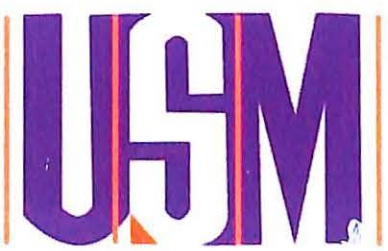
THE IN-VITRO EFFECTS OF 'GAMAT' EXTRACT OF STICHOPUS SPECIES ON HUMAN OSTEOBLAST CELL LINE

By

DR SHAHRULAZUA BIN AHMAD

MBBS (Lon) MRCS (Edin)

Dissertation Submitted In Partial Fulfilment Of The Requirements For The Degree Of Master Of Medicine (ORTHOPAEDICS)



UNIVERSITI SAINS MALAYSIA

NOVEMBER 2006

ACKNOWLEDGEMENTS

Praise and humblest gratefulness go to Allah, God the Almighty, for giving me the strength and determination to complete this work. It takes both rain and sunshine to make a rainbow. This dissertation project was the end-product of hard work and expertise of many, either directly or indirectly. My gratitude to all of them is beyond words.

I am forever indebted to my supervisors, Professor (Dr.) Ab. Rani Samsudin (Dean of Pusat Pendidikan Sains Pergigian) and Dr. Mohd Iskandar Mohd Amin (Head of Orthopaedic Department), for their relentless trust, support, encouragement, and invaluable guidance given during this dissertation project. My special appreciation goes to Prof. Rani, who has willingly become my supervisor and mentor, despite his name not being included in the research grant and regardless of his busy time schedule. His patience in teaching me the art of cell culture and his unceasing optimism were the key factors that moved this project forward. Without his support, this dissertation project would not have been made possible. I also would like to thank Dr. Amran Ahmed Shokri who is the co-supervisor in this study for his ideas and support in improving this project. It was his vision that commenced this project.

My sincere gratitude goes to Professor Zulmi Wan specifically for being so understanding and for his continual guidance and moral support in all my work including this dissertation project. Furthermore, I would like to extend my thanks to my other teachers, Dr. Halim, Dr. Wan Faisham, Dr Imran, Dr. Razak, Dr. Vishvanathan, Dr.

Norazman, Dr. Nawfar, Dr. Mohd Paiman, Dr. Bal Kishan, Dr. Sallehuddin, Dr. Tengku Muzaffar, and also my fellow orthopaedic colleagues who have given me the confidence and encouragement in all my undertakings.

I also would like to acknowledge the invaluable technical guidance and advice rendered to me by the staffs of the Craniofacial Lab, PPSG, in particular Cik Siti Fadilah Abdullah and Puan Nor Shamsuria Omar. They have been instrumental in helping to bring this project to fruition.

Additionally, I would like to express my special gratitude to Dr. Mohd Ayub Saddiq for his untiring contribution in the statistical analysis of this project. My knowledge in biostatistics has tremendously improved under his special coaching.

Numerous staffs from the Chemical Pathology Department and the Microbiology Department have also contributed to this project. Their input too is deeply appreciated.

A special note of thanks goes to my dearest beloved wife and best friend, Marina Abdul Rashid, for her unwavering faith in me. She has changed my life and made it more meaningful. Last but not least, I would like to dedicate this dissertation to my fondly beloved father and mother, the late Ahmad Othman and Atkah Yaacob, for raising me to the person I am now and who have inspired me in every aspect of my life.

Finally, my appreciation as well goes to the USM Short Term Research Grant (Number 304/PPSP/6131399) for the funding of this dissertation project.

TABLE OF CONTENTS

LIST OF ABBREVIATIONS

ALP	—	alkaline phosphatase
CO_2	—	carbon dioxide
^0C	—	degree Celsius
Cl^-	—	Chloride
DMEM	—	Dulbecco's Modified Eagle Medium
FBS	—	Fetal Bovine Serum
ISO	—	International Standardization Organization
kGγ	—	kiloGray
K^+	—	Potassium
µl	—	microlitre
mg	—	milligram
MTT	—	(3-[4,5-dimethylthiazol-2-yl])-2,5-diphenyl tetrazolium bromide
Na^+	—	Sodium
95% CI	—	95% Confidence Interval
O.D.	—	optical density
PBS	—	Phosphate Buffered Solution
WHO	—	World Health Organization

GLOSSARY

Cell line	—	Cells population derived from a primary culture after the first passage
Confluent	—	All the available growth area is utilized and all the cells make close contact with one another
Cytotoxicity	—	Cell killing property of a substance
Cytotoxic dose (IC_{50})	—	Concentration of the tested substance which reduces the number of cells by 50% as compared to the untreated cell population
Gamat	—	Sea cucumber of Stichopus sp1
Gray ($G\gamma$)	—	Standard International unit of absorbed dose
Negative control	—	Standard culture media
Passage	—	Also called subculture, i.e. to transfer or transplant cells of an ongoing culture to a new culture vessel in order to propagate the cell population or set up replicate cultures for study
Positive control	—	50% ethanol solution
Primary culture	—	The culture that is derived from intact or dissociated tissues or organ fragments. A culture is regarded as a primary culture until it is passaged, after which it is termed a cell line
Standard culture media	—	Mixture of DMEM, 10% FBS, and 1% penicillin-streptomycin

ABSTRAK

Timun laut yang dikenali tempatan sebagai 'gamat', banyak digunakan dan telah dibuktikan mempunyai pelbagai faedah perubatan. Walau bagaimanapun, kesan gamat terhadap sel osteoblast manusia masih belum pernah dikaji. Oleh yang demikian, satu penyelidikan 'in-vitro' ke atas ekstrak produk gamat yang dihasilkan secara komersial daripada Stichopus spl telah dijalankan dengan meggunakan ujian MTT dan ALP untuk menyelidik kesan yang dikenakannya ke atas pertumbuhan dan fungsi aktiviti sel osteoblast manusia.

