SHORT TERM EFFECT OF HUMAN LYMPHOCYTES VIABILITY UPON EXPOSURE OF PRODUCTS WITH PARABENS

by

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LIST OF ABBREVIATIONS

ADI	Acceptable Dose Intake
AR	Androgen receptor
ASEAN	Association of Southeast Asian Nations
bw	Body weight
^{o}C	Degree celsius
CO_2	Carbon dioxide
CYN	Cylindrospermopsin
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic acid
DPS	Director of Pharmaceutical Services
DPX	Di-n-butyl phthalate in Xylene
DOX	Doxycycline
ER	Estrogen receptor
FAO/WHO	Food and Agriculture Organization/World Health Organization
g	Gram
GMP	Good manufacturing practice
HIV	Human immunodeficiency virus
JECFA	Joint of Expert Committee on Food Additive
kg	Kilogram
LD_{50}	Lethal dose for acute toxicity
mg	Milligram
ml	Millilitre
mm	Millimetre

mm^3	Cubic millimetre
MTT	3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide
nm	Nanometre
NO	Nitric oxide
NOAEL	No observed adverse effect level
NK	Natural killer
NPCB	National Pharmaceutical Control Bureau
PBMCs	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PIF	Product information file
RBC	Red blood cell
ROS	Reactive oxygen species
SED	Safety and Environmental Detriment
SEM	Standard error of mean
TiO_2	Titanium dioxide
UV	Ultraviolet
ZnO	Zinc oxide
µg/ml	microgram/millilitre
µl	Microliter
µm	Micrometre

KESAN JANGKA PENDEK PERUBAHAN SEL LIMFOSIT MANUSIA TERHADAP PENDEDAHAN PRODUK YANG MENGANDUNGI PARABEN

ABSTRAK

Produk penjagaan diri digunakan untuk kebersihan diri dan mempunyai sebatian kelas kimia yang meluas. Semenjak tahun 1930-an, paraben telah digunakan dalam kosmetik sebagai bahan pengawet. Walau bagaimanapun, paraben telah menjadi kontroversi kerana terdapat laporan mengenai aktiviti estrogenik. Tujuan kajian ini adalah untuk menentukan kesan perubahan limfosit dan morfologi terhadap produk yang mengandungi paraben pada kepekatan yang berbeza dengan pendedahan jangka masa yang pendek. Sampel darah telah diambil daripada tujuh subjek yang sihat dan limfosit diasingkan menggunakan *EasySep Direct Human Total Lymphocyte Isolation Kit.* Asai MTT telah digunakan untuk menentukan kesan peningkatan limfosit. Smear darah dan *Wright`s stain* digunakan untuk menentukan morfologi limfosit. Hasil kajian yang signifikan ($p < 0.001$) dan sederhana positif ($r = 0,351$) terhadap hubungan antara kepekatan paraben dengan peratusan perubahan limfosit. Perubahan sel yang dicatatkan dalam methyl paraben adalah sehingga 120%. Peningkatan peratusan limfosit berlaku disebabkan oleh antigen yang berada pada reseptor kemudian mengaktifkan limfosit. Hal ini menunjukkan bahawa produk yang mengandungi paraben mempunyai kesan ketoksikan kepada limfosit selepas dikenakan pendedahan dalam jangka masa yang pendek.

SHORT TERM EFFECT OF HUMAN LYMPHOCYTES CELL VIABILITY UPON EXPOSURE OF PRODUCTS WITH PARABEN

ABSTRACT

Personal care products are commonly used in personal hygiene and constitutes of broad classes of chemical compounds. Since 1930s, paraben have been used in cosmetic as preservatives. However, paraben effect has become controversial due to report on estrogenic activity. The aims of this study are to determine the effect of lymphocyte viability and morphological after being administered products with paraben at different concentrations for short term exposure. Blood sample were drew from seven healthy subjects. Lymphocytes were isolated using EasySep Direct Human Total Lymphocyte Isolation Kit. The MTT assay was used to determine lymphocytes viability. Blood smear and stained with Wright`s stain were used to determine the morphological changes of the lymphocytes. The result showed significant ($p < 0.001$) and moderate positive ($r = 0.351$) correlation between paraben concentration with the percentage of lymphocyte viability. The cell viability recorded for methyl paraben was up to 120%. The lymphocytes rapidly proliferate due to antigen that bind to the receptors and activates the lymphocytes. This showed that products with paraben have toxicity effects towards lymphocytes even after being exposed in short period of time.

CHAPTER 1

INTRODUCTION

1.1 Study background

Personal care products are used in personal hygiene and becoming important not only in women but also to men. It constitutes of broad classes of chemical compounds (Basaglia *et al.*, 2011). According to Aarflot (2013), cosmetics are products used to be rubbed, pour, sprinkled or sprayed on, introduced into, or otherwise applied to human body for cleansing, beautifying, promoting attractiveness, or altering the appearance. Skin moisturizers, perfumes, lipsticks, fingernail polishes, eye and facial makeup preparations, cleansing shampoos, permanent waves, hair colors, and deodorants, as well as any substance purposely use as a component of a cosmetic product are the products included in the definition of cosmetic.

Cosmetic Technical Working Group, (2009) stated that personal care products and cosmetics must comply with government safety regulation. In Malaysia, cosmetic products are regulated under the Control of Drugs and Cosmetic Regulations 1984. The National Pharmaceutical Control Bureau (NPCB) responsible to review and register the cosmetic products. Starting from 1st January 2008, notification procedures are used in conformance with the harmonization of cosmetic regulations in the ASEAN region. Under the Control of Drugs and Cosmetics (amendment) Regulations 2007, the company that responsible for placing a cosmetic product in the local market must notify the Director of Pharmaceutical Services (DPS) through National Pharmaceutical Control Bureau (NPCB) prior to product

manufacture or importation (Cosmetic Technical Working Group, 2009). Good Manufacturing Practice (GMP) is a standard that should be followed by manufacturers of registered pharmaceutical or traditional products and notified cosmetics to ensure that the products manufactured are safe, beneficial and have quality (Cosmetic Technical Working Group, 2009).

