CYTOTOXICITY TESTING OF CHITOSAN DERIVATIVES ON PRIMARY CULTURED FIBROBLAST

by

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CERTIFICATE

This is to certify that the dissertation entitled

"Cytotoxicity testing of chitosan derivatives on primary cultured fibroblast"

is a bonafide record of research work done by

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ABSTRACT

Chitosan has been used in several studies as wound healing accelerator. This study was designed to determine the cytotoxicity of chitosan on primary cultured human fibroblasts. Human skins were obtained from surgical specimen of consented donor and primary cultured fibroblasts were established. The fibroblasts were incubated in the absence or presence of increasing concentrations of Oligo Chito 1% and Oligo Chito 5% for 24, 48 and 72 hours. Subsequently, MTT reagent was added and 4 hours later the purple crystals produced were dissolved in DMSO. Then, cells viability was read using ELISA plate reader. Cells viability was used as a marker for toxicity. The results showed that cell viability was dependent on concentration. The cell viability decreased when high concentrations of chitosan were used. Prolonged incubation period also caused decreased cell viability. Analysis of chitosan effects on cell culture is useful as a screening tool for their potential *in vivo* activity as wound healing agents.

ABSTRAK

Chitosan telah digunakan dalam beberapa kajian untuk mempercepat penyembuhan luka. Kajian ini dilakukan untuk menentukan ketoksikan chitosan terhadap kultur primer sel fibroblas manusia. Kulit manusia diperoleh daripada spesimen pembedahan penderma yang telah bersetuju dan kultur primer fibroblas dilakukan. Fibroblas tersebut dieramkan dengan kehadiran dan tanpa kehadiran Oligo Chito 1% dan Oligo Chito 5% pada kepekatan yang meningkat selama 24, 48 dan 72 jam. Selepas itu, reagen MTT ditambahkan dan 4 jam kemudian hablur ungu yang terbentuk dilarutkan di dalam DMSO. Kemudian, viabiliti sel dibaca menggunakan ELISA. Viabiliti sel digunakan sebagai penanda kepada ketoksikan. Keputusan menunjukkan bahawa viabiliti sel adalah bergantung kepada kepekatan. Terdapat penurunan dalam viabiliti sel apabila chitosan dengan kepekatan yang tinggi digunakan. Masa pengeraman yang panjang juaga menyebabkan penurunan dalam viabiliti sel. Analisis kesan chitosan ke atas kultur sel adalah berguna sebagai saringan untuk aktiviti potensial bahan tersebut secara *in vivo* sebagai agen penyembuh luka.

1.0 INTRODUCTION

1.1 CHITOSAN

Chitosan and chitin are polysaccharide polymers containing more than 5,000 glucosamine and acetylglucosamine units, respectively, and their molecular weights are over one million Daltons. Chitin is found in fungi, arthropods and marine invertebrates. Commercially, chitin is derived from the exoskeletons of crustaceans (shrimp, crab and other shellfish). Chitosan is obtained from chitin by a deacetylation process. Chitin, the polysaccharide polymer from which chitosan is derived, is a cellulose-like polymer consisting mainly of unbranched chains of N-acetyl-D-glucosamine. Deacetylated chitin, or chitosan, is comprised of chains of D-glucosamine.

1.2 CHITOSAN DERIVATIVES

Chitosan has very strong functional properties in many areas. However, the water insoluble property of chitosan is disadvantageous for its wide application. Therefore, chitosan derivatives with water-soluble and functional property have been developed (Je *et al,* 2006).

1.3 PRIMARY CULTURE OF FIBROBLASTS

Fibroblasts are connective tissue cells. The cells are flat and elongated with cytoplasmic processes at each end. The nuclei are flat, oval and vesicular. Fibroblasts which differentiate into chondroblasts, collagenoblasts, and osteoblasts, form the fibrous

tissues in the body including tendons, aponeuroses, supporting and binding tissues of all sorts.

Cell culture is a process where cells are removed from animal tissue or whole animals. The cells are supplied with nutrients and growth factor so that they will continue to grow. The growth will continue until some parameter such as nutrient depletion limit their further growth.

Two types of cell cultures are cell lines and primary culture. A primary culture is established when the cells taken directly from animal tissues are added to growth medium. Primary cell cultures are derived from rat, mouse and human. After a specified period of time, cells in primary culture normally will stop to grow therefore new culture need to be established by inoculating some of the cells into fresh medium. This step is known as sub-culturing or passaging. A secondary culture is established after the first passage of the primary culture. Cell population that continues to grow through many sub-cultures is known as cell line (Butler *et al*, 1996).

There are some advantages using primary cell culture. The major advantage is the ability to control physical environment which is difficult to control *in vivo*. Besides, we also can get homogenous population as well as fewer compounds needed than in animal model. Cells also have not been modified in any way either enzymatic or physical dissociation. Furthermore, in toxicological testing, the use of cell culture techniques may allow greater understanding of the effects of particular compound on a specific cell type.

The disadvantages of primary cultures are the mixed nature of each preparation, limited lifespan of the culture and the potential contamination problems.

1.4 CYTOTOXICITY STUDY

Cytotoxicity can be determined by either qualitative or quantitative means. In qualitative evaluation, the cells are examined microscopically to assess the changes in cellular properties such as the general morphology, vacuolization, detachment, cell lysis and membrane changes.

While in quantitative evaluation, cell death, inhibition of cell growth, cell proliferation or colony formation are measured. The number of cells, amount of protein, release of enzymes, release of vital dye, reduction of vital dye or other measurable parameter may be quantified by objective means.

1.5 MTT ASSAY

Measurement of cell viability and proliferation form the basis for numerous in vitro assays of cell population's response to external factors. Furthermore, the use of colorimetric assays to determine cell survival has permitted the procedure to become highly automated, thus yielding a rapid test system.

The tetrazolium salt, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), is taken up into cells and reduced by mitochondrial dehydrogenase enzyme to yield a purple formazan product which is largely impermeable to cell membranes, thus resulting in its accumulation within healthy cells. Solubilisation of the cells results in the liberation of the product which can readily be detected using a simple colorimetric assay. The ability of cells to reduce MTT provides an indication of the mitochondrial integrity and activity which, in turn, may be interpreted as a measure of viability and/or cell number (Margaret EW, 1990).

There is still other assay that can be used to measure cells proliferation but MTT assay is chosen in this study because of its proven technology. The utilization of MTT method has been documented in the literature for many different applications. Besides, we also can get accurate measurements as the spectrophotometric procedure can detect slight changes in cell metabolism, making it much more sensitive than trypan blue staining. This method is also easy to be used. The procedure is relatively simple and uses equipment already available in most laboratories. Furthermore, safer reagents are used and there is no need to manipulate radioactive substances. This method is also rapid in processing. Assays are run in a 96-well plate and read with a microtitre plate reader, allowing high-throughput handling of samples.

