of killing for CM increased to 85 %. The result are shown in Table 4.6.

Table 4.6: Cytotoxicity of 2 mg/ml SM, CM and TM on MRC-5 cells after incubation period of 3 Days .

No.	Material	Spectrophotometer (Optical Density)		% of Killed
			Average	
1. 2.	Cell	0.094 0.086	0.09	0 %
3. 4.	Pure HA (SM)	0.121 0.111	0.116	0 %
5. 6.	Positive Control (CM)	0.009 0.017	0.013	85 %
6. 7.	Test Material (TM)	0.084 0.079	0.0815	9.44 %

7 days incubation period

For the 7 day incubation period all the CM, SM and TM showed cytotoxicity to MRC-5 cell line. SM started to show a percentage killed of 20.9 %. The percentage killing for TM and CM increased to 46.27 % and 93.66 % respectively. Cytotoxicity effect was highest with CM, lowest with SM and moderate with TM for this period of incubation time. The results are shown in Table 4.7.

Table 4.7: Cytotoxicity of 2 mg/ml SM, CM and TM on MRC-5 cells after incubation period of 7 Days.

No.	Material	Spectrophotometer (Optical Density)		% of Killed
			Average	
1. 2.	Cell	0.138 0.130	0.134	0 %
3. 4.	Pure HA (SM)	0.124 0.088	0.106	20.9 %
5. 6.	Positive Control (CM)	0.015 0.002	0.0085	93.66 %
6. 7.	Test Material (TM)	0.064 0.080	0.072	46.27 %

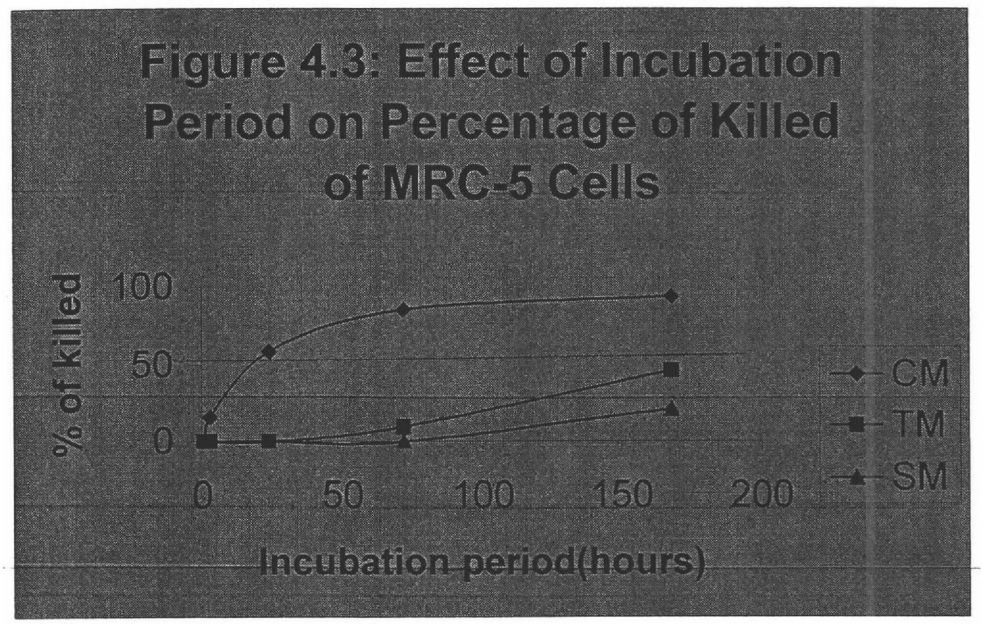
Summary of Result

Cytotoxicity of 2 mg/ml of CM on MRC-5 cells at 1 hour, 3 hours, 24 hours, 3 days and 7 days is shown in Table 4.8a and Figure 4.2.

Table 4.8a: Positive control (CM): Percentage of killing versus time

Time: Hour/ Day	% of Killed
1 Hour	0.99 %
3 Hours	16.00 %
24 Hours	58.53 %
3 Days	85.56 %
7 Days	93.66 %

The minimal cytotoxicity effect was at 1 hour (0.99 %) and maximum effect at 7 days (93.66 %). Full result as showed in Table 4.8a and Figure 4.2.



