STUDY OF LOW VOLTAGE ELECTROPORATION TECHNIQUE ON HeLa CELL TREATED WITH NEOLAMARCKIA CADAMBA EXTRACTS

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ABSTRACT

Electroporation is a process of creating pores on cell by exposing the cell with electric field. This process increases the permeability of the cell. Neolamarckia Cadamba is a natural source that is widely used in the medical arena due to its pharmacological properties. Neolamarckia Cadamba known as *kelempayang* to Malaysian people. Its mainly planted in the states of Pahang, Perak, Sabah and Sarawak. Electrochemotherapy is a combination of the electroporation process and chemotherapeutic drugs. The Standard electric field parameters for Electrochemotherapy (SEFPECT) are 1000V/cm of pulse amplitude, 100µs of pulse duration, 8 numbers of pulses and 1Hz of frequency. Electrochemotherapy process causes transient burns to patient due to the intensity of pulse amplitude used, which is 1000V/cm. The use of chemotherapeutic various drugs gives side effect to the patient. To overcome the problem, this research suggests to lower the pulse amplitude and longer pulse duration plus combining natural extract of Neolamarckia Cadamba Leaves. In this study, the combination of electroporation process and natural extract is conducted in vitro for 48 hours. The cell used is cervical cancer cell (HeLa cell) and the natural extract from Neolamarckia Cadamba Leaves. The experiment was started to determine the best parameter of pulse amplitude and pulse duration that gives lower viability percentage of HeLa cell. The pulse amplitude started with a 200V/cm, 400V/cm, 600V/cm, 800V/cm and 1000V/cm and the pulse duration at 100µs, 500µs, 1ms, 2ms and 5ms and the result revealed that the best parameter at 600V/cm of pulse amplitude and 5ms of pulse duration. Then the IC_{50} value of Neolamarckia Cadamba Leaves extract were tested and found to be at 40µg/ml. The findings of these studies have shown positive respond on the combination of these two methods. This was proven by the reduction in the percentage of anti proliferation of HeLa cell where with electroporation 30.08% cell can survive and by combining electroporation and extact of Neolamarckia Cadamba leaves only 2.12% HeLa cell can survive.

ABSTRAK

Elektroporasi adalah proses mewujudkan liang-liang pada sel dengan mendedahkan sel dengan medan elektrik. Proses ini meningkatkan kebolehtelapan sel. Neolamarckia Cadamba adalah sumber semulajadi yang banyak digunakan di arena perubatan kerana sifat farmakologinya. *Neolamarckia Cadamba* dikenali sebagai kelempayang oleh rakyat Malaysia. Ia kebanyakannya ditanam di negeri Pahang, Perak, Sabah dan Sarawak. Elektrokimoterapi adalah gabungan dari proses elektroporasi dan ubat kemoterapi. Parameter bidang medan elektrik untuk Elektrokimoterapi (SEFPECT) adalah 1000V/cm nadi amplitud, 100μs nadi denyut, 8 nadi dan 1Hz frekuensi. Proses elektrokimoterapi menyebabkan kesan terbakar sementara kepada pesakit kerana intensiti nadi amplitud yang digunakan, iaitu 1000V/cm. Penggunaan pelbagai ubat kimoterapi memberi kesan sampingan kepada pesakit. Untuk mengatasi masalah ini adalah dengan cara menurunkan nadi amplitud dan panjangkan tempoh nadi denyut serta menggunakn ekstrak sumber semulajadi daun *Neolamarckia Cadamba*. Dalam kajian ini, gabungan proses elektroporasi dan ekstrak semula jadi dilakukan secara in vitro selama 48 jam. Sel yang digunakan adalah sel kanser serviks (sel HeLa) dan ekstrak semula jadi dari daun *Neolamarckia Cadamba*. Eksperimen ini bermula dengan menentukan parameter nadi amplitud dan nadi denyut yang terbaik memberikan peratusan daya tahan yang lebih rendah kepada sel HeLa. Nadi amplitud bermula dengan 200V/cm, 400V/cm, 600V/cm, 800V/cm dan 1000V/cm dan nadi denyut pada 100μs, 500μs, 1ms, 2ms dan 5ms dan hasilnya menunjukkan bahawa parameter terbaik pada 600V/cm nadi amplitud dan 5ms nadi denyut. Kemudian nilai IC_{50} yang ditunjukkan oleh ekstrak daun *Neolamarckia Cadamba* didapati berada pada 40μg/ml. Penemuan kajian ini telah menunjukkan tindak balas positif mengenai gabungan kedua-dua kaedah, ini dibuktikan dengan penurunan kadar peratusan percambahan sel HeLa apabila didedahkan medan elektrik hanya 30.08% sel HeLa Bertahan hidup manakala apabila sel HeLa didedahkan dengan gabungan medan elektrik dan ekstrak hanya 2.12% sel hidup.