2.0 REVIEW OF LITERATURE

2.1 CHITOSAN

2.1.1 Structure

Being a structural component of shellfish, insects and the cell walls of bacteria and mushrooms, chitin or poly-β-(1-4)-N-acetyl-D-glucosamine is the most abundant polymer found in nature after cellulose. Chitosan is the N-deacetylated product and has a molecular weight ranging from 1-3 x 10^5 Da (Begona and Ruth, 1997). The chemical structure of chitosan can be seen in Figure 1.

Chitosan prepared from chitin through alkaline hydrolysis is the most preferable research subject. The existence of two hydroxyl groups and one amino group in the monomer unit and its solubility in aqueous acidic solutions offer wide possibilities for various transformations of chitosan aimed at obtaining more easily processible products or for the synthesis of new compound with unusual properties (Vasnev *et al.*, 2005).

Most biological activities of chitosan are attributed to their free amino group.

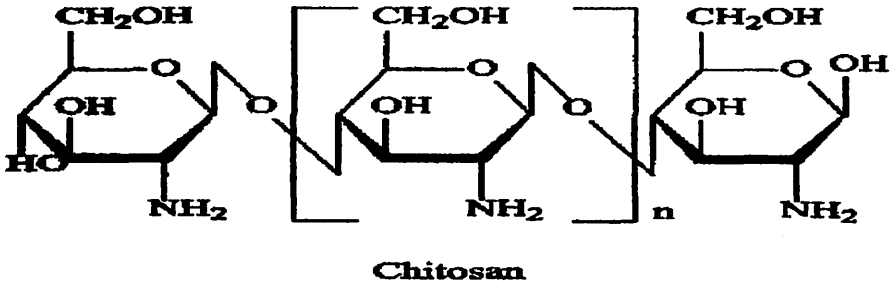

Figure 1: Chemical structure of chitosan (http://www.pdrhealth.com/)

2.1.2 Properties and functions

The discovery that chitin and a water-soluble product of chitin acetylation, chitosan, posses unique properties, such as, antibacterial, antiviral, intoxic and inallergic, high radiation resistance, the capability for the immunological protection against pathogens, biocompatibility, biodegradability and etc. gave impetus to further research on this material (Vasnev *et al*, 2005). Most chitosans are only soluble in aqueous acidic solutions below pH 6.5, where the primary amino groups of chitosan are protonated (Mao *et al*, 2005).

The biological properties including bacteriostatic and fungistatic properties are particularly useful in wound treatment (Paul and Sharma, 2004).

Chitosan also has been applied in drug delivery including as an absorption enhancer for hydrophilic macromolecular drug and a gene delivery system (Mao *et al*, 2005).

2.1.3 Effects of chitosan in wound healing

In a study done by Ueno *et al* (2001) on skin of dogs, they proved that chitosan played a major role in accelerating wound healing. Chitosan promotes the migration of the inflammatory cells which are capable of the production and secretion of a large repertoire of proinflammatory products and growth factors at a very early phase of healing. Chitosan also promotes the granulation phase of healing. Chitosan induces activation in peritoneal macrophages. Moreover, chitosan stimulation will increase the production of biological mediators by macrophages. With regard to the effects of chitosan on fibroblast culture, the study showed that high concentration chitosan cultures supplemented with 10% FCS showed a significant ($P<0.05$) reduction in the rate of L929 fibroblasts cells proliferation.

However, the inhibition of cell proliferation by high concentrations of chitosan was not shown in cultures without FCS.

Howling *et al* (2001) performed in vitro study to evaluate the various chitin and chitosans, to assess their ability to influence the proliferation of human fibroblasts as an index for their potential as wound healing agents. The screening of the biopolymers for their effects on fibroblast proliferation showed that chitosan "Seacure CL313", and its shorter chain length fraction, CL313A, had the greatest mitogenic activity at all concentrations tested. The other chitosan samples showed lesser degrees of mitogenic activity and some samples, such as chitin 50A, had antiproliferative effects. The deacetylation level of chitosan seems to be a key factor in the mitogenic activity on fibroblasts with the molecular mass of the biopolymer being somewhat less important (Howling *et al*, 2001).

The mechanism by which chitosan CL313A stimulates fibroblast cell growth is unknown although it has been postulated that it may function in similar way to hyaluronan (Mattioli-Belmonte *et al*, 1997).

Ueno *et al* (1999) had concluded the efficacy of chitosan for wound healing. Chitosan accelerated infiltration of polymorphonuclear cells into the wound area. Chitosan also increase effusion which forms thick fibrin and activates the migration of fibroblasts into the wound area. Besides, chitosan also play important roles in stimulation of the migration of the macrophages and stimulation of proliferation of fibroblasts. Chitosan also stimulate production of type III collagen.

2.2 WOUND HEALING

Wound repair is a complex process involving an integrated response by many different cell types controlled by a variety of growth factors. During the initial inflammatory phase fibroblasts start to enter the wound where they synthesize and later remodel new extracellular matrix material, of which collagen is the main component. The dermal response is only one aspect of cutaneous wound repair however, the outermost and vital barrier layer, the epidermis which is composed of several layers of keratinocytes, must also be restored. In injured skin, basal layer keratinocytes migrate from the wound edge and from injured epidermal appendages (hair follicles and sweat glands) into the defect, moving over the newly formed dermal scaffolding. They proliferate, stratify and differentiate to produce a neoepidermis to cover the wound and restore the skin's barrier function (Singer and Clark, 1999).

In the early phase of wound healing, there are two stages as major components of wound healing. The first stage is the inflammatory stage, and the second is the stage of new tissue formation. At the inflammatory stage, infiltrating neutrophils clean foreign agents in the area. At the new tissue formation stage, fibroplasias begin by the formation of granulation tissue within the wound space. This tissue consists of a loose matrix of collagen, fibronectin and hyaluronic acid (Ueno *et al*, 1999)

2.3 CYTOTOXICITY TESTING

Cytotoxicity can be assessed by direct or indirect methods (Cook and Mitchell, 1989). These cytotoxicity assays are based on the used of various parameters quantifying cell death, or the effects of the products on cell metabolism (Corrine *et al*, 1998). The common methods used to determine cytotoxicity are the neutral red uptake and dimethylthiazole-diphenyl tetrazolium bromide (MTT) metabolism (Mosmann, 1983), lactate dehydrogenase, N-acetyl glucosaminidase or alkaline phosphatase release (Scaife, 1985), ATP intracellular dosage (Kemp *et al*, 1985) and the release of inflammation mediators such as prostaglandin E2 or the cytokines (Triglia *et al*, 1981).