Statistical Analysis

Investigation into the effect of incubation time (hour) of CM on % of killed of MRC-5 cells.

Correlations

		KILLED	CM
Pearson Correlation	KILLED	1.000	.839
	CM	.839	1.000
Sig. (1-tailed)	KILLED	.	.038
	CM	.038	.
N	KILLED	5	5
	CM	5	5

Model Summary

Model	R	R Square	Adjusted R Square	Std. Error of the Estimate	Change Statistics				
					R Square Change	F Change	df1	df2	Sig. F Change
1	.839	.704	.606	25.8759	.704	7.151	1	3	.075

a Predictors: (Constant), CM

ANOVA

Model		Sum of Squares	df	Mean Square	F	Sig.
1	Regression	4788.277	1	4788.277	7.151	.075
	Residual	2008.680	3	669.560		
	Total	6796.957	4			

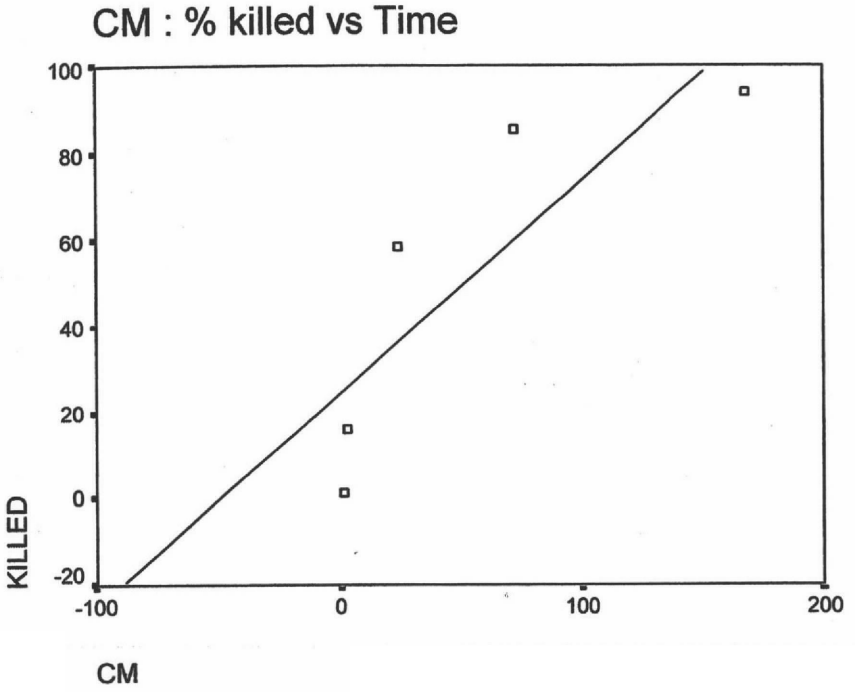
a Predictors: (Constant), CM
b Dependent Variable: KILLED

Coefficients

Model		Unstandardized Coefficients		Standardized Coefficients	t	Sig.	95% Confidence Interval for B	
		B	Std. Error	Beta			Lower Bound	Upper Bound
1	(Constant)	24.475	15.229		1.607	.206	-23.988	72.938
	CM	.494	.185	.839	2.674	.075	-.094	1.082

a Dependent Variable: KILLED

Figure 4.4 : CM; Effect of incubation time (Hours) of CM on % of killed of MRC-5 cells



Killed = % of killed
CM = Time of incubation (Hours)

Interpretation from simple linear regression analysis:

Positive control (CM)

At different times the R was 0.704, this means highly correlation. Scatter graph (Fig. 4.4), Showed positive correlation. The P value were 0.075, slight not significance. Confidence interval were –0.094-1.082, This include 0 means slightly not significance. β= 0.494.

Summary of Result

Cytotoxicity of 2 mg/ml of SM on MRC-5 cells at 1hour, 3hours, 24 hours, 3 days and 7 days.