Penyelidikan saringan dijalankan dengan mencampurkan sel osteoblast kepada larutan kawalan negatif, larutan kawalan positif, dan juga kepada kepekatan berganda larutan gamat yang disediakan di dalam media kultur standard daripada 1.6mg/ml kepada 100mg/ml. Keputusan yang diperolehi setelah tempoh pengeraman selama 72 jam menunjukkan bahawa terdapat perhubungan 'songsang' di antara kepekatan larutan gamat dan kesannya ke atas pertumbuhan sel osteoblast. Nilai IC_{50} dianggarkan pada 75mg/ml. Terdapat kesan promosi positif ke atas fungsi aktiviti sel osteoblast apabila larutan gamat berkepekatan 1.6mg/ml, 3.1mg/ml, 6.2mg/ml, 12.5mg/ml, and 25mg/ml digunakan. Larutan ethanol 50% adalah lebih cytotoksik daripada gamat 100mg/ml meskipun pertumbuhan dan fungsi aktiviti sel osteoblast yang ditunjukkan oleh gamat 100mg/ml adalah yang terendah berbanding dengan kepekatan larutan gamat yang lain.

Ujian susulan kemudian dijalankan setelah memilih empat jenis kepekatan, iaitu, 1mg/ml, 5 mg/ml, 10mg/ml, dan 20mg/ml, bagi mengkaji kesan pelbagai tempoh pengeraman ke atas sel osteoblast. Untuk tujuan ini, sel osteoblast terlebih dulu didudukkan di atas piring mikro selama 24 jam sebelum dicampurkan dengan pelbagai kepekatan larutan gamat dan larutan kawalan negatif. Ujian MTT dan ALP dilakukan setelah satu jam, satu hari, 3 hari, 5 hari, dan 7 hari tempoh pengeraman. Keputusan yang diperolehi menunjukkan pertumbuhan dan fungsi aktiviti sel osteoblast bertambah dengan bertambahnya tempoh pengeraman. Walau bagaimanapun, kesan yang dipamerkan oleh setiap kepekatan larutan gamat berbeza pada tempoh pengeraman yang berlainan. Terdapat perencatan pada pertumbuhan sel osteoblast apabila kepekatan larutan gamat bertambah di kebanyakan tempoh pengeraman. Larutan gamat berkepekatan 1mg/ml sahaja yang menunjukkan kesan positif pada pertumbuhan sel tetapi ini hanya dilihat berlaku pada 3 hari pengeraman. Kesan ke atas fungsi aktiviti sel osteoblast diperhatikan bertambah apabila gamat berkepekatan 5mg/ml dan 10mg/ml dibandingkan dengan larutan kawalan negatif. Meskipun begitu, pemerhatian ini tidak konsisten pada tempoh pengeraman berlainan dan kepentingannya tidak dapat dipastikan.

Sebagai kesimpulannya, walaupun kesan ekstrak gamat ke atas fungsi aktiviti sel osteoblast adalah tidak konsisten, kajian menunjukkan bukti kukuh yang ekstrak gamat merencatkan pertumbuhan sel osteoblast manusia.

ABSTRACT

Title

The in-vitro effects of 'Gamat' extract of Stichopus species on human osteoblast cell line

Abstract

"Gamat", a local term for sea cucumber, is widely used and had been shown in various studies to have many therapeutic effects. However, its action on osteoblast cells had never been investigated before. Hence, in-vitro study utilising a commercially produced gamat extract of Stichopus sp1 as the test substance was performed to elucidate the effects on the osteoblast cell line proliferation and functional activity, using MTT colorimetric assay and alkaline phosphatase (ALP) assay respectively.

In the preliminary study, osteoblast cells were mixed with a series of two-fold dilutions of gamat concentration in standard culture media from 100mg/ml down to 1.6mg/ml, and with negative as well as positive controls. Results that were noted after 72 hours incubation period showed an inverse relationship between the gamat concentration and its effect on the osteoblast cell proliferation. The exact IC_{50} was estimated to be approximately 75mg/ml. There was a positive promoting effect of gamat extract on osteoblast cell functional activity when 1.6mg/ml, 3.1mg/ml, 6.2mg/ml, 12.5mg/ml, and 25mg/ml of gamat concentrations were used. Although gamat at 100mg/ml showed the

lowest osteoblastic proliferation and functional activity when compared to other concentrations, it was not as cytotoxic as 50% ethanol solution.