Some ingredients in cosmetics and personal care products can cause short term and long term effect. Allergic reactions or sensitivity in certain individuals is a short term effect for consumers while long term effect may cause cancer or other serious illness. Preservatives are chemicals with antimicrobial activity to prolong products` shelf life and protect the consumer from potential infection (Leena, 2011). According to Leena (2011), sorbic acid, benzoic acid, benzyl alcohol and hydroxybenzoates (parabens) are some of the common preservatives used in cosmetic industry. Paraben are effective antibacterial or fungicides for microorganisms in a wide variety of cosmetics. However, paraben has become controversial due to their reported estrogenic activity (Wang & Zhou, 2013). Figure 1.1 shows the structure of paraben which R indicates an alkyl group such as methyl, ethyl, propyl or butyl.

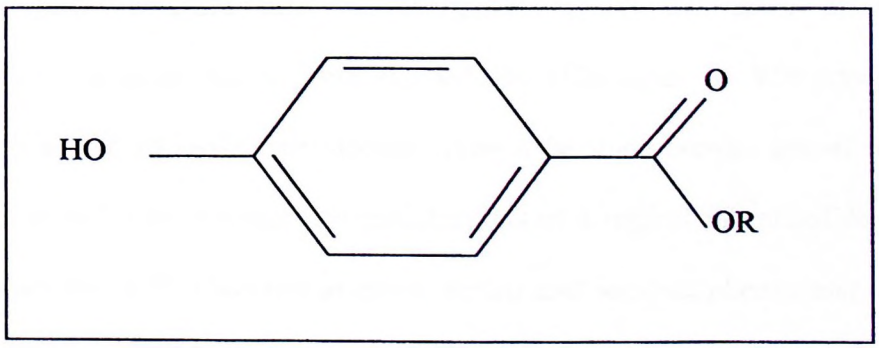


Figure 1.1: Paraben Structure.

Source: Aarflot, (2013)

1.2 Problem statement

Consumers have a freedom to choose what products to buy without awareness of the ingredients (Dodson *et al.*, 2012). Products labeling is one of the requirement from (PIF) audited on 2011. PIF containing information about the products including the product description, the Cosmetic Product Safety Report, details of methods of manufacture in accordance with good manufacturing practice and, where justified, proof of the effect claimed (ASEAN Cosmetic Directive, 2007). This is a legal requirement and the files are open to inspection by the competent authorities.

Since 1930s, paraben has been used in cosmetic and pharmaceutical products (Kirchhof & Gannes, 2013). Paraben are used over 22 000 cosmetics (Andersen, 2008). The average of cosmetics personal paraben exposure is estimated to be 76 mg/day while personal care products accounting for 50 mg/day and 25 mg/day from pharmaceutical products (Cashman & Warshaw, 2005). More controversial appear when scientific evidence shown that parabens can accumulate in the body and their effects can build up over time.

Paraben can be allergenic and this sensitization commonly manifests as an eczematous rash, urticaria and bronchospasm which the rates of reported sensitization to paraben range from 0.5%-3.5% (Cashman & Warshaw, 2005). Paraben also effect on male reproductive system because human sperm were not viable when exposed to paraben at concentrations of 1 mg/ml (Kirchhof & Gannes, 2013). Paraben has been detected in urine, serum and seminal plasma and there are also detected in breast tissue from patients with breast cancer (Darbre *et al.*, 2004). Table 1.1 shows the percentage of adverse effects in proportion to the population using the products on daily/weekly basis.

Table 1.1: Percentage adverse effect in proportion to the population using the products.

Product	Female (%)	Male (%)
Deodorant	38	39
Eye make-up	35	
Moisturizer	32	20
Depilatory agent	28	
Hair shampoo	21	18
Hair spray/gel	17	17
Shaving form		9
Sunscreens	9	3

Source: Norwegian Scientific Committee, (2009)

1.3 Research objective

1.3.1 General objective

The general objective of this study is to evaluate the short term effect of human lymphocytes viability upon exposure with the products containing paraben.

1.3.2 Specific objectives

1. To determine the effect of products containing paraben at different concentration on lymphocyte viability.
2. To determine the effect of products containing paraben on lymphocyte morphology in short term exposure.

1.4 Hypothesis

Null hypothesis

There is no significant relationship between the lymphocytes viability with the concentration level of products with paraben in short term exposure.

Alternative hypothesis

There is significant relationship between the lymphocytes viability with the concentration level of products with paraben in short term exposure.

Null hypothesis

There is no morphological change after short term exposure of products with paraben at acceptable dose intake (ADI).

Alternative hypothesis

There is a morphological change after short term exposure of products with paraben at acceptable dose intake (ADI).

1.5 Significance of study

Consumer's enables to know the short term of cytotoxic effect of products with paraben towards human lymphocytes and ensure that products are safe in daily live. Increased or decreased human lymphocytes count that was isolated from healthy human blood sample will indicate the degree of cytotoxic caused by products that contain paraben. The different in concentration levels of products will give different level of cell viability.

The high concentration level of paraben that used in products for a continuous exposure will give effects to the human health. Paraben have the potential to cause harmful effect either locally or subsequently elsewhere in the body. Furthermore, this study provides scientific evidence regarding products toxicity and safety.

CHAPTER 2

LITERATURE REVIEW

2.1 Dermal absorption

Purpose of dermal absorption is to transport the chemicals from the outer surface of the skin into the body. According to Environmental Health Criteria 235 (2006), the rate of dermal absorption depends largely on the outer layer of the skin called the stratum corneum. When in contact with the skin, the compound will penetrates into dead stratum corneum and subsequently reach the epidermis, dermis and vascular network (Sanco, 2004) (Figure 2.1). Sanco, (2004), stated that the stratum corneum provides the greatest barrier function against hydrophilic compound, whereas the epidermis is most resistant to highly lipophilic compound.