Table 4.8b: Standard Material (SM): Percentage of killed versus time

Time: Hour/ Day	% of Killed
1 Hour	0 %
3 Hours	0 %
24 Hours	0 %
3 Days	0 %
7 Days	20.9 %

The cytotoxic effect was only observed after 7 days of incubation period. The % of killed was 20.9 %. No cytotoxic effect was observed for incubations periods of less than 7 days. Result showed in Table 4.8b and Figure 4.2.

Statistical Analysis

Investigation into the effect of icubation time (hour) of SM on % of killed of MRC-5 cells.

Correlations

		KILLED	SM
Pearson Correlation	KILLED	1.000	.913
	SM	.913	1.000
Sig. (1-tailed)	KILLED	.	.015
	SM	.015	.
N	KILLED	5	5
	SM	5	5

Model Summary

					Change Statistics				
Model	R	R Square	Adjusted R Square	Std. Error of the Estimate	R Square Change	F Change	df1	df2	Sig. F Change
1	.913	.833	.778	4.4051	.833	15.008	1	3	.030

a Predictors: (Constant), SM

ANOVA

Model		Sum of Squares	df	Mean Square	F	Sig.
1	Regression	291.234	1	291.234	15.008	.030
	Residual	58.214	3	19.405		
	Total	349.448	4			

a Predictors: (Constant), SM
b Dependent Variable: KILLED

Coefficients

		Unstandardized Coefficients		Standardized Coefficients	t	Sig.	95% Confidence Interval for B	
Model		B	Std. Error	Beta			Lower Bound	Upper Bound
1	(Constant)	-2.349	2.592		-.906	.432	-10.599	5.902
	SM	.122	.031	.913	3.874	.030	.022	.222

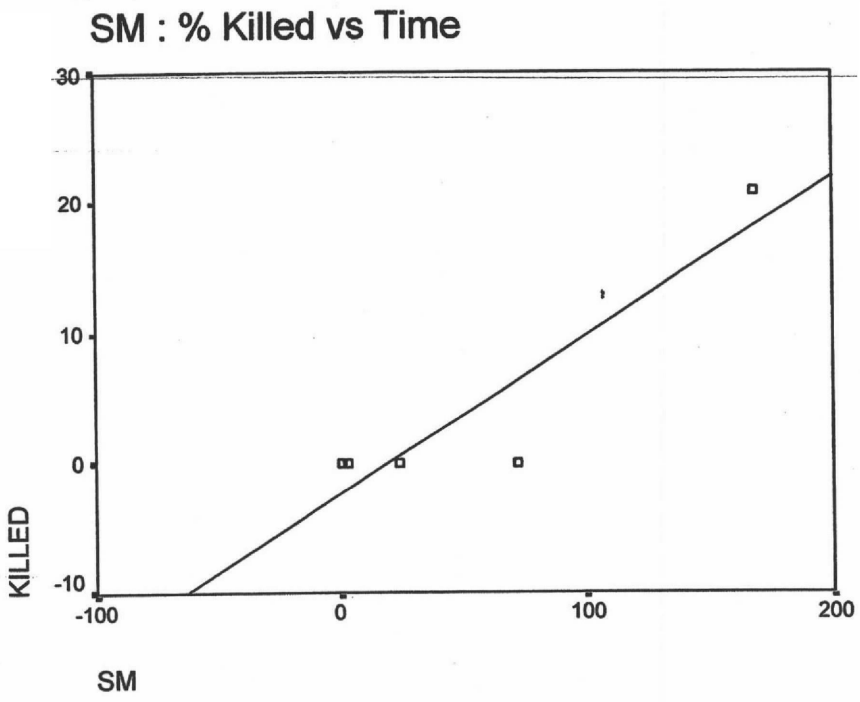
a Dependent Variable: KILLED

Interpretation from simple linear regression analysis:

Standard material (SM)

With different times the R was 0.913, this indicate highly correlation. The scatter graph (Fig. 4.6), showed positive correlation. The P value was 0.03; this means that the time (hour) has a slightly more significance effect on percentage of killed when treated with SM. Confidence interval was 0.022-0.222, this significance. $\beta = 0.122$.

Figure 4.6: SM ; Effect of incubation time (Hours) of SM on % of killed of MRC-5 cells



Killed = % Killed
SM = Incubation time (Hours)

Summary of Result

Cytotoxicity of 2 mg/ml of TM on MRC-5 cells at 1hour, 3hours, 24 hours, 3 days and 7 days.