2.4 MTT ASSAY

Tetrazolium salts are used extensively in cell proliferation and cytotoxicity assays, enzyme assays, histochemical procedures, and bacteriological screening. In each, tetrazolium salts are metabolically reduced to highly colored end products called formazans (Michael *et al*, 1996).

The MTT assay is widely used in cell proliferation and cytotoxicity assays. Most cellular bioreduction of MTT is associated with enzymes of the endoplasmic reticulum and involves the reduced pyridine nucleotides NADH and, to a lesser extent, NADPH. Succinate is a weak electron donor for mitochondrial MTT reduction (Michael *et al*, 1996).

The common characteristics of MTT assay are that the microplate assays require no cell transfers. It measures cell proliferation under defined growth condition. Besides, it is also primarily dependent on net rate of glycolytic NAD(P)H production by cells. Mitochondria is not involved in cellular reduction of this assay but it is inefficiently

reduced by mitochondrial succinate dehydrogenase. MTT assay produce a visible end point that can be quantified using an ELISA plate reader. The type of product produced by MTT assay is a single reagent and the formazan formed requires solubilization. In addition, the color is stable for days. The final endpoint of this assay allows single measurement only. MTT assay is metabolically reduced by all viable cells and not use in conjunction with an intermediate electron receptor. The reduction occurs intracellularly and it is catalyzed by enzymes of the endoplasmic reticulum. This assay is useful in biochemical assays with biochemical factors such as NAD(P)H and succinate. However, MTT assay cannot be used consecutively or simultaneously with other cell assays (Michael *et al*, 1996).

3.0 OBJECTIVES

The objectives of this study are:

1) To establish primary fibroblasts culture from human skin.

2) To determine effects of concentration of chitosan on fibroblasts viability.

3) To determine effects of incubation period on fibroblast viability.

4.0 MATERIALS AND METHODS

4.1 Equipment and instruments

The equipments that have been used in this research project are listed below. The equipments and instruments are located in the Tissue Culture Laboratory, National Tissue Bank and supplied by Reconstructive Sciences Unit (PPSP).

1. Laminar air flow- Holten Lamin Air HB2448
2. CO2 incubator- Heto Cell House 150
3. Incubator- LEEC Compact Incubator
4. Inverted microscope- LEICA DMIL
5. Centrifuge- UNIVERSAL 16R
6. Liquid nitrogen
7. Haemocytometer
8. ELISA plate reader

4.2 Materials

4.2.1 Chitosan

Chitosan derivatives of different concentrations namely Oligo Chito 1% and Oligo Chito 5% were used in this study. These materials were in the form of solution and supplied by SIRIM.

4.2.2 Skin material for primary culture of fibroblast

Skin material for primary culture of fibroblasts is obtained from surgical specimens of consented donor supplied by Reconstructive Sciences Unit (PPSP).

4.2.3 Chemicals

1) Dulbecco's Modified Eagle Medium (DMEM)

DMEM that is used contain high glucose content, with L-glutamine and pyridoxine hydrochloride, without sodium pyruvate and supplemented with 10% FBS and 1% PSA.

2) Defined Keratinocyte Serum Free Media (DKSFM)

3) Dulbecco's Phosphate Buffer Saline (DPBS)

DPBS without calcium chloride and magnesium chloride is used in this study.

4) Dispase

2.4 units dispase is prepared in every 1 ml of DKSFM.

5) Collagenase I

200 units of Collagenase I is prepared in every 1 ml of DMEM.

6) MTT reagent

MTT reagent is prepared from tetrazolium salts. 5mg of tetrazolium salts is dissolved in 1 ml phosphate buffer saline.

7) Optical Grade Dimethyl Sulfoxide (DMSO)

8) Trypsin EDTA (0.25%)

9) Fetal Bovine Serum (FBS)

10) Penicillin Streptomycin

11) 70% alcohol

4.3 METHODS

All procedures including preparation of reagent and tissue were done under aseptic technique. All subsequent steps were performed in a laminar air flow cabinet.

4.3.1 Storing skin samples for primary cultured fibroblast

- ***Materials***

1. Defined Keratinocyte Serum Free Media (DKSFM)
2. Skin sample

- **Procedure**

The skin samples were placed in Defined Keratinocyte Serum Free Medium (DKSFM) supplemented with 5μg/ml gentamycin. Skin samples can be stored in DKSFM at 4^0C for approximately 5 days without significant loss of viable cells.

4.3.2 Separating dermal from epidermal layer

- ***Materials***

1. Dulbecco's Phosphate Buffer Saline (DPBS)
2. 70% alcohol
3. Defined Keratinocyte Serum Free Media (DKSFM)
4. Dispase (2.4 units/ml in DKSFM)
5. Collagenase I (200 units/ml in DMEM)

- **Procedure**

Skin samples were trimmed carefully to remove excess blood and fat tissues. Then, the skins were washed three times with DPBS without Ca^{2+} or Mg^{2+} (supplemented with 5% PSA or 20μg/ml gentamycin), 70% alcohol and rinsed again in DPBS. Skin samples were cut into fragment about 3mmx3mm to facilitate the digestion process. After that the skin samples were transferred into 2.4 units/ml dispase which was dissolved in DKSFM and incubated for 18 hours at 2 to 8^0C. Dispase is important for separating epidermis and dermis.

After incubation in dispase, dermal layer was separated from the epidermal layer. The dermal layer was placed in the Petri dish containing *Clostridium histolyticum* collagenase A to liberate fibroblasts. The specimens were incubated overnight at 37^0C in humidified atmosphere containing 5% CO_2.

4.3.3 Processing dermal layer

- ***Materials***

1. Dulbecco's Modified Eagle Medium (DMEM)
2. Dulbecco's Phosphate Buffer Saline (DPBS)

- **Procedure**

Following incubation, fibroblast cells from dermis layer were aspirated using 2ml pipette to aid in cell dissociation. The cell suspension was transferred into 50ml centrifuge tube and centrifuged at 2000 to 3000 rpm for 10 minutes. Supernatant was removed and

cells pallet was resuspended in 20 ml of DPBS. Then suspension cells were sieved by 70μm cell strainer and spinned at 1000 rpm for 10 minutes. This step was done twice.

Next, the resulting pallet was gently resuspended in 2 to 10 ml DMEM complete media and fibroblasts concentration was determined by cell strainer. Primary cells were seeded into 75T culture flask at a density approximately $1x10^6$ cells/flask. Cultures were incubated at 37^0C in humidified atmosphere containing 5% CO_2. Fluids of cultured fibroblasts were changed with fresh complete medium for every 2 to 3 days.