A follow-up study was subsequently performed by choosing four gamat concentrations namely, 1mg/ml, 5 mg/ml, 10mg/ml, and 20mg/ml, to investigate the effect of different incubation periods on the osteoblast cells. For this purpose, the osteoblast cells were first seeded for 24 hours before mixed with the various gamat concentrations and negative control. MTT and ALP assays were carried out after one hour, one day, 3 days, 5 days, and 7 days incubation periods. The results showed that both the osteoblast cell proliferation and functional activity increased as the incubation time increased. However, the effect of each gamat concentration varied with different incubation periods. Most of the time, there was a decrease in osteoblast cell proliferation with an increase gamat concentration. Only gamat concentration at 1mg/ml exerted a significant promoting action on the cell proliferation but this was only observed at the 3 days incubation period. Overall, there was a trend showing a relatively greater promoting effect on osteoblast cell functional activity by gamat at 5mg/ml and 10mg/ml than the negative control but the significance of this finding was questionable and not consistent at the different incubation periods.

In conclusion, the study indicated that whilst the effect of gamat extract on the osteoblast cell functional activity was rather inconsistent, there was strong evidence that gamat decreased the cell proliferation in a concentration-dependent manner.

CHAPTER ONE

INTRODUCTION AND LITERATURE REVIEW

1.1 General Introduction

Many natural products are currently used as complimentary medicine. One example is "gamat" which is widely used in Malaysia, either orally or topically. Gamat is a local term for sea-cucumber extract and is used to treat variety of illnesses such as asthma, chest pain, hypertension, sinus, cold extremities, cuts, burns, worm infestations, sexual impotence, low back pain, rheumatism, bone pain, and joints pain by the local people, especially the Malays (Taiyeb-Ali et al., 2003). Its usage is now growing in popularity as a result of commercialisation. Various formulations of gamat, for examples, oral liquid, ointment, gel, and powder, are now marketed by many private companies (Figure 1.1).

Figure 1.1: Examples of gamat products in the market

1.2 Literature Review

1.2.1 Complementary medicine

The term "complementary" or "alternative" medicine is often used interchangeably with "traditional medicine". It is essentially a product of industrialisation adaptation of what is previously called traditional medicine (WHO, 2003). Traditional medicine refers to health practices, approaches, knowledge, and beliefs, which incorporate plant, animal, and mineral based medicines, as well as spiritual therapies, manual techniques and exercises, applied to treat, diagnose and prevent illnesses or maintain well-being (WHO, 2003).

There has been growing interest and expenditure in complementary medicine, both in developing and developed countries, particularly in the past 20 years (WHO, 2001).

More than 50% of the population in Europe, North America and other industrialized regions has used complementary medicine at least once (WHO, 2003). In United States, $17 billion was spent on traditional remedies in year 2000, whereas in United Kingdom, annual expenditure on alternative medicine is US$ 230 million (WHO, 2003). In Malaysia, the sales of complementary medicines were relatively high, estimated to be about one billion ringgit annually, compared to the sale value of 900 million ringgit of allopathic medicine (WHO, 2001).

Clearly, the use of complementary medicines needs to be regulated. Strong evidences based on the safety, efficacy and quality of the products and practices should be established as inappropriate use can have hazardous effects to patients' health. Furthermore, with its growing popularity and great commercial benefit, there is a threat to biodiversity through over harvesting of the raw material, which may lead to the extinction of endangered species and the destruction of natural habitats and resources. In particular, serious concerns have been raised on the dwindling populations of Malaysian sea cucumbers as a result of over fishing for medicinal purpose (Baine, 1999; Conand and Battaglene, 1999). At present, the requirements for protection provided under international standards for patent law and by most national conventional patent laws are inadequate to protect patients' safety and biodiversity (WHO, 2003).

1.2.2 Sea cucumber

Sea cucumbers, also called Holothurians, are marine invertebrates. There are roughly about 1200 Holothuroidea species (Ponomarenko, 2001). As their name suggests, they are cucumber shaped with an elongated, muscular, flexible body with a mouth at one end and the anus at the other. Around the mouth there is a number of tentacles (modified tube feet) used in food collecting as they move slowly over ocean floor. Sea cucumbers come in many sizes, from small species only a few centimetres in length to long snakelike animals which may stretch up to 2 meters. They have a unique defence mechanism against predatory which allow them to detach parts of their body or eviscerate their internal organs, all of which can be regenerated later. This observation consequently has led to the public's belief of its healing strength.

Stichopus is one of the examples of a holothurian of the order Aspidochirotes, family Stichopodidae, which is widely distributed across the tropical Indo-Pacific region (Figure 1.2). It is mainly found in shallow water, on reef flats and slopes with considerable hydrodynamic energy. The species' density is relatively low, but sometimes reaches up to several specimens per square metre (Uthicke, 1994; Conand, 1993; Conand et al., 1998). Similarly to nine other Aspidochirotes sea cucumbers, they can reproduce both sexually and asexually (Uthicke 1994, 1997; Conand et al., 1998; Tan Shau-Hwai and Yasin, 2000). Asexual reproduction is achieved by transverse fission resulting in two animals that each regenerates the missing part (Uthicke, 1997; Conand et al., 1998).