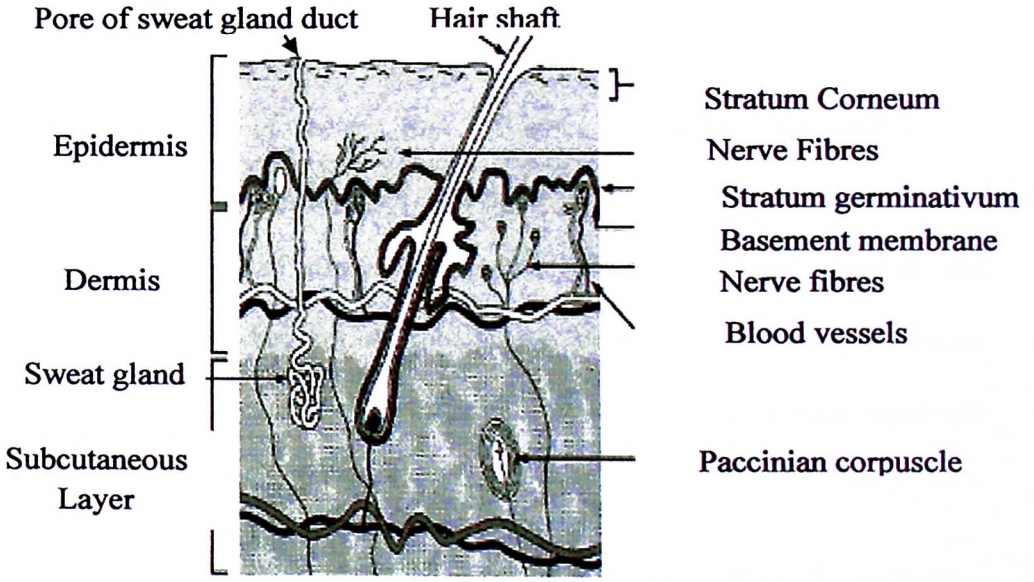


Figure 2.1: A schematic diagram of the skin structure

Sources: Environmental Health Criteria 235, (2006)

There is an inverse relationship between concentration of dose and percentage of absorption. At low concentrations of dose, the percentage absorption is lower than the percentage absorption at high concentrations.

Chemicals used commonly have potential result in systemic toxicity if penetrate through the skin. Assessment for systemic toxicity has traditionally been based on the median lethal dose (LD_{50}) value. LD_{50} is an estimate of the dose of a test substance that kills 50% of the test animals. Substances that have systemic toxic effects must be absorbed through the body and distributed by the circulation to sites in the body where it exerts toxic effects. These chemicals enter the blood stream and affect any organ in the body. Suriati *et al.* (2005) stated that parabens used in cosmetic products can cause an adverse effect because their adsorption in the bloodstream by-passes the gastrointestinal tract and might be broken down into toxic component.

There are a number of factors that influence the dermal absorption of a substance for e.g. skin integrity whether damaged or intact, location of exposure, thickness and water content of stratum corneum, skin temperature, physical and chemical properties of the hazardous substances, concentration of a chemical, duration of exposure and the surface area of skin exposed to a hazardous substance (Environmental Health Criteria 235, 2006).

Dermal absorption occurs *via* diffusion, the process whereby molecules spread from areas of high concentration to areas of low concentration. Three mechanisms by which chemicals diffuse into the dermal have been proposed. First, intercellular absorption (Figure 2.2 a) which is the stratum corneum consists of cells known as corneocytes and the spaces between the corneocytes are filled with

substances such as fats, oils, or waxes known as lipids and some chemicals can penetrate through these lipid-filled intercellular spaces through diffusion. Second mechanism is the transcellular absorption (Figure 2.2 b) which molecules diffuse directly through the corneocytes and the third mechanism is the appendageal absorption (Figure 2.2 c) that uses hair follicles and glands to diffuse into the dermal (Environmental Health Criteria 235, 2006).