TM started to have cytotoxicity effect at 3 days incubation period with percentage of killed 9.44 %. The cytotoxicity increased more at 7 days with % of killed 46.27 %. Result shown in Table 4.8c and Figure 4.2.

Table 4.8c:Test Material (TM): Percentage of killed versus time

Time: Hour/ Day	% of Killed
1 Hour	0 %
3 Hours	0 %
24 Hours	0 %
3 Days	9.44 %
7 Days	46.27 %

Statistical Analysis

Investigation into the effect of incubation time (hour) of TM on % of killed of MRC-5 cells.

Correlations

		KILLED	TM
Pearson Correlation	KILLED	1.000	.973
	TM	.973	1.000
Sig. (1-tailed)	KILLED	.	.003
	TM	.003	.
N	KILLED	5	5
	TM	5	5

Model Summary

Model	R	R Square	Adjusted R Square	Std. Error of the Estimate	Change Statistics				
					R Square Change	F Change	df1	df2	Sig. F Change
	.973	.946	.928	5.3753	.946	52.698	1	3	.005

a Predictors: (Constant), TM

ANOVA

Model		Sum of Squares	df	Mean Square	F	Sig.
1	Regression	1522.625	1	1522.625	52.698	.005
	Residual	86.681	3	28.894		
	Total	1609.306	4			

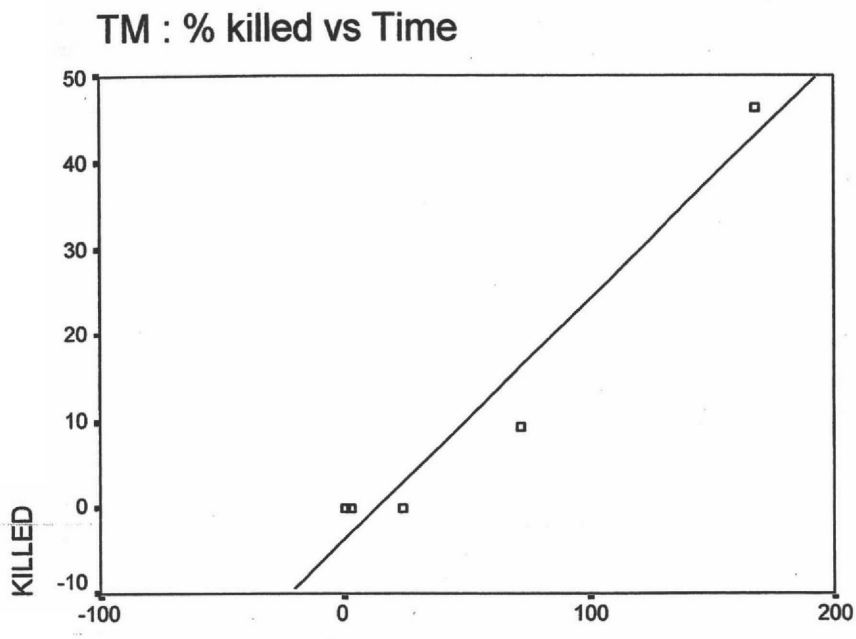
a Predictors: (Constant), TM
b Dependent Variable: KILLED

Coefficients

Model		Unstandardized Coefficients		Standardized Coefficients	t	Sig.	95% Confidence Interval for B	
		B	Std. Error	Beta			Lower Bound	Upper Bound
1	(Constant)	-3.786	3.163		-1.197	.317	-13.854	6.281
	TM	.279	.038	.973	7.259	.005	.156	.401

a Dependent Variable: KILLED

Figure 4.5: TM; Effect of incubation time (Hours) of TM on % of killed of MRC-5 cells



Killed = % Killed
TM = Incubation time (Hours)

Interpretation from simple linear regression analysis:

Test material (TM)

For TM with different times, the R was 0.9736 and this was highly correlation. The scatter graph (Fig. 4.5) showed positive correlation. The P value was 0.005, significance. Confidence interval were 0.156-0.401, its' significance.β was 0.279.