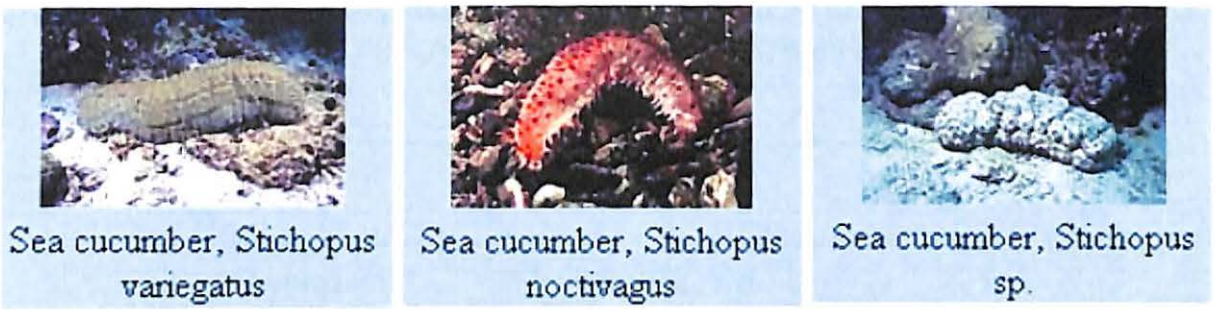


Figure 1.2: Examples of a few Stichopus species
(photos taken from *http://www.reefimages.com/Seastars/Seastars4.htm*)

Ridzwan (1978) found that among the 27 species of sea cucumber in Malaysian coastal areas, there are only 3 species belonging to the genus Stichopus. However, much progress has been made on the taxonomy, and ecology of sea cucumbers since. Recent surveys now showed that there are 37 species of sea cucumber in Malaysian coastal areas, with more species from the genus Stichopus have been found and yet to be determined (Conand and Battaglene, 1999). For the purpose of my study, water based extract of Stichopus sp1 was used and its phylogenesis is summarised below (Table 1.1):

Table 1.1: Phylogenesis of the Stichopus species used in the study
(http://www2.bishopmuseum.org/HBS/invert/holothuroidea.htm)

Phylum	**Echinodermata**
Subphylum	Eleutherozoa
Class	Holothuroidea
Order	Aspidochirotida
Family	Stichopodidae
Genus	Stichopus
species	sp1

1.2.3 Sea cucumber and its medicinal properties

Data on the properties and the effects of sea cucumber medicine is scanty. It is only lately that attempts have been made to study the sea cucumber scientifically but much of the local works done have remained unpublished. The main focal points are usually targeted on the healing properties of the sea cucumber on soft tissues.

For instance, Hassan et al. (1994a) showed in their study that an orally administered water extract of Stichopus sp1 displayed faster healing rate on guinea-pig cutaneous wound when compared to oral saline and topical disinfectant povidone-iodine as controls. Subsequently, Noor Ibrahim and Lim (2000) established that methanol extracts of

Holothuria atra and Stichopus variegatus also enhanced healing of induced incisional cutaneous wound on guinea pigs, as well as improving the quality of the resultant scar. Using oxoferin, and povidone-iodine as controls, their histological studies showed that the wound treated with extract of Holothuria atra had a higher concentration of connective tissues on the sixth day, and the electron microscopic scan demonstrated that the wound treated with the extracts exhibited a smooth and uniform healed skin closely resembling normal skin.

The healing effect of gamat on gastric ulcer disease has also been shown. Yanti et al. (2000) induced gastric ulcers in rats by giving reserpine 10mg/kg intraperitoneally. The ulcer healing when treated with crude extract of Stichopus variegatus administered orally was compared with treatment using distilled water as control. Based on the gross and histological observations of the rat gastric tissues, they found that Stichopus variegatus extract managed to accelerate the healing of gastric ulcer. Nihayah et al. (2000) additionally confirmed that the methanol extract of Stichopus variegatus accelerated the healing of gastric ulcers in rats which were induced by oral administration of 100% acetic acid. They further suggested that the observations could be due to increased regeneration of mucosal epithelial cells and increased gastric mucous production.

Further study on soft tissue healing was done by Vasanthan (2002) by investigating the effect of Gamat on the rate and quality of healing in perforated tympanic membranes of guinea pigs. Surgical perforations were created in both eardrums of the guinea pigs, and gamat was placed in one ear whilst the other ear served as a control. The eardrums were harvested 3 weeks later for evaluation. The results showed that the tympanic membrane

treated with gamat demonstrated an increase in granulation tissue and fibrous layers when examined histologically, suggesting that gamat did have an effect on healing process. However, it was unclear whether this observation had any clinical significance since both groups of eardrums, treated or untreated, had already healed at the 3 weeks period.

In a prospective, randomized, double-blinded clinical study, Taiyeb-Ali et al. (2003) also demonstrated that the application of 'Gamadent' toothpaste that contained all the basic constituents of a toothpaste with the addition of Stichopus spl extract when compared to a placebo, was statistically significant in improving parameters such as Plague Index, Gingival Index, Papilla Bleeding Index, and Probing Pocket Depth, during the healing phase in patients with chronic gingivitis, and early periodontitis.

There have also been studies to look into the various components of sea cucumber extract and the possible bioactive components by which the extract works. However, the precise actions of sea cucumber extract on tissue healing still remain unclear. In their study, Hassan et al. (1994a) analysed the composition of heavy metals in the crude water extract of Stichopus spl using Atomic Absorption method. They observed the presence of iron, manganese, copper, and zinc, which might be contributing to the healing effect of the Stichopus spl as these constituents are important in physiological enzymatic reaction. Furthermore, they found no plumbum, nickel and cobalt in the extract, and that all the heavy elements detected were well below the safety levels, suggesting that chronic consumption of the extract was harmless and might not lead to heavy metal toxicity.

Fredalina et al. (1999) in contrast, studied on the fatty acid profile of crude extracts of Stichopus chloronotus using gas chromatography technique. They found the presence of various fatty acids including myristic, palmitic, stearic, linoleic, arachidic, eicosapentaenoic, docosahexaenoic, and oleic, which were potentially playing the active role in tissue repair.