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LIST OF SYMBOL AND ABBREVIATIONS

ECM 830 - Square Wave Pulse Generator

EP - Electroporation

HeLa - Cervical Cancer Cell

SEFPECT - Standard electric field parameters for Electrochemotherapy

CHAPTER 1

INTRODUCTION

1.1. Overview

This chapter consists of background of research followed by problem statement, aim, objectives and scope of study.

1.2. Background of Research

HeLa cells are human cervical cancer cells (the cervix is found at the top of the vagina and the entrance of the uterus). The cells were first cultured or grown in the laboratory in 1951 and were the first type of human cancer cell to be cultured continuously for experiments. There are many different strains (subtypes) of HeLa cells that are now used (J. Zhang et al., 2015).

Electroporation or electropermeabilization is a molecular biology technique in which an electrical field is applied to cells in order to increase the permeability of the cell membrane, allowing chemicals, drugs, or DNA to be introduced into the cell (C. Rosazza et al., 2016). In molecular biology, the process of electroporation is often used to transform bacteria, yeast, or plant protoplasts by introducing new coding DNA. When bacteria and plasmids are mixed together, the plasmids can be transferred into the bacteria after electroporation. Several hundred volts across a

distance of several millimeters are typically used in this process. Afterwhich, the cells have to be handled carefully until they are able to divide and produce cells that contains reproduced plasmids. This process is approximately ten times more effective than chemical transformation (A. Natarajan et al., 2012).

Electroporation is also highly efficient for the introduction of foreign genes into tissue culture cells, especially mammalian cells. For example, it is used in the process of producing knockout mice, as well as in tumor treatment, gene therapy, and cell-based therapy (C. R. Tracy et al., 2010). The process of introducing foreign DNA into eukaryotic cells is known as transfection. Electroporation is highly effective for transfecting cells in suspension using electroporation cuvettes. Electroporation has proven efficient for use on tissues in vivo, for in utero applications as well as in ovo transfection. Adherent cells can also be transfected using electroporation, providing researchers with an alternative of trypsinizing their cells prior to transfection (A. Rizk & B. M. Rabie, 2013).

Compound Extract Test as Anti-Proliferation (Neolamarckia Cadamba Leaves) also well-known as "Anthocephalus Cadamba", is a precious plant in Ayurvedic medicine. This Rubiaceae family tree, is commonly called as Kadam by the Indians and Kalempayan by Malaysians which it grows naturally in Australia, India, China, Malaysia, Indonesia, Philipines, Vietnam, and Singapore. Moreover, Neolamarckia Cadamba tree can grow up to 45 m tall, with a diameter up to 100-160 cm but normally less. Neolamarckia Cadamba has an umbrella shape crown and tiers arrangement of branches (C. P. Khare, 2007)

Importantly, Neolamarckia Cadamba is selected as one of the timber species for forest rehabilitation projects in Malaysia, due to its short rotation period and early commercial returns within 8-10 years. Whilst in India, the leaves are traditionally used in treating illnesses. It has been mentioned and described in many Indian medicine for the treatment of anemia, fever, uterine and liver complications, diabetes, diarrhea and much more. It also has antioxidant, anti-inflammatory also wound healing, anti-tumor, and anti-cancer properties. (A. Kumar et al., 2015).