4.3.4 Maintaining primary cultured fibroblast

4.3.4.1 Washing and changing the medium

- **Materials**

1. Dulbecco's Modified Eagle Medium (DMEM)
2. Dulbecco's Phosphate Buffer Saline (DPBS)

- **Procedure**

The culture medium was changed every two days because this rate enabled the maintenance of ideal conditions of pH between 7.6 and 7.8 without non-physiologic upheavals. This pH stability aimed a balance between cellular proliferation and cellular biosynthesis activity of the fibroblasts. The culture medium was aspirated and the flasks were rinsed with 10ml of DPBS. Then, the DPBS was aspirated and 10ml of DMEM with gentamycin was instilled into the flasks.

4.3.4.2 Subculture of fibroblasts

- **Materials**

1. Dulbecco's Modified Eagle Medium (DMEM)
2. Dulbecco's Phosphate Buffer Saline (DPBS)
3. Trypsin EDTA (0.25%)

- **Procedure**

Subculture (passage) of fibroblasts was done with 80% cellular confluence in T75 or T25 flasks. After aspirating the culture medium, fibroblasts grown in the flask were rinsed with 10ml of DPBS. Afterwards, DPBS was aspirated and trypsin with EDTA was instilled in the flask and maintained in humidified incubator for four to five minutes. Fibroblasts were observed detached from the dish surface with spherical morphology, opposite to the fusiform morphology usually observed in fibroblasts or with cytoplasmatic prolongation of fibroblast found scattered in the culture surface. The flask was tapped gently to make sure that all cells detached from the surface. Next, 20ml of DMEM was instilled into the flask and resuspended. Fibroblast cultures were split into two T75 flasks and maintained at 37^0C under 5% CO_2. In this experimental culture, fibroblasts between fourth and seventh passage were used in order to avoid the influence of plasmatic factors and senescent changes in the cellular morphology.

4.3.4.3 Cryopreservation

- ***Materials***

1. Trypsin EDTA (0.25%)
2. Fetal Bovine Serum (FBS)
3. Optical Grade Dimethyl Sulfoxide (DMSO)

- **Procedure**

Fibroblasts also can be stored in liquid nitrogen at -196^{0}C. Culture medium was aspirated and cells were rinsed with DPBS. Next, trypsin with EDTA was instilled in the flask and incubated in CO_2 incubator for four to five minutes. Fibroblasts were observed to detach from the dish surface with spherical morphology and tapped gently to make sure that all cells detached from the surface. The cellular suspension was centrifuged for 1000 rpm in 10 minutes. Supernatant was removed and the cell pallet was resuspended with freezing medium.

Freezing medium was freshly prepared containing 90% Fetal Bovine Serum (FBS) and 10% optical grade dimethyl sulfoxide (DMSO) was prepared for one T75 flask.

4.3.5 Treatment of chitosan to primary cultured fibroblast

- ***Materials***

1. Dulbecco's Phosphate Buffer Saline (DPBS)
2. Trypsin EDTA (0.25%)
3. Dulbecco's Modified Eagle Medium (DMEM)
4. Trypan blue

5. Oligo chito 1%
6. Oligo chito 5%

- **Procedure**

Cells need to be seeded in 96-well microtitre plate. Before that, cells were washed with DPBS . Then, 3.5 ml trypsin was added and incubated in CO_2 incubator until all cells were detached from the surface of flask. Culture medium was added to neutralize enzymatic action of trypsin. The cellular suspension was centrifuged for 1000 rpm in 10 minutes. Supernatant was disposed while cell pallet was resuspended with 10 ml DMEM. A cell suspension was put onto the Neubeauer haemocytometer grid to count the cells in a standard seeding density. Trypan blue was added to the cell suspension before counting. The dye penetrates the membrane of non-viable cells which were stained blue and which could therefore be distinguished from viable cells. Then, cells were seeded into 96-well microtitre plate at a density of 6×10^4 cells/ml.

On the next day, the cells were treated with test materials that were Oligo chito 1% (1 hour) and Oligo chito 5% (1 hour). The chitosan solutions were added to the plates at various concentrations. 100% of test material was added at the first line of microtiter plate. Then, serial dilution was done. There were 6 replicates for each concentration. The final line was negative control where no test material was added.

The plates were incubated for 24, 48 and 72 hours.

4.3.6 MTT assay

- **Materials**

1. MTT reagent
2. Optical Grade Dimethyl Sulfoxide (DMSO)

- **Procedure**

After 24 hour, 10µl MTT reagent was added and 4 hour later the purple crystals produced were dissolved in 100µl of DMSO. Absorbance was read at 570nm using ELISA plate reader. The procedure was repeated for 48 hour and 72 hour plates.

4.3.7 Statistical analysis

The data obtained were subjected to repeated measure ANOVA (RM ANOVA) and multifactorial ANOVA using SPSS 12.0 for Windows.

5.0 RESULTS

On the first day of primary cultured fibroblast **[Figure 2]**, the fibroblast showed rounded morphology as it failed to attach to the flask surface. This morphology also can be seen when trypsinization was done. The cells detached from the surface.

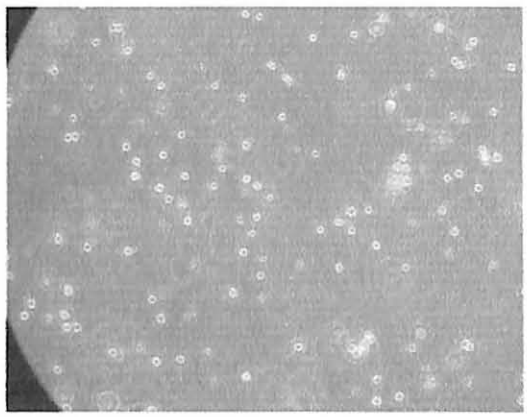

Figure 2: Day 1 of primary cultured fibroblast

While on the third day of culture **[Figure 3]**, some of fibroblast cells attached to the flask surface. They turned into slightly oval shape. But there were some cells which still did not attach as can be seen from their round shape.