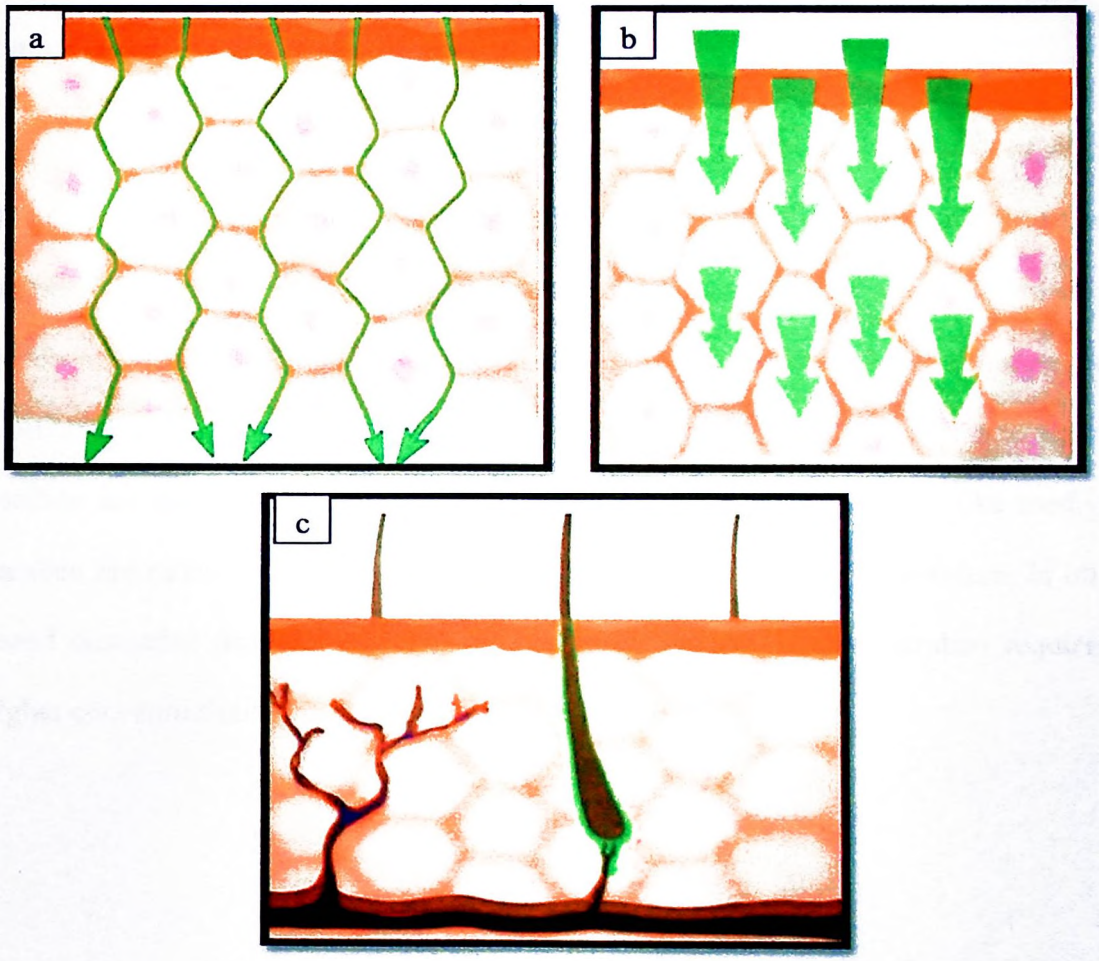


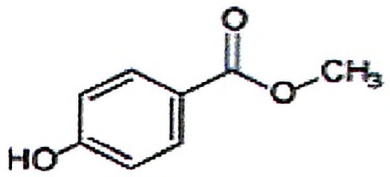
Figure 2.2: Dermal absorption (a) Intercellular Absorption (b) Transcellular Absorption (c) Appendageal Absorption (hair follicles, glands)

Sources: Environmental Health Criteria 235, (2006)

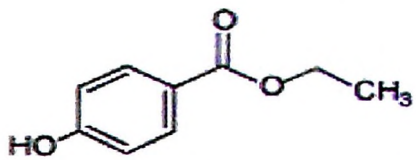
2.2 Paraben

Paraben are esters of p-hydroxybenzoic acid and commonly used as preservatives in cosmetic, pharmaceutical and industrial products due to their effectiveness, inexpensive, colorless, odorless, neutral pHs and wide spectrum of antimicrobial activity (Wang & Zhou, 2013). According to Frederiksen *et al.* (2011), methyl paraben, ethyl paraben, propyl paraben, and butyl paraben are commercially important parabens and as preservatives.

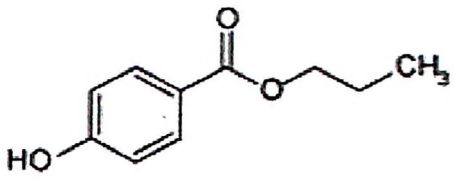
Penetration of paraben through the skin increases with the increasing of alkyl chain. As alkyl chain length increase, water solubility decrease, oil solubility and antimicrobial activity also increase (Cashman & Warshaw, 2005). Figure 2.3 shows the chemical structure of paraben with different alkyl chain length. All paraben are soluble in oil-phase, but those with longer chain paraben like propyl and butyl paraben are more soluble in oil, and those with shorter chain paraben like methyl paraben are more soluble in water (Cashman & Warshaw, 2005). Therefore, in oil-based cosmetics formulations such as cream and lotion, methyl paraben requires higher concentration to maintain effectiveness.



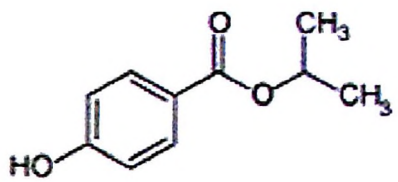
Methyl paraben



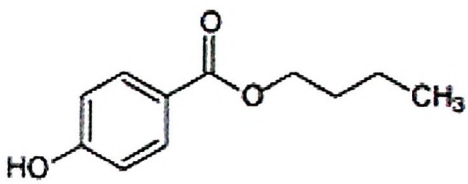
Ethyl paraben



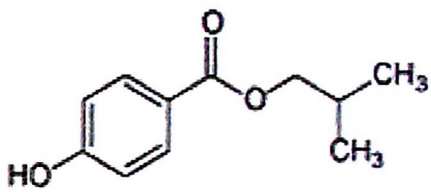
n-Propyl paraben



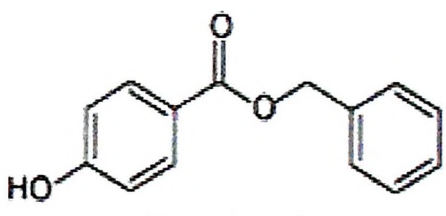
Isopropyl paraben



n-Butyl paraben



Isobutyl paraben



Benzyl paraben

Figure 2.3: The chemical structure of paraben

Source: Aarflot, (2013)

According to Aarflot, (2013) paraben has caused concern due to estrogenic and antiandrogen properties. Estrogen is female sex hormone which is important in menstrual and estrous reproductive cycle. Androgens present in males and females that play role in male traits and reproductive activity (Sarrel, 2011). Many chemicals interfere with normal, hormonally regulated biological processes to adversely affect the development and or reproductive function in animals and humans by mimicking

or inhibiting endogenous hormone action, modulating the production of endogenous hormones, or altering hormone receptor populations (Okubo *et al.*, 2001).

Paraben has the capability to bind with human estrogen receptor (ER) and then regulate gene expression and cell growth in estrogen responsive cells through ER mediated mechanism (Figure 2.4). Paraben can act as ER agonists and androgen receptor (AR) antagonists. Paraben also disturb lysosomal and mitochondrial mechanisms, cause to DNA damage and amplify UVB- induced damage through production of reactive oxygen species (ROS) and nitric oxide (NO). Paraben may have effect on health status even at lower concentrations due to capability binding to ERs (Aarflot, 2013).