1.3. Problem Statement

Statistically, cancer is among the leading causes of morbidity and mortality worldwide with approximate 18 million cancer cases recorded based on World Health Organization in 2018. This number is expected to increase by about 63% over the next 2 decades. In Malaysia, the numbers of cancer cases are approximately at 43 thousand recorded in 2018. This number is expected to increase by 47% over the next 2 decades. In Malaysia the most common cancer diagnosed in 2018 are breast, cervical, prostate, lung and colorectal cancer (F. Bray, et al., 2018).

Cancer can start at any part of the body. It starts when cell growth is out of control and crowds out the normal cell, thus making it hard for the body to function as it should. Cancer cells spread to other parts of the body. For instance, cancer cells in the lung can spread to the bones and grow. When cancer cells spreads, it is called metastasis. Some cancers cells grow and spread fast and others grow slowly. Most cancer form a lump called a tumor or a growth. But not all lumps are cancerous. Lumps that are cancerous are called malignant which have the potential to be dangerous (G. Cooper & R. E. Hausman, 2000). An alternative cancer chemoprevention is a medical intervention that aims to reduce the incidence and burden of cancer via the development of agents to prevent, reverse and delay the process of carcinogenesis (W. P. Steward & K. Brown, 2013).

The current chemotherapy side effect on human depends on the cancer type, drug taken and dosage. The treatment is not controllable (J. B. Wolinsky, et al., 2012). This study suggests an alternative treatment by used natural extract of Neolamarckia Cadamba leaves. This natural extract has phenolic found inside the leaves that have anti-cancer properties.

1.4. Aim

The aim of this research is to investigate the effect of electroporation and natural extracts of Neolamarckia Cadamba Leaves on HeLa cell cultured

1.5. Objectives

This study embarks on the following objectives:

a) To identifiy electric field parameter due to Electroporation process towards HeLa cell.

b) To analyze the effect of Neolamarckia Cadamba Leaves on HeLa cell

c) To evaluate the combined effect of Electric Field and Neolamarckia Cadamba Leaves on HeLa cell as anti-cancer mechanism.

1.6. Scope of study

In order to achieve the objectives of this research, the following scope of work were identified as followed:

a) HeLa cell lines were acquired and cultured in a physiological environment similar to mammalian cell. The cells were maintained at temperature of 37 ^{o}C similar to human temperature and humidified environment at 5% CO2 in order to maintain the pH of the medium as in the human blood plasma (J. T. Lee, et al., 2011).

b) Investigation of the best electric field parameter effect on HeLa cell lines proliferation in vitro followed by the standard electric field parameter for electrochemotherapy (SEFPECT) not more than 1000V/cm of pulse amplitude using the following parameter:
Pulse Amplitude: 200V/cm, 400V/cm, 600V/cm, 800V/cm and 1000V/cm
Pulse Duration: 100µs, 500µs, 1ms, 2ms and 5ms

c) Investigation of anti-cancer properties and determination 50% Inhibitory Concentration (IC_{50}) of Neolamarckia Cadamba leaves effect on HeLa cell lines proliferation, this is by considered the following range of concentration: 1µg/ml, 5µg/ml, 10µg/ml, 20µg/ml, 30µg/ml, 40µg/ml, 50µg/ml, 60µg/ml, 70µg/ml, 80µg/ml, 90µg/ml and 100µg/ml

d) Analyse the Combined effect of electric field and Neolamarckia Cadamba leaves extract on HeLa cell lines

e) Experiment to be repeated minimum thrice or more to ensure the reading is accurate and to get an average value.