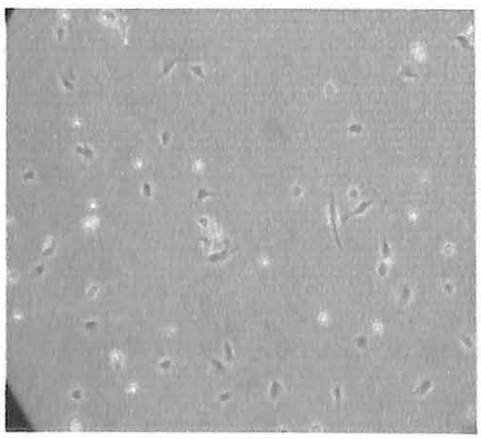

Figure 3: Day 3 of primary cultured fibroblast

On the sixth day **[Figure 4]**, the cells exhibit fusiform morphology usually observed in fibroblasts. Fibroblasts with cytoplasmatic prolongation were found scattered in the culture surface.

Figure 4: Day 6 of primary cultured fibroblast

Later, on the day 8 **[Figure 5]**, confluent monolayer of fibroblasts were seen. The cells were elongated and they were close to each other. All spaces on the surface were occupied. At this stage, the cells can be used for treatment or sub cultured into other flasks.

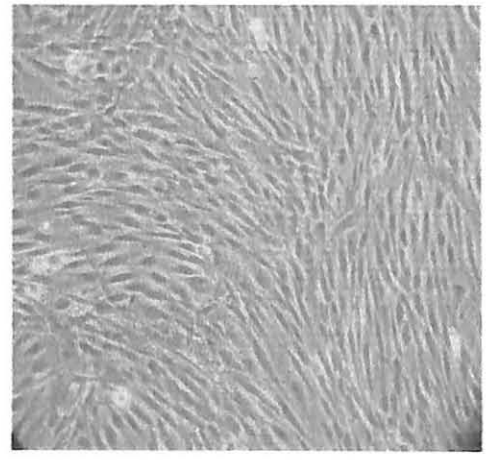

Figure 5: Day 8 of primary culture fibroblast

The graph **[Figure 6]** showed that the percentage of viability decreased as the concentration of Oligo Chito 1% increased. The viability gradually reduced at 25% dilution and there was no viable cell when 100% of Oligo chito 1% is applied. It showed that cells viability was reduced as the incubation period prolonged.

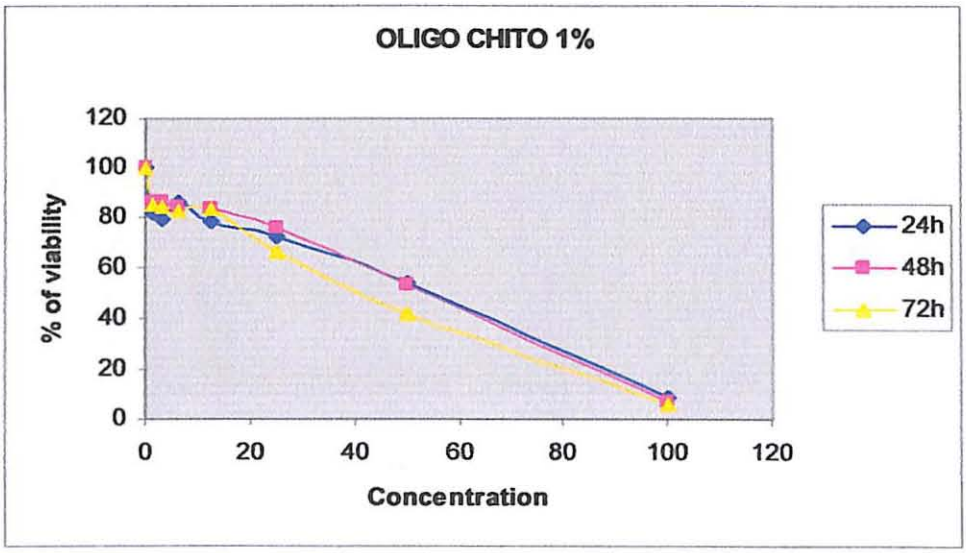

Figure 6: Percentage of viability versus concentration of Oligo Chito 1%

For Oligo chito 5%, the cells viability decreased markedly **[Figure 7]**. The cells viability is 100% when no Oligo chito 5% was applied. However, the viability decreased when the test compound was diluted at 12.5%. Cells viability was low compared to Oligo Chito 5%. Cells viability also reduced as incubation period was prolonged.

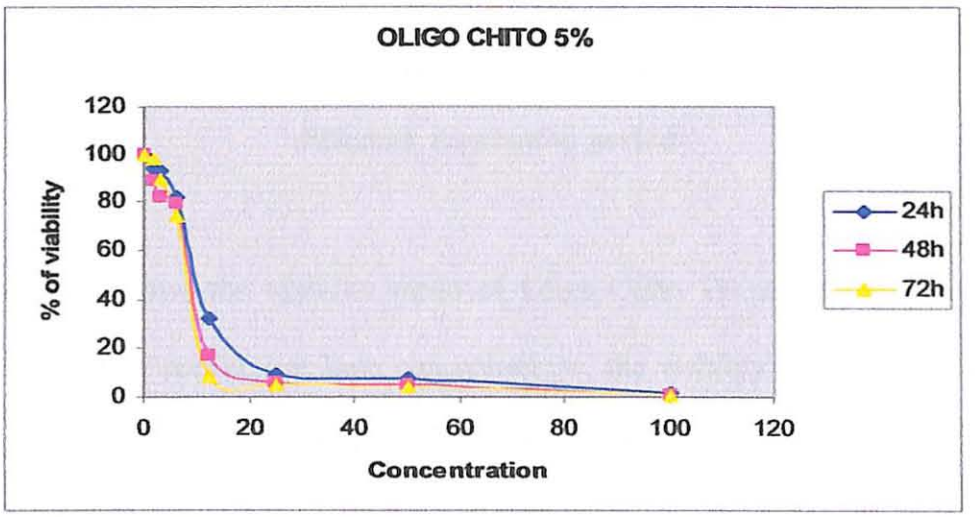

Figure 7: Percentage of viability versus concentration of Oligo Chito 5%

Conc		Mean (sd)
24 hours	1%	0.41233 (0.157859)
	5%	0.28104 (0.230475)
48 hours	1%	0.48327 (0.193639)
	5%	0.34794 (0.298240)
72 hours	1%	0.45788 (0.193840)
	5%	0.32023 (0.297238)

Table 1 : Mean and standard deviation of Oligo chito 1% and Oligo Chito 5% at different incubation period

This table shows the viability mean of Oligo Chito 1% and Oligo Chito 5% at different incubation period. For both concentrations, the viability was slightly low at 24 hours of incubation. Then, the viability increased at 48 hours and decreased again at 72 hours of incubation.