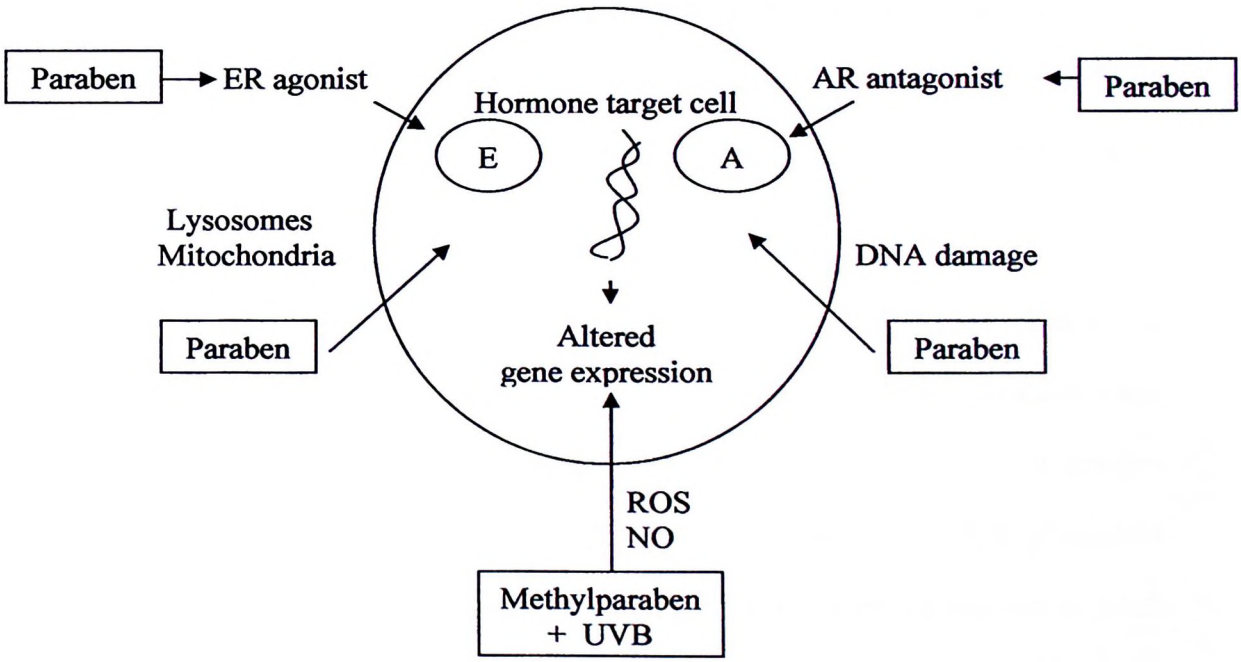


Figure 2.4: Paraben molecular pathways in cells.

Source: Aarflot, (2013)

2.3 Paraben use and regulations

According to Kirchhof & Gannes (2013) concentrations of methyl paraben up to 1.0% were found in personal care products tested with lipstick containing the highest concentration ranging from 0.15% to 1.0%. Concentrations of paraben used in food products up to 0.1% (Kirchhof & Gannes, 2013). Suriati *et al.* (2005) stated that the maximum concentration level of paraben for cosmetic is 4 g per kg of cosmetic product (0.4%) for single ester and 8 g of parabens per kg of cosmetic product (0.8%) for mixture ester. While maximum concentration level of paraben in food and pharmaceutical products are 0.1% and 0.2% (Suriati et al., 2005).

According to Yazar et al. (2010), from 204 cosmetic products bought from stores in Stockholm, Sweden, 44% of the products tested contained parabens. Methyl paraben was detected in 41% of the products, propyl paraben was detected in 25% of the products, ethyl paraben in 22%, butyl paraben in 14% and isobutyl paraben in 13% of the products (Yazar et al., 2010).

Janjua *et al.* (2008) study about the contents of different types parabens in cosmetic products. Through a survey from 215 cosmetic products, parabens were detected in 77% of rinse-off products and 99% of leave-on products. All product groups were found to contain methyl paraben which was present in 98% of paraben containing cosmetics. Rinse-off products is a product that must be remove or wash after using it such as shampoo and face cleanser, while leave-on products like body lotion, face cream and lip-care products.

2.4 Paraben`s safety

In 1974, the Joint of Food and Agriculture Organization/World Health Organization (FAO/WHO) Expert Committee on Food Additive (JECFA) had established an

acceptable daily intake (ADI) for methyl paraben is 0-10 mg/kg body weight/day. LD_{50} is the amount of a toxic agent (as a poison, virus, or radiation) that is sufficient to kill 50% of a population of animals usually within a certain time. Paraben lethal dose for acute toxicity (LD_{50}) is 2-8 g/kg (Anton *et al.*, 2011). No observed adverse effect level (NOAEL) is the highest tested dose of a substance that has been reported to have no harmful (adverse) health effects on people or animals. NOAEL value of 1000 mg/kg bw/day was accepted for all esters of parabens (Scientific Committee on Consmetic Products, 2005). The cumulative value of 17.4g/day is used for the calculation of Safety and Environmental Detriment (SED) (Scientific Committee on Consumer Safety, 2010).

2.5 Lymphocyte

Blood is preferable utilized for investigation of metabolism due to its direct potential effects (Aarflot, 2013). Lymphocytes (Figures 2.5 and 2.6) are the smallest white cells, measuring 6-15 μm in diameter that have round, densely staining, acentric nuclei, very little cytoplasm that may contain a few lysosome like granules and once activated, the amount of cytoplasm increases.