Azizah et al. (2001) conducted a study on the nutritional and antioxidative properties of raw sea cucumber (Holothuria sp.), and presented their results in the 16th Scientific Conference of Nutrition Society of Malaysia. They showed that the raw sea cucumber could be a good supply of quality protein, having contained 1.75% protein (23.6% in dry weight basis), and most essential amino acids except for methionine, tryptophan, and lysine. The raw sea cucumber only contained 2% fat (25.98% in dry weight basis), and much of it consisted of polyunsaturated fatty acid (67.46% of the total lipid), as well as eicosapentaenoic (EPA), docosahexaenoic acid (DHA), and linolenic acid at 38.04%, 21.46% and 3.84% respectively. Furthermore, they found that the petroleum ether extract of raw sea cucumber also exhibited appreciable antioxidative activity.

The presence of antioxidant substances such as superoxide dismutase, in the coelomic fluid of 3 species of holothuroid namely, Bohadschia mamorata, Stichopus badionotus Selenka, and Stichopus variegatus Semper have also been shown by Hawa et al. (1999). These antioxidants may possibly play a vital role to defy the damaging effects of oxygen free radicals formed in human body, hence helpful in combating common illnesses such as premature ageing, cancer and other degenerative diseases.

In addition to the studies on the healing property, many researches have been performed to investigate the effects of sea cucumber on bacteria. Ridzwan et al. (1995) conducted in-vitro tests using agar absorption method to screen for the presence of antibacterial activity in 3 species of sea cucumbers and to determine the best extraction method that would show such activity. Seven species of bacteria were used for these purposes: 4 gram positive (Streptococcus faecalis, Streptococcus viridens, Streptococcus pneumoniae, Staphylococcus aureus) and 3 gram negative bacteria (Escherichia coli, Shigella sonnei, Proteus mirabilis). They found that both the lipid extract and the methanol-solvent extract from Holothuria atra (H.atra), Holothuria scabra (H.scabra), and Bohadshia argus (B.argus) showed no antibacterial activity. On the contrary, phosphate-buffered saline (PBS) extract from H.atra and B.argus showed inhibition of all gram positive and negative organisms tested. Comparisons were also made between extracts from the outer layer of H.atra and its inner part, and it was found that the extract from the outer layer showed less bacterial growth inhibition property. Futhermore, Ridzwan and his colleagues also showed that the antibacterial property of sea cucumber was concentration-dependent.

Additionally, Villasin and Pomory (2000) demonstrated that methanol-acetone extract of the body wall of sea cucumber Parastichopus parvimensis also possessed antibacterial activity against Bacillis subtilis and Escherichia coli.

The disparity in the antibacterial activity by difference sea cucumber extraction methods was also shown by Ahmad et al. (2000) who investigated the antimicrobial activity of Holothuria edulis species extracts against Escherichia coli (E.coli) and Staphylococcus aureus (S.aureus) using plate sensitivity technique. They found that the

lipid-based extract did not have any antibacterial effect on these 2 organisms. In contrast, the water-soluble extract produced significant activity against S.aureus when the extract concentration of 50, 100, and 200mg/ml were used. When protinase K enzyme was added to the water-soluble extract, they found that the antibacterial activity was lost, and so they suggested that the antibacterial component in the extract was possibly a peptide.

The different effects of antibacterial property with extracts of different body parts of a sea cucumber were moreover confirmed by Haug et al. (2002). They performed a study whereby extracts of sea cucumber Cucumaria frondosa taken from the coelom, eggs, intestinal organs, muscle, tentacles, respiratory tree, and body wall, were tested for antibacterial activity against Vibrio anguillarum, Escherichia coli, Staphylococcus aureus, and Corynebacterium glutamicum. They found that the various tissues extracts possessed antibacterial activity but the main effect was observed in extracts of coelomocyte, body wall, and eggs. They indicated that besides protein, several different compounds were also involved in the antibacterial activities.

Nevertheless, results of studies on antibacterial property of sea cucumber were not always consistent. An example of this was the study carried out by Zainal Abidin et al. (2000). They investigated the antimicrobial activities of 10 mg/ml concentration of water-soluble extracts of Stichopus badionotus, Stichopus chloronotus, Stichopus variegatus, Holothuria edulis, and Holothuria atra. All the extracts were tested against Listeria monocytogenes ATCC 7644, Lactobacillus sp., Nocardia sp., Staphylococcus aureus, Bacillus subtilis, Salmonella typhi, Salmonella enteriditis, Escherichia coli 0157, Shigella sp., Proteus sp., and also Actinomycete species. In contrast to the findings of the

aforementioned studies, they indeed found that only the extract of Stichopus variegatus and Holothuria atra demonstrated inhibitory effect against Nocardia sp., and that the antibacterial activity against Nocardia sp. remained the same even when the concentration of S. variegatus extract was increased from 10 mg/ml to 20 mg/ml and 40 mg/ml.