Time	Mean difference (95% CI)	F test	P value
24 hrs, 48 hrs	-0.069 (-0.90,-0.48)	267.171	< 0.001
24 hrs, 72hrs	-0.042 (-0.63,-0.22)	267.171	< 0.001
48 hrs, 72 hrs	0.027 (0.008,0.045)	267.171	0.002

Table 2 : Effects of different time of incubation and viability

Table 2 compares the effects of different time of incubation and viability. There is a significant difference between 24 hours and 48 hours as p value is less than 0.001. There is also a significant difference between 24 hours and 72 hours but no difference can be seen between 48 hours and 72 hours.

Concentration	Mean (SE)	Mean difference (95% CI)	P value
Oligo chito 1%	0.451 (0.009)	0.135 (0.110,0.160)	< 0.001
Oligo chito 5%	0.316 (0.009)	-0.135 (-0.160,-0.110)	< 0.001

Table 3: Effects of concentration on viability of fibroblasts

Effects of concentration on viability of fibroblasts can be seen from this table. From mean, viability of Oligo Chito 5% is less than Oligo Chito 1%. There is a significant difference between these two concentrations indicated by P value less than 0.001.

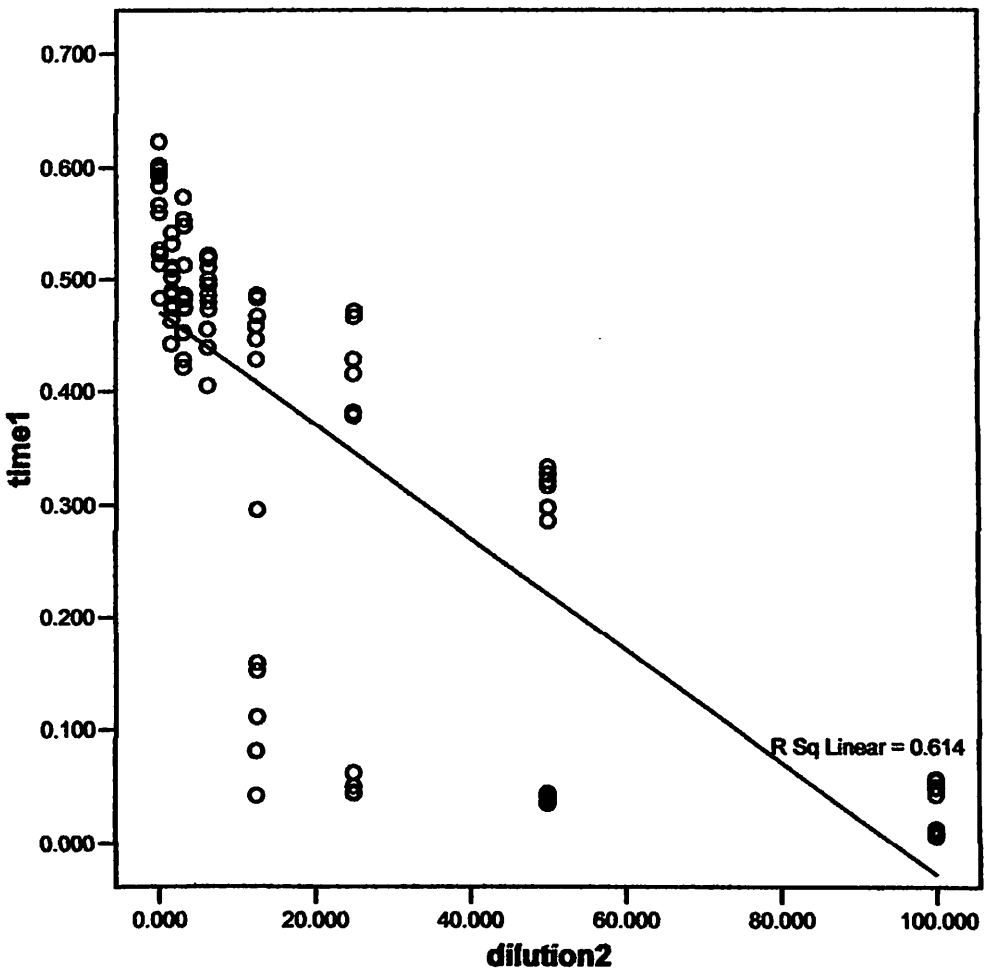

Figure 8: Correlation between dilution and viability at 24 hours of incubation

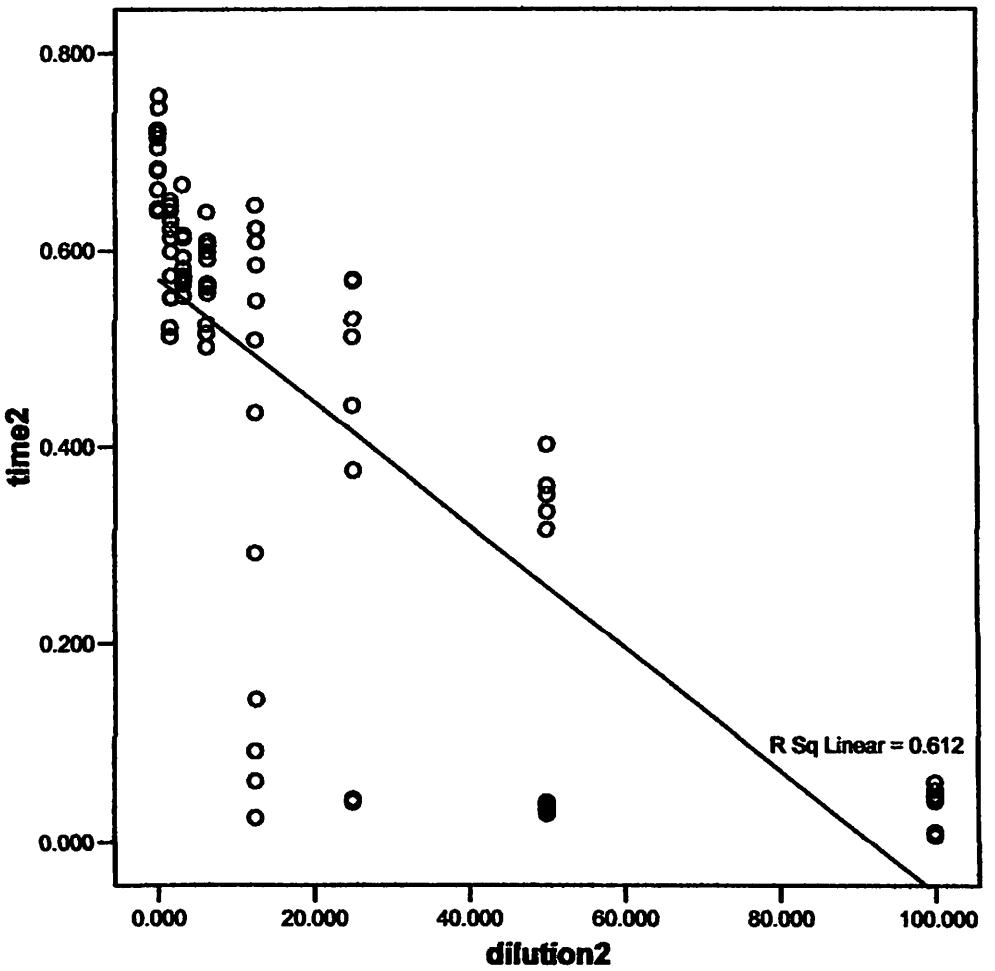

Figure 9: Correlation between dilution and viability at 48 hours of incubation

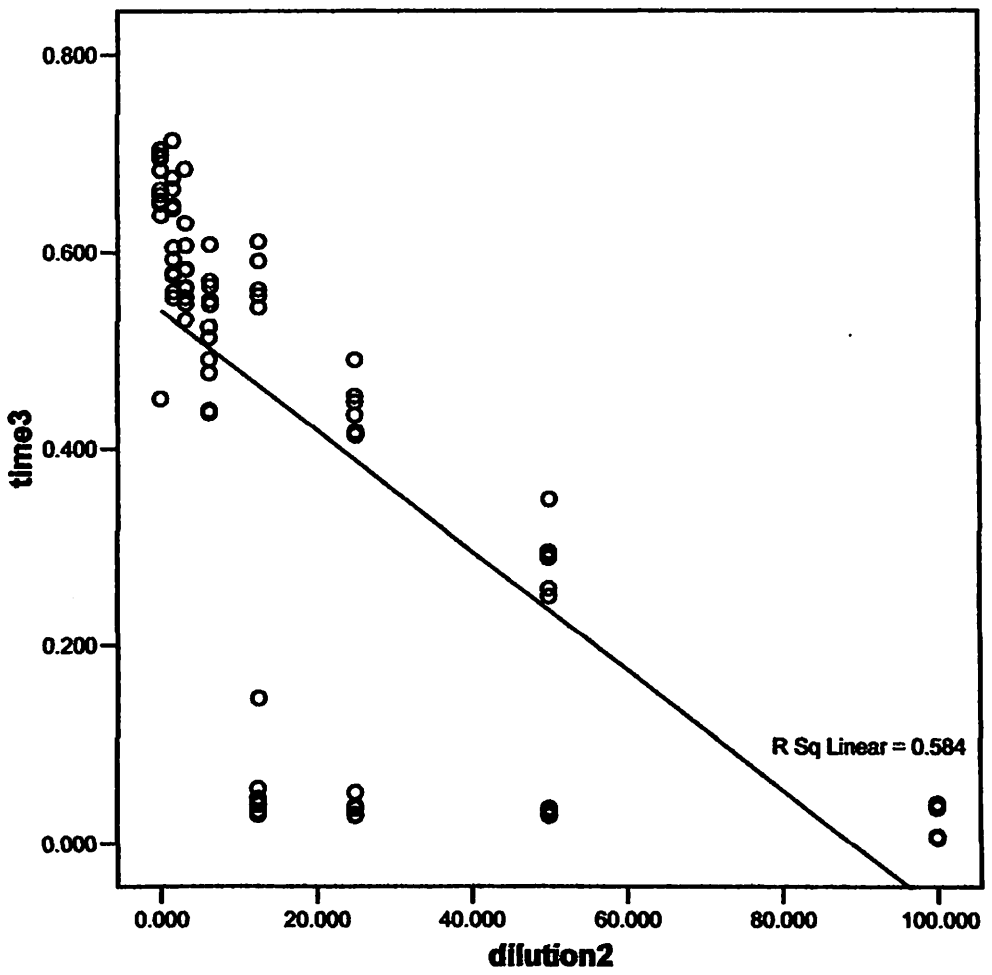

Figure 10: Correlation between dilution and viability at 72 hours of incubation

From these 3 graphs **[Figure 8, Figure 9, Figure10]**, we can conclude that there is an association between dilution and viability of fibroblasts cells. Dilution is directly proportional to cells viability. When the test compound is diluted, the concentration will decrease. Decreased concentration allows high cells viability.

6.0 DISCUSSION

Chitosan is the N-deacetylated product of chitin. As one of the most abundant naturally occurring compounds, chitosan has attracted people's attention on the account of its unique physiochemical characteristics and its bioactivities. However, because of its poor solubility in water, the use of chitosan is limited in many fields. Hence, many chitosan derivatives have been synthesized in order to improve its aqueous solubility. As a wound healing agent, a cytotoxicity testing should be done on chitosan to ensure that it is not harmful for human bodies.