Research question

To investigate the effect of time (hours) when treated with CM , TM and SM on % of killed. Also to investigate the effect of TM concentration on % of killed.

Table 4.9 : Summary of statistical value.

Biomaterial	**R^2**	**P Value**	**Confidence interval**	**β**	**R**
CM (Time)	0.704	0.075	-0.094-1.084	0.494	0.839
TM (Time)	0.946	0.005	0.156-0.401	0.279	0.973
SM (Time)	0.833	0.030	0.022-0.222	0.122	0.913
TM (Concentration)	0.946	0.000	7.635-13.448	10.541	0.972

CHAPTER 5

DISCUSSION AND CONCLUSSION

5.1 Positive control (CM)

A positive control is a basic requirement in vitro biomaterial testing. In this study, the positive control used was latex rubber. As mentioned by Etorre positive control generally used was latex tubing (Etorre 1983). Positive control material is material which is tested in accordance with ISO 10993 which provides a reproducible cytotoxic response (ISO 10993 1992). A concentration / weight of 2mg latex rubber was used as the positive control in every stage of the experiment. The result showed that an increased in killing cells during exposure of the embryonic lung fibroblast cells to latex rubber showed an exponential pattern of cells killed.

The cell lines used in this study was embryonic lung fibroblast with less then 50 passage. The cells show a normal behavior of proliferation.

Application of toxic positive control killed the cell lines in expected pattern and thus on our observation confirm that the cell lines used in this study are reliable from the physiological point of view.

variations are unexplained. The β was 0.122 means that the rate of percentage of killed cells per hour (treated with SM) was 0.122%/hour.

5.3 Test material (TM)

The main interest in this study is to look for biocompatibility of our local product HA base material which is the test material in the in vitro experiment. Results showed that at the concentration of 2mg/ml of the TM, applied to the human embryonic lung fibroblast cells, about 10 % of cells were killed at 3 days and nearly 50 % cells were killed at 7 days . This showed that TM seem to be more toxic then the standard HA . Statistical analysis prove that the percentage of cells killed with time when exposed to the test material is significant, Showing P value < 0.05.

Interpretation from simple linear regression analysis shows that the P value and confidence interval were significance and the R and scattered graph shows high correlation. This shows that there is significant effect of time (hour) or concentration when treated with TM on the percentage of killing.

R square was 0.946 means 94.6 % of the variation that affect the percentage of killed may be explained by its' lineal relationship with time (hours). Other variations are unexplained. The β was 0.279 means that the rate of percentage of killed per hour (treated with TM) was 0.279 %/hour. Compare to SM is 0.122 %/hour. The results and statistical analysis showed that value added HA slightly more toxic then pure HA.

For different concentrations of TM , statistic shows that the R square were 0.946 means 94.6 % of killed also may be explained by its' lineal relationship with concentration (mg/ml). The β were 10.541 means that every TM concentration increase about 1 mg/ml, the percentage of killed cells will be 10.541%.

This statistical analysis further confirm that our locally produce material is less biocompatible then the standard HA in vitro study.

It is known that zirconia is an extra element that has been added to this test material (Modified HA). This was to increase the strength of the HA structure. We postulated that the zirconia element may have leached into the tissue culture media causing a cytotoxicity effect to the cell lines. In this respect Li *et al* (1993) has evaluated the biocompatibility of various ceramic powders including zirconia with human fibroblast in vitro study.

In that study, cultured human fibroblast was exposed to different types of ceramic powder: zirconia (ZP), alumina (A), Tricalcium phophate (TCP) and Hydroxyapatite (HA), at various concentrations.

The cell viability at the different exposure times was measured by the colony formation (expressed as colony forming efficiency (CFE), neutral red uptake and colorimetric tetrazolium (MTT) reduction).

Alumina and HA showed no cytotoxic effect at studied doses (1-500 μg/ml) while zirconia and tricalcium phosphate inhibited cell viability, with 50 % of CFE reduction at the concentration of about 50 μg/ml.

In order to study the cytotoxicity mechanism of the zirconia powder, two further experiments were carried out. The cellular response to the sintered zirconia

concentration of test material clearly shows an increase in the cells death. On the other hand, an increase of duration of exposure for both standard HA and test material did show an increase in the cytotoxicity for both materials with the test material being more toxic than the standard material.