Besides the abovementioned antibacterial property, there are also other properties demonstrated by sea cucumber, namely, antifungal, antiviral, antitumour, anti-anaphylactic, anti-inflammatory, antinociceptive, antipruritic, and antipyretic effect. Shimada (1969) reported that the holotoxin, that was, the steroidal glycosides from the sea cucumber species of Stichopus japonicus, contained antifungal and antitumour properties. Murray et al. (2001) in addition, isolated a new triterpene glycoside, patagonicoside A (1) from the sea cucumber Psolus patagonicus and showed that this compound possessed substantial antifungal activity against the pathogenic fungus Cladosporium cucumerinum. Moreover, Hedge et al. (2002) showed in their study that the aqueous methanolic extract of sea cucumber Telenata ananas sp. contained two triterpene glycosides. These glycosides displayed antagonistic activity on chemokine receptor CCR5, which would normally act as cofactor for HIV-1 attachment and entry into target cells. The blockade of CCR5 by the extract would therefore disrupt the viral transmission, and so, would be clinically important for HIV treatment.

Evaluation of antitumour properties of sea cucumber Holothuria sp. and Bohadschia marmorata vitiensis was furthermore performed by Oon et al. (2000) using MTS tetrazolium dye assay on the human breast cancer cell line, MCF-7. The results showed that

at the extract concentration of 0.5 microgram/ml, these sea cucumbers reduced the number of cancer cells by 20% as compared to the untreated cell population.

The anti-anaphylactic effect of sea cucumber extract was demonstrated by Hassan et al. (1997). Guinea-pigs were first sensitized with a dose of crude egg ovalbumin, given subcutaneously and intraperitoneally. Three weeks later, an induction of systemic anaphylactic reaction was performed in the sensitized guinea-pigs by giving a bolus intraperitoneal injection of ovalbumin. In groups pre-treated with Stichopus spl extract (100 mg/kg) at one, 2, and 3 hour before the induction made, the onset of anaphylactic symptoms and death were distinctly delayed in a time-dependent manner, with the effect most marked in one hour pre-treatment group. The effects of ovalbumin challenge in isolated sensitized guinea-pigs hearts pre-treated with intraperitoneal Stichopus spl (100 mg/kg) at one hour prior to the experiment were also made. The results showed that there was no change in both coronary perfusion pressure (Cpp) and cardiac developed tension (Cdt) at least for the first 5 minutes post-induction, in contrast to the untreated group which showed an immediate persistent increase in the Cpp and a decrease in the Cdt. Another study carried out by Ridzwan and his colleagues (1990) additionally showed that the extract of Holothuria scabra and Stichopus variegates could inhibit anaphylaxis induced by histamine in guinea-pigs.

In order to investigate the anti-inflammatory property, Hassan et al. (1997) performed a study on rats and examined the ability of orally administered Stichopus spl extract to inhibit carrageenan-induced hind paw oedema. They found that the extract showed a dose-dependent inhibitory effect on the oedema, which became significant at a

dose of 3 mg/kg. The maximum inhibitory effect was seen with Stichopus spl extract at a dosage of 300 mg/kg and this showed the same efficacy to the inhibitory effect achieved with indomethacin 1mg/kg given intraperitoneally.

The antinociceptive effect of sea cucumber extract was demonstrated by Hassan et al. (1994b) through hot-plate test on mice. They showed that the Stichopus spl extract possessed marked analgesic activity, equivalent to three-quarters of the efficacy demonstrated by morphine, with the effect lasting for about 6 hours. The analgesic effect of Stichopus spl extract was also shown in another study using writhing tests on mice, which confirmed a dose-dependent response, and further illustrated that the extract at 100 mg/kg dosage was equipotent to aspirin at similar dosage (Hassan et al., 1994c).

The antipruritic property of sea cucumber extract was shown by studying scratching response in mice which had been given oral administration of Stichopus spl (Hassan et al., 1997). Itchy sensation was induced in mice by giving intradermal injection of substance P and scores were recorded whenever the mice scratched the injected site by their hind paws by means of an automatically adjusted video camera and specially-programmed computer software. The results showed that there was a significant reduction in the number of scratching in animal group pre-treated with Stichopus spl extract, and suggested that the extract might thus acted as an antagonist to the neuropeptides such as substance P.

For the determination of antipyretic property of sea cucumber extract, mice were injected intraperitoneally with 8 mg/kg of lipopolysaccharide and rectal temperatures were followed to maximum steady pyrexia before the animals were given 0.2 ml of either oral

Stichopus spl extract or aspirin as control (Hassan et al., 1997). The results confirmed that administration of the extract attenuated the rise in temperature in a dose-dependent manner.

An attempt to elucidate the mechanism of action of sea cucumber extract in the treatment of asthma had also been carried out by Merican et al. (1986). The effect of holothurian extract on the tension and duration of twitches of cat soleus muscle was performed in order to determine whether the extract had β-adrenoceptor agonist or phosphodiesterase inhibiting properties. However the results showed that the extract had neither of these actions that could substantiate its claimed anti-asthmatic properties.

1.2.4 Sea cucumber and human bone

There is not much information until today about the effect of sea cucumber extract on human bone despite its widespread use either orally or topically, by people especially in this country. In particular, we do not know whether the extract has any beneficial effect or whether it is actually toxic to the human bone cell. With the growing interest in the therapeutic use of sea cucumber extract, there is therefore a pressing need to perform a study to fill up this gap in knowledge which will have a serious clinical implication to the people suffering with various bone conditions.

Extensive literature research was done to unveil any study that relates sea cucumber and animal or human bone. Published literature reports on this subject are however scanty. Only recently, a study performed by Kariya et al. (2004) succeeded in isolating and

characterising two types of fucan sulphate from chloroform/methanol extract of the body wall of Stichopus japonicus. From their in-vitro study, these fucan sulphates were found to inhibit osteoclastogenesis more than 95% at 50μg/ml concentration. Consequently, this property may explain the symptomatic relief when sea cucumber extract is used in the treatment of bone diseases such as rheumatoid arthritis and osteoporosis since both are associated with increased resorption of bone tissue by osteoclast cells.