Tissue culture for establishing and maintaining cells derived from skin, such as dermal fibroblasts, is a well established technique to evaluate cytotoxicity effects of biomaterials. The advantage of studying cells from skin *in vitro* is that it allows investigators to explore better the mechanisms of disease and understand how cells respond to certain stimulatory or inhibitory signals (Je *et al*, 2006). Cell cultures have advantages over animal experimentation since they afford highly defined culture conditions, thereby avoiding the complex homeostatic mechanisms that occur in vivo.

Dermal fibroblasts are the main cell type involved in matrix production for the wound healing process, and therefore were chosen for this cytotoxicity study. They are also can be cultured easily. Besides, fibroblasts have variable consumption related to its stage population's growth cycle. This property is important in choosing appropriate biochemical indicator of cytotoxicity since it should be central to cellular metabolism (Corrine *et al*, 1998).

Cytotoxicity testing is a rapid, standardized, sensitive, and inexpensive mean to determine whether a material contains significant quantities of biologically harmful extractables. The high sensitivity of the tests is due to the isolation of the test cells in cultures and the absence of the protective mechanisms that assist cells within the body.

Testing for cytotoxicity is a good first step toward ensuring the biocompatibility of a medical device. A negative result indicates that a material is free of harmful extractables or has an insufficient quantity of them to cause acute effects under exaggerated conditions with isolated cells. On the other hand, a positive cytotoxicity test result can be taken as an early warning sign that a material contains one or more extractable substances that could be of clinical importance. In such cases, further investigation is required to determine the utility of the material (Wally and Arscott, 1998).

Generally, the determination of cell viability is an assay to evaluate the in vitro cytotoxicity of biomaterials. The predictive value of in vitro cytotoxicity tests is based on the concept that toxic chemicals affect the basic functions of cells. Such functions are common to all cells, and hence the toxicity can be measured by assessing cellular damage. MTT assay is the method commonly used for this purpose.