In a study done by Li J. *et al.*, release of the ions from the powders was also very important since the zirconia and alumina tested had similar particle size (Li *et al.*, 1993) .

This observation clearly shows that the effects of corrosion or leaching of ions from materials to environment may be the main factor for these adverse biocompatibility phenomena. Also the effect of the particle size must be included.

5.4 Conclusion

The present *in vitro* evaluation indicated that the value added HA was more toxic han pure HA but the toxicity of value added HA was slight compared to the positive ontrol.

In this screening we can conclude that there is a toxicity effect by both value dded HA and pure HA but the value added HA had more toxicity effect. The toxicity effect was more on value added HA when the concentration was increased by 1 mg/ml ompared to the pure HA which had no effect. Statistics showed a strong correlation etween the effect of TM concentration on the percentage of killing. In this testing, the onclusion can be made that value added HA is less biocompatible than commercialized ure HA.

This toxicity may be caused by both the particle size and degradation (leaching). Further studies should be carried out to determine whether there is particle size effect or leaching effect when using powder as compared to the block materials.

The *in vivo* evaluation should be done to assess the reaction to this value added HA as compared to the pure HA. It is also recommended that the sample size be increased for a good statistical analysis.

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Nevertheless it may be possible that the powder form of the test material although showing cytototoxicity effect *in vitro*, may be more biocompatible *in vivo*, because of the present circulation of the body, buffer mechanism and neutralization process which is present *in vivo*.

Evaluation under the *in vitro* condition can provide rapid and inexpensive data on biological interaction. However, the questions must always be raised whether the *in vitro* test is truly measuring what will occur in the much more complex environment *in vivo*. *In vitro* test minimizes the use of animals in research, which is a desirable goal. However, this must be tempered with an appreciation that the results of in vitro tests may not be relevant to the implant situation (Ratner , 1996).

One must not forget this that in study *in vitro* embryonic lung fibroblast which have been subcultured many time (but less then 50 times) thus the cell physiology of fibroblast may have changed. This changes may lead to the cell being more sensitive, less resistant and more vulnerable to the effect of foreign element.

This above phenomena is a well characteristic observation as mentioned by Caroline Mac Donald that normal cells have a limited lifespan in culture, approximately 50 generations for a normal human fibroblast. Most cells are capable of between 10 and 75 generations before reaching senescence (Mac Donald, 1994).

Silvio also mentioned in his notes that senescence may arise at any time but for human diploid fibroblasts, it is most likely between 30 and 60 cell doublings or 10 to 20 weeks, depending on the doubling time (Silvio, 1999).

A final observation seen in the result of this experiment showed that while the increase concentration of standard HA did not increase its cytotoxicity, the increase in

concentration of test material clearly shows an increase in the cells death. On the other hand, an increase of duration of exposure for both standard HA and test material did show an increase in the cytotoxicity for both materials with the test material being more toxic than the standard material.

In a study done by Li J. *et al.*, release of the ions from the powders was also very important since the zirconia and alumina tested had similar particle size (Li *et al.*, 1993) .

This observation clearly shows that the effects of corrosion or leaching of ions from materials to environment may be the main factor for these adverse biocompatibility phenomena. Also the effect of the particle size must be included.

5.4 Conclusion

The present *in vitro* evaluation indicated that the value added HA was more toxic than pure HA but the toxicity of value added HA was slight compared to the positive control.

In this screening we can conclude that there is a toxicity effect by both value added HA and pure HA but the value added HA had more toxicity effect. The toxicity effect was more on value added HA when the concentration was increased by 1 mg/ml compared to the pure HA which had no effect. Statistics showed a strong correlation between the effect of TM concentration on the percentage of killing. In this testing, the conclusion can be made that value added HA is less biocompatible than commercialized pure HA.

This toxicity may be caused by both the particle size and degradation (leaching). Further studies should be carried out to determine whether there is particle size effect or leaching effect when using powder as compared to the block materials.

The *in vivo* evaluation should be done to assess the reaction to this value added HA as compared to the pure HA. It is also recommended that the sample size be increased for a good statistical analysis.

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