Nonetheless, there have been many studies to show the presence of chondroitin sulphate in the body wall of sea cucumber. Kariya et al. (1990) isolated glycosaminoglycan from the body wall of Stichopus japonicus and showed that the disaccharide unit of the glycosaminoglycan was composed of 22.4% chondroitin sulphate E, 11.2% chondroitin, 10.4% chondroitin 4-sulphate, and 56% chondroitin 6-sulphate. Chondroitin sulphates have been shown in various literatures to be involved in osteoarticular metabolism (Bali et al., 2001). In an in-vivo study, oral administration of chondroitin sulphates had been shown to raise the total calcium pool and intestinal absorption of calcium (Bali et al., 2001). Another study showed that chondroitin sulphate, in association with collagen, could also promote in-vitro mineralization (Bali et al., 2001). Therefore, chondroitin sulphate supplied in the sea cucumber extract may potentially induce an increased capacity for an injured bone to regenerate during osteogenesis.

In Universiti Sains Malaysia Hospital, Shaifuzain (2005) undertook a study evaluating the effect of tibial bone fracture healing after oral administration of Stichopus sp1 extract in rabbit models. He discovered that at 3 weeks post administration of the extract (1mg/kg), the architecture of organised callus formation was significantly better

when evaluated by histological examination as compared to those given the higher concentration of extract (10mg/kg) and the controls (i.e. no extract given). The results suggested that the sea cucumber extract had a potent effect on bone healing when used at a low dose. This may be further explored pharmaceutically to treat bone diseases such as osteoporosis, which are associated with relative decrease in the rate of bone formation as compared to bone resorption. However it remains to be elucidated whether the enhanced organization of formed callus observed after the administration of oral sea cucumber extract was a direct effect on bone cells, or indirectly through some mechanisms affecting the bone metabolism, such as through the chondroitin-calcium-mineralization relationship as explained earlier.

Consequently, we decided to study the effect of the sea cucumber extract at the cellular level in order to ascertain whether or not, the extract has any direct effect on human osteoblast cells. The results will thus provide scientifically proven facts to enable consumers to make informed decision on the usage of sea cucumber extract and at the same time, map the path of future research on this substance.

1.2.5 Osteoblast cell

Osteoblast is a mature differentiated cell responsible for the formation and mineralization of bone matrix. The cells express various characteristic features such as high alkaline phosphatase activity, and synthesis of type 1 collagen and bone matrix proteins. Mature osteoblasts in vivo have a definite life span, as most are eliminated by apoptosis

process and only a small proportion (about 20%) become osteocytes (Hughes and Aubin, 1998). The osteoblast pool is replaced by further differentiation of preosteoblast. The other cell type of osteoblast lineage is the bone lining cells.

1.2.6 Human Cell Line Cultures

Cell culture offers the avenue for studying cells' intrinsic functions such as proliferation, differentiation, adhesion, migration, matrix synthesis, and death. In addition, one may also observe the interaction of the cells with each other, and with their environment. Studies utilising cell cultures allow the elimination of other confounding factors that are often difficult to be controlled if performed in-vivo, such as diurnal variation and episodic hormonal fluctuation. Additionally, the cells physicochemical environment can be easily controlled and monitored for examples, pH, temperature, osmotic pressure and tension of gases. The amount of a test substance used may also be greatly reduced compared to in vivo studies. As much information can be obtained from cell culture studies, the use of preliminary study using animal models can thus be avoided. Nonetheless, cell culture is not without any limitation. The dissociation of cells from their normal three-dimensional architecture may lead to loss of certain cell interaction and behaviour. Furthermore, the influence of body's neural and hormonal homeostatic regulation on the cells is absent and cannot be investigated using cell culture techniques.

Cell lines describe the cells that arise from a primary culture after first successful subculture (Abdul, 2003). There are two categories of cell line: primary cultures and

permanent cell lines. Primary culture cell line has the disadvantage that it has limited lifespan, and usually consists of heterogenous mixture of cells which may include other cell lineage such as fibroblasts. It has the advantage that the cells are less susceptible to time-dependent changes that occur in culture as the cells adapt to the in-vitro environment, grow at different rates and mutate with successive cell divisions (Majeska and Gronowicz, 2002). Thus, primary cell line culture provides a reasonable approximation to their in-vivo phenotypic characteristics.

Permanent cell lines describe cells of a known origin with an unlimited lifespan in culture. In contrast to primary culture, permanent cell lines usually consist of uniform cells and clonal by design, though individual sublines of the clonal population still showing a range of phenotypes (Majeska and Gronowicz, 2002). The disadvantages of permanent cell lines are that there will inevitably be phenotypic drift and loss with each cell division, and although there may be immortalized cells, they will no longer represent their in-vivo phenotype after a certain period of time.

In addition to the above categories, there are also two types of cell line: adherent (monolayer) cells and non-adherent (suspension) cells. As the name implies, adherent cells anchor to the surface of the container where they are cultured. An example of adherent cell is osteoblast cell.