The chitosan solutions were suspended in culture medium to evaluate the cytotoxicity of the solution. In the MTT method, the concentration of formazan generation is directly proportional to the cells viability. This is because the reduction of MTT into formazan reflects the action of mitochondrial enzymes in metabolically active cells. The purple formazan reaction product is largely impermeable to cell membranes, thus resulting in its accumulation within healthy cells. The concentration of formazan was measured

spectrophotometrically (Chen XG *et al*, 2006). Comparison of results between control and test culture provide an indication of the cytotoxic effect of the test compounds.

The results showed that there is significant difference ($P<0.001$) between two concentrations of chitosan used. Oligo chito 5% is more toxic compared to Oligo chito 1%. In test compounds concentration as low as 1.56%, a negligible cytotoxicity was observed whereas already at a concentration 12.5% a significant cytotoxicity occurred. Moreover, there is an association between dilution and viability of fibroblasts. Dilution is directly proportional to the cells viability. When the test compound is diluted, the concentration will decrease. Decreased concentrations allow high cells viability.

This is supported by the study done by Mao (2004) who suggested that cytotoxicity is concentration dependent. In a study performed by Guggi *et al* (2004), the results of MTT assay revealed a concentration dependent decrease of mitochondrial activity for all the tested compounds. An explanation for this effect might be given by the various concentration dependent mechanisms of cell resistance and/or sensitivity.

Besides, the important finding of this study is the effects of different incubation time on cells viability. The results showed that there was a significant difference ($P<0.001$) between 24 hours and 48 hours, and 24 hours and 72 hours respectively. The cells viability was slightly low at 24 hours, and then it increased at 48 hours and decreased again at 72 hours of incubation. The results of chitosan cytotoxicity showed that it was less cytotoxic to the cell cultures tested after 48 h, while the cytotoxic effect increased after 72-h exposure.

In this aspect, there are various findings obtained by researchers. The study performed by Yang *et al* (2001) had suggested that 72 hour exposure is more sensitive and

24 hour exposure in quiescent cells is more likely to display more resistance to toxicity. While Mao *et al* (2005) found that complexation of trimethyl chitosan (TMC) with insulin decreased the cytotoxicity of TMC after 24 hour of incubation with cells. The study suggested that electrostatic interaction between TMC and insulin decreased the interaction of the positively charged amino groups of TMC with the anionic components of the glycoproteins on the cell membrane, leading to higher cell viability.

7.0 CONCLUSION

There are several important findings obtained from this study. The effects of 2 different concentrations of chitosan derivatives had been determined. Oligo chito 5% was more toxic to fibroblast viability even at low concentration. This indicates that cytotoxicity is concentration dependent. There was also correlation between dilution and cells viability. Besides, incubation period also influenced the cells viability.

A more elaborative study with more specific method is required in order to evaluate the cytotoxicity effects of chitosan derivatives on primary cultured fibroblasts. More detailed study on chitosan should be done especially in Malaysia. This will increase the knowledge about this chitin deacetylation product and increase the possibility to manipulate it in biomedical fields. Hopefully, chitosan will be widely used in Malaysia as wound healing accelerator.

8.0 LIMITATIONS

In every study, there will be some limitations. The major limitation of this study is the cost. This study is very costly as it needs a lot of instruments such as CO_2 incubator liquid nitrogen storage and ELISA reader.

Besides, cell culture is time consuming and technically demanding. The confluent monolayer can be obtained only after few days before proceeding to the subsequent steps. Every steps need to be done carefully to get satisfied result. Therefore, high patience is needed in this study.

Other limitations of this study include the high chance of the cell culture contamination. High concentration of test compound also caused precipitation in the cultured cells.

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APPENDICES

Figure 11: Laminar air flow (Holten Lamin Air HB2448)

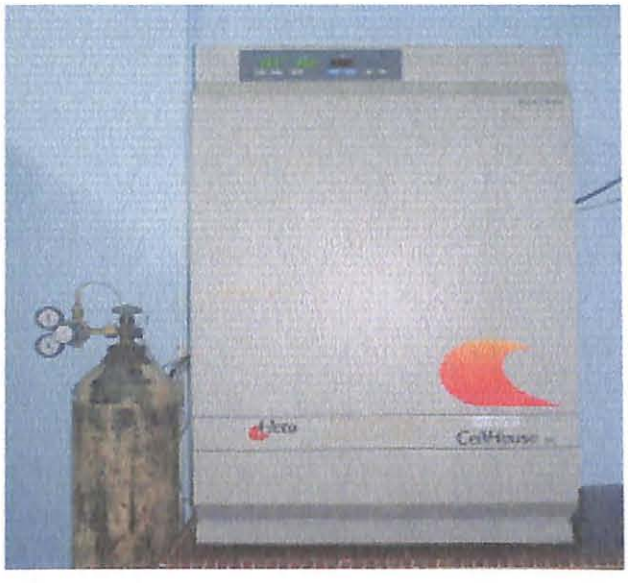

Figure 12: CO2 incubator (Heto Cell House 150)

Figure 13: Incubator (LEEC Compact Incubator)

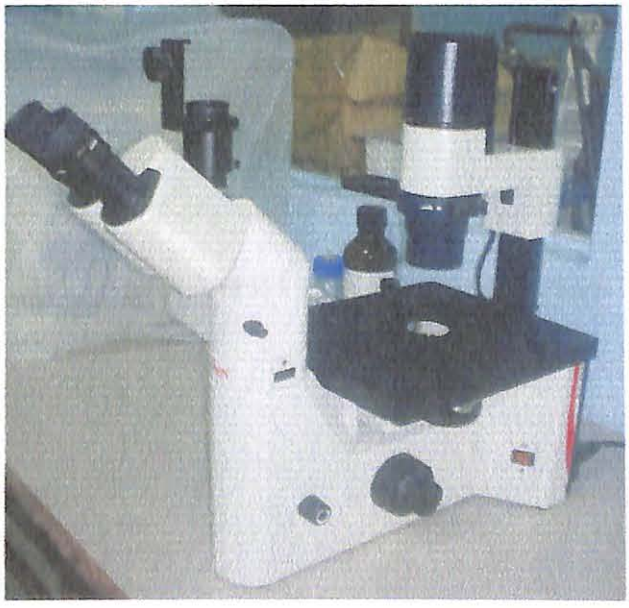

Figure 14: Inverted microscope (LEICA DMIL)

Figure 15: Centrifuge (UNIVERSAL 16R)

Figure 16: Liquid nitrogen storage units

Figure 17: Oligo Chito 5% (left) and Oligo Chito 1%