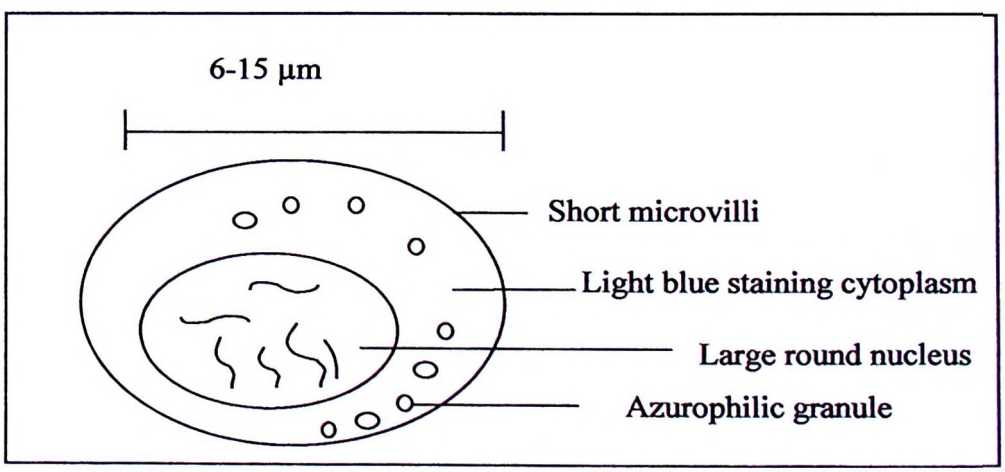


Figure 2.5: Lymphocyte structure

Source: Gareth, (2008)

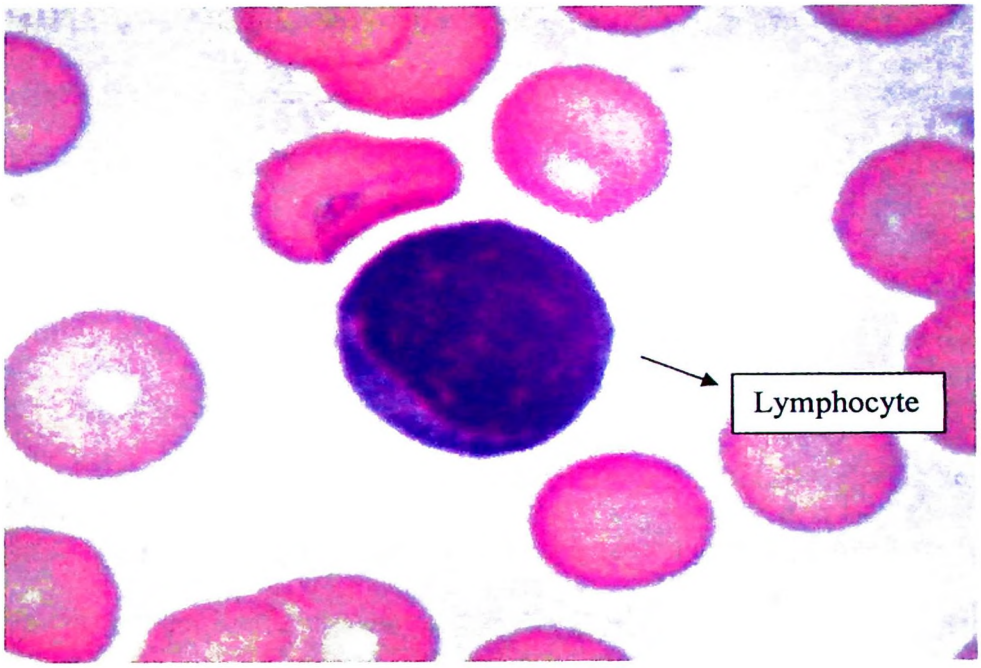


Figure 2.6: Normal lymphocytes are quit small (on average 9 μm diameter). Contain very little cytoplasm and occasional azurophilic granules

Source: Gargani, (2012)

Lymphocytes circulate between tissue, lymphatics and the blood which produce antibodies and kill foreign or virally altered cells. The lifespan of lymphocytes varies depending on their interaction with antigens.

There are three types of lymphocytes which are B cells, T cells and natural killer (NK) cells (Gareth, 2008). B cells and NK cells tend to be larger than T cells. According to Gargani (2012), T helper cells release a chemical, called a cytokine when activated by an antigen. These chemicals then stimulate B lymphocytes to begin immune response. B cell produces proteins that fight antigens, called antibodies when activated.

Lymphocytes are the second most frequently occurring leukocytes in the blood which normal adults have an absolute number of circulating small lymphocytes of 1.5 to 4.0 × 10^9/liter, or 35% of the total leukocytes with a range of

20 to 40% (Bell & Sallah, 2005). The typical small lymphocyte as observed with Romanovsky polychromatic stains such as Giemsa or Wright has an ovoid or kidney-shaped nucleus that stains purple, has densely packed nuclear chromatin and occupies about 90% of the cell area and have a narrow rim of blue cytoplasm.

According to Bain (2003), abnormalities of lymphocytes are rare and mostly caused by viral infections. Increased numbers of lymphocytes showing a variable degree of morphological abnormality are indicative of a neoplastic process, either a lymphoid leukaemia or a lymphoma. Large numbers of atypical lymphocytes (Figure 2.7), similar to those seen in infectious mononucleosis, can also occur in infection by cytomegalovirus, hepatitis A virus, and adenovirus and during the parasitic infection toxoplasmosis (Bain, 2003).

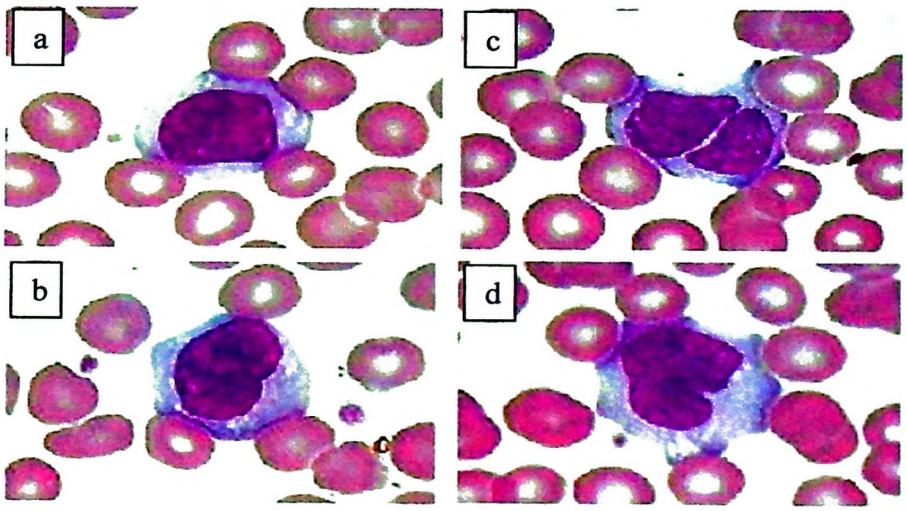


Figure 2.7: Atypical lymphocytes which can present with an enlarged (a) either slightly (b) to deeply intended (c) nucleus (d) fully binucleated

Lymphocytosis occurs in acute viral illnesses such as infectious mononucleosis and certain chronic infections such as tuberculosis, mumps and hairy cell leukemia (Bell & Sallah, 2005). Mature small lymphocytes are increased in blood of chronic lymphocytic leukemia in adults.