1.2.7 Measurement of cytotoxic effect and cell viability using MTT assay

International Standard (ISO 10993-5) has outlined the principles of cytotoxic assay which include the inclusion of positive and negative control materials, extraction conditions, selection of appropriate cell lines and cell media, and also important elements of the test procedures such as testing by direct or indirect contact. The standard cytotoxic evaluation could be categorised into: 1) qualitative assessment of cell morphology, 2) quantitative measurement of cell growth, and 3) quantitative measurement of specific cellular metabolism.

In this study, MTT assay was used to investigate any cytotoxic effect of sea cucumber extract on human osteoblast cell, and also to determine the effect of the extract on osteoblast cell viability and proliferation (Mosmann, 1983; Di Silvio, 2003a). In the past, this assay has been extensively used to study the action of various substances on osteoblast cell lines and human bone cell cultures (Majeska and Gronowicz, 2002).

MTT {(3-[4,5-dimethylthiazol-2-yl])-2,5-diphenyl tetrazolium bromide}is a colourless substance which is converted to a blue formazan by mitochondrial dehydrogenase. This enzyme is present only in intact living cells, hence the blue colour produced (i.e. the amount of formazan) should be directly proportional to the number of viable cells present. Activated cells produce more formazan than resting cells, which allows the measurement of activation even in the absence of proliferation. Thus the signal

generated is also proportional to the degree of activation of the cells (Mosmann, 1983; Di Silvio, 2003a).

Cytotoxicity simply means the cell killing property of a substance, whereas "cytotoxic dose" is defined as the concentration of the tested substance which reduces the number of cells by 50% as compared to the untreated cell population (Abdul, 2003). The principle of MTT assay as a cytotoxic test is that dead cells would not be able to metabolize the various tetrazolium salts. However, the disadvantage of using this metabolism assay is that it underestimates the cellular damage since it overlooks cell apoptosis, which is an active mode of cell death requiring the metabolism of cells (Abdul, 2003).

Advantage of using MTT assay is that no radioisotope is used. Furthermore, the substrate does not interfere with measurement of the results. This allows the assay to be read with no removal or washing steps, which increases the speed of the assay and helps to minimize variability between samples (Mosmann, 1983). MTT assay is also very sensitive as it can detect cell numbers as low as 4×10^2 per well against background (Clifford and Downes, 1996).

1.2.8 Measurement of cell functionality using ALP assay

The high alkaline phosphatase expression of osteoblast phenotype enables its use as a marker of differentiating osteoblasts in vitro. The alkaline phosphatase is localized to the osteoblast cell membrane and is covalently bound to phosphatidyl inositol (PI)

phospholipid complexes. The enzyme can therefore be released from the cells by PI-specific phospholipase C enzyme (Hughes and Aubin, 1998). The advantage of using alkaline phosphatase as a marker in osteoblast cell culture assay is that the enzyme is expressed relatively early in osteoblastic differentiation, which allows its amount to be measured within a short period of incubation time.

Alkaline phosphatase assay is possibly the most frequently used test in characterizing osteoblast function (Hughes and Aubin, 1998; Oreffo et al., 1998). In addition, it has often been used in conjunction with MTT assay to determine osteoblast viability (Majeska and Gronowicz, 2002).

This assay however, does not measure the amount of the enzyme protein present directly. Instead, the enzyme alkaline phosphatase that is present in the osteoblast cells will cleave the phosphate group from the colourless p-nitrophenyl phosphate in the substrate reagent, to produce p-nitrophenol which is yellow at alkaline pH and can be monitored at or around 405nm (Oreffo et al., 1998; Di Silvio, 2003b).

CHAPTER TWO

2.1 AIM

- To investigate the effects of gamat extract of Stichopus species on human osteoblast cell lines.

2.2 OBJECTIVES

1. To determine the proliferation and viability of human osteoblast cells when grown in culture media added with Stichopus sp1 extract at different concentrations.
2. To determine the functional activity of human osteoblast cells when grown in culture media added with Stichopus sp1 extract at different concentrations.
3. To determine the optimum safety dose of Stichopus sp1 extract on human osteoblast cells.

2.3 RESEARCH HYPOTHESIS

"Gamat extract of Stichopus sp1 at an appropriate concentration will increase the rate of osteoblast cell proliferation and functional activity".

The above hypothesis was constructed based on the literature review findings (described in Section 1.2.4), that relate the extract of sea cucumber and human bone. The study done by Kariya et al. (2004) already proved that the extract of sea cucumber Stichopus japonicus had a direct effect on bone cells (in their case, osteoclasts) via its fucan sulphate component. Furthermore, the results of the study done by Shaifuzain (2005) showed that the bone fracture healing was qualitatively enhanced when extract of Stichopus spl was given to his animal models. Thus, there might be a direct effect of the sea cucumber extract on the animal osteoblast cells causing increased osteoblastic proliferation and activity, which was manifested as increased organization of formed callus in his study. However, as pointed out, this effect could be dose-dependent (Shaifuzain , 2005).

2.4 IMPORTANCE AND BENEFITS OF THE RESEARCH

1. To scientifically establish the effect of a local natural product, Gamat, on osteoblast cell line, in keeping to the World Health Organization's recommendations on traditional medicine (WHO, 2003). This will have an immediate relevance with respect to health benefit or risk to consumer.
2. The potential for gamat promoting an increase in osteogenic cell differentiation and proliferation will offer new avenue for treatment of bone defects.
3. Gamat may potentially be used in many areas of current orthopaedic and rheumatology practice, such as:-
    1. Promoter in fracture healing.