Decrease numbers of atypical lymphocytes are seen in many other viral infections (including acute infection by the human immunodeficiency virus—HIV) and in some bacterial, rickettsia and infections protozoa. Other reactive changes, in addition to those typical of infectious mononucleosis, occur in lymphocytes both during infection and during exposure to other antigenic stimuli (Gargani, 2012).

2.6 Lymphocytes viability

According to Suman & Jamil (2006), all anti-cancer drug that have been used in their research may be toxic to normal cells by inhibiting the viability of the cells. Healthy and unhealthy blood samples that were collected in heparinized syringe have been used to extract lymphocyte by using Ficoll density gradient technique. MTT assay have been used to determine the lethal concentration of the drugs. The lymphocytes were treated with different concentration of each drugs to determine the comparative toxicity in short term lymphocyte culture for two hours and carried out in triplicates and the averages level recorded. As a result of the tests, the different dosage levels of drugs can produce the toxicity which the lymphocytes viability had decreased after concentration drug used increased. MTT assays can be used to identify the best dosage levels that affected the lymphocyte percentage viability.

Das et al. (2006) also evaluated the toxic effects of profenofos in the soil extract on lymphocytes isolated from the fresh blood of clinically healthy humans. This research used Ficoll-density gradient centrifugation to separate lymphocyte under sterile conditions and used Neubauer`s chamber to count the cells. The cytotoxic effects of the profenofos pesticide were determined by the loss of membrane integrity by Trypan blue dye exclusion method. Through these experiments, the final volume of lymphocytes was adjusted to achieve a cell density

of $2x10^6$ cells/ml and then treated with varying concentrations of profenofos. After two hours of incubation, the viability of treated and untreated samples was determined using 0.4% Trypan blue solution. This experiment indicated a clear dose-dependent cytotoxic effect of the pesticide on lymphocyte which the percent viability of the cells decreased with higher concentration of the pesticide.

Krishnamurthi et al. (2006) stated that DNA is one of the most critical cellular targets for hazardous chemicals and wastes. In their study, human venous blood was collected from healthy donors using a plastic syringe containing sufficient heparin and the viability of leukocyte 98% determined by trypan blue exclusion assay. This study also evaluates the cytotoxicity of the soil extract on leukocytes. $1x10^6$ human polymorphonuclear leukocytes were incubated with different concentrations of the soil extract at different times (1h, 2hrs, 3hrs, 4hrs, 5hrs and 6 hrs). Significant cytotoxicity was not observed after one hour exposure to the doses, but significant cell death was seen when the cells have incubated with 100 and 200 µl doses at 4^{th} hours onward and significant cytotoxicity was observed from the 5^{th} hour onward after treated with 50 µl of the extract.

According to Bulla *et al.* (2014), the period of cell culture can affect the cell viability. The analysis of research showed significance difference of culture viability and banding resolution between times. Resolution is fundamental for identification of chromosomal alterations, in which higher resolution may detect smaller alterations. A difference between zero and 48 hours was observed and the mean value of the bands was higher at times zero and 24 hours while at the 48^{th} hours after blood collection, significant reduction of band resolution was observed. These data highlight the importance of the biological material quality, as viability is lower when the culture is grown after 24 hours.

Sekeroglu *et al.* (2012), stated that Doxycycline (DOX) is the antibiotic used in the treatment of many infections showed a toxic effect on lymphocyte culture after treated with DOX at three concentrations (2, 4 and 6 μg/ml) for 48 hours. Mitotic index values decreased linearly as DOX concentration increased. Lymphocyte cultures have over half their metaphases in second division at 48 hours and the best period of cultivation of human peripheral lymphocytes for chromosomal analyses in subjects exposed to mutagens and carcinogens is 44–48 hours.

In other study by Poddar *et al.* (2004) they demonstrated that the iron compound have a direct effect on human lymphocytes following mitogenic stimulation. Exposure to ferric chloride induced significantly high levels of chromosomal aberrations (%) and damaged cell (%) as compared to control. Mitotic index was reduced significantly as compared to control and the reduction was greater after exposure for 24 hours.

CHAPTER 3

METHODOLOGY

3.1 Study design

This study was approved by the Research Ethics Committee (Human) Universiti Sains Malaysia. Human sample bloods were taken from seven healthy subjects. The lymphocytes were extracted by EasySep Direct Human Total Lymphocyte Isolation Kit and tested by MTT assay for lymphocytes viability. Blood smear and stained with Wright`s stain used to determine the morphological changes of lymphocytes. The flow chart of research process as follows as shown in figure 3.1:

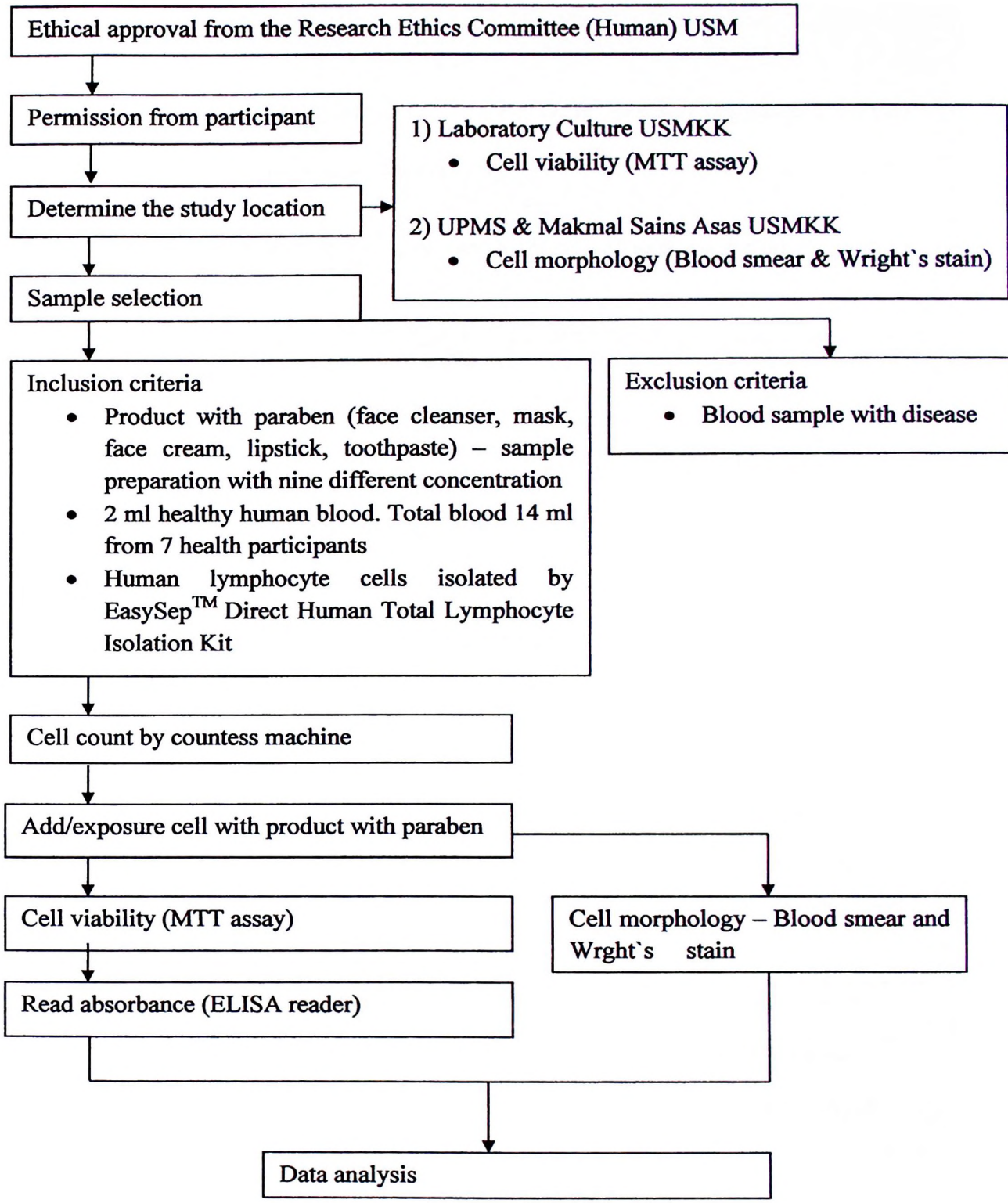


Figure 3.1: Flow chart of research process

3.2 Ethic approval

The study design was approved by the Research Ethics Committee (Human) USM/JEPeM (14110473) (Appendix D).

3.3 List of chemicals, biological materials, instruments and apparatus

Table 3.1: List of chemicals

No	Chemicals	Company/sources
1	70% Ethanol	HmbG
2	Trypan blue	aMResco
3	Phosphate-Buffered Saline (PBS)	Gibco
4	Methyl paraben	Sigma
5	Dimethyl Sulfoxide (DMSO)	Trevigen
6	Fetal Bovine Serum (FBS)	Gibco
7	Wright` stain	Lobachemie
8	MTT formazan	Sigma
9	Disterene (DPX)	Richard-Allan Scientific

Table 3.2: List of biological materials

No	Biological materials	Company/source
1	Blood	Healthy human
2	Lymphocytes	Isolated from blood

Table 3.3: List of instruments and apparatus

No	Instruments & apparatus	Company/source
1	Centrifuge tube	Partnar
2	Micro centrifuge tube	Monotaro
3	Pipette tips	Mc Farlan
4	Micro pipette	Gilson
5	Dry oven	Sheldon
6	Biosafety cabinet	Biological Safety Cabinet, NUAIRE-NU425-400E
7	Heparin tube	TUD
8	Easysep Direct Human Total Lymphocyte Isolation Kit	Easysep
9	96-well micro titer plate	Cellstar
10	Countess machine	Invitrogen
11	Countess slide	Invitrogen
12	Light microscope	Olympus BX41 camera
13	Incubator	Sheldon
14	ELISA reader	Chemoscience
15	Slide	Menzel-glaser
16	Differential countess	Gemmy Industrial Corp.

3.4 General method

3.4.1 Sterilization

Instruments were autoclaved by using Sun Clave at 15 psi (121°C) for 15 minutes. Then the instruments were oven dried for 1-2 days. In addition, UV light was used for sterilizing in biosafety cabinet.

3.4.2 Aseptic techniques

It is important to carry out the experiment aseptically in order to avoid contamination of the cell. Thus, laboratory work was carried out using a biosafety cabinet (Biological Safety Cabinet, NUAIRE-NU425-400E). Every item that comes into contact with the samples cells must be sterile. This includes direct contact (e.g., a pipette used to transfer cells) as well as indirect contact (e.g., flasks or container used to hold a sterile reagent prior to aliquot the solution into the sterile media). Single-use sterile disposable item such as centrifuge tube and pipette tips were used. Apart from that, 70% ethanol was used for a quick sterilization of surface and equipment.

3.4.3 Preparation of 3-(4, 5-dimethyl-2-thiazolyl)-2, 5-diphenyl-2H-tetrazolium bromide (MTT) solutions

MTT solutions were prepared by adding 30 mg MTT formazan in 6 ml PBS. Light sensitive MTT was wrapped with aluminum foil and kept at 4°C. Before use, the bottle is pre-warmed for 5 minutes at 37°C and gently mix by inverting the bottle (creating bubbles were avoided).