CHAPTER 2

LITERATURE REVIEW

2.1. Overview

This chapter focuses on previous research done by other researchers that is similar and related to this research. This chapter is important to give an idea, guide and knowledge to the researcher on the commencement of the experiment.

2.2. Living Cell

Based on Mariana (2007) cells are called "Building blocks of life". The author described that cells are the functional and structural unit of all organisms that are living in this world. Cells are divided into two types that are prokaryotic cells and eukaryotic cells. The characteristics of prokaryotic cells can be described by the size that is usually less than 5μm and smaller compared to eukaryotic cells. This type of cell does not have a nucleus. Prokaryotic cells mass typically is around 1 nano gram. Examples of prokaryotic cells are bacteria and archaea. Figure 2.1 showed the structure of the prokaryotic cell.

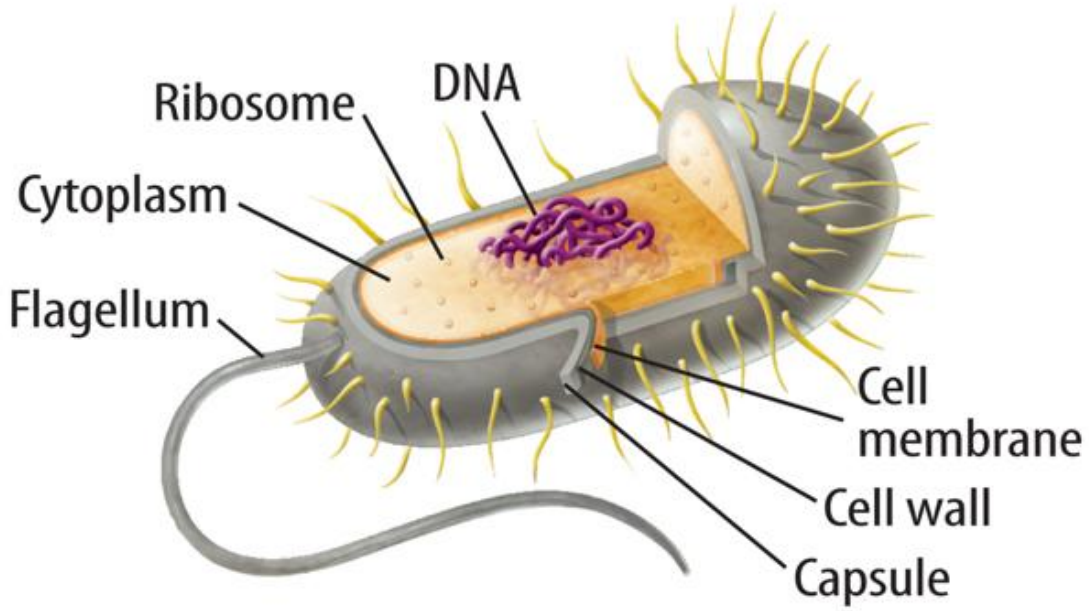

Figure. 2.1 Prokaryotic cell

(http://legacy.hopkinsville.kctcs.edu/instructors/Jason-Arnold/VLI/Module%202/m2cellstructure/m2cellstructure4.html and http://7marshscience.blogspot.com/2015/09/)

For a eukaryotic cell, the characteristics are opposed to a prokaryotic cell. Eukaryotic cells usually have more complex structures. The structures of eukaryotic cells are called organelles. Organelles contain many structures such as the nucleus, endoplasmic reticulum that consists of rough endoplasmic reticulum (contain ribosomes) and smooth endoplasmic reticulum (does not contain ribosomes), Golgi apparatus, lysosomes, mitochondria, chloroplast (found inside plant cells), vesicles, vacuoles and many more. Animal cell, plant cell, fungi, and protista are examples of eukaryotic cells. Figure 2.2 shows the structure of the eukaryotic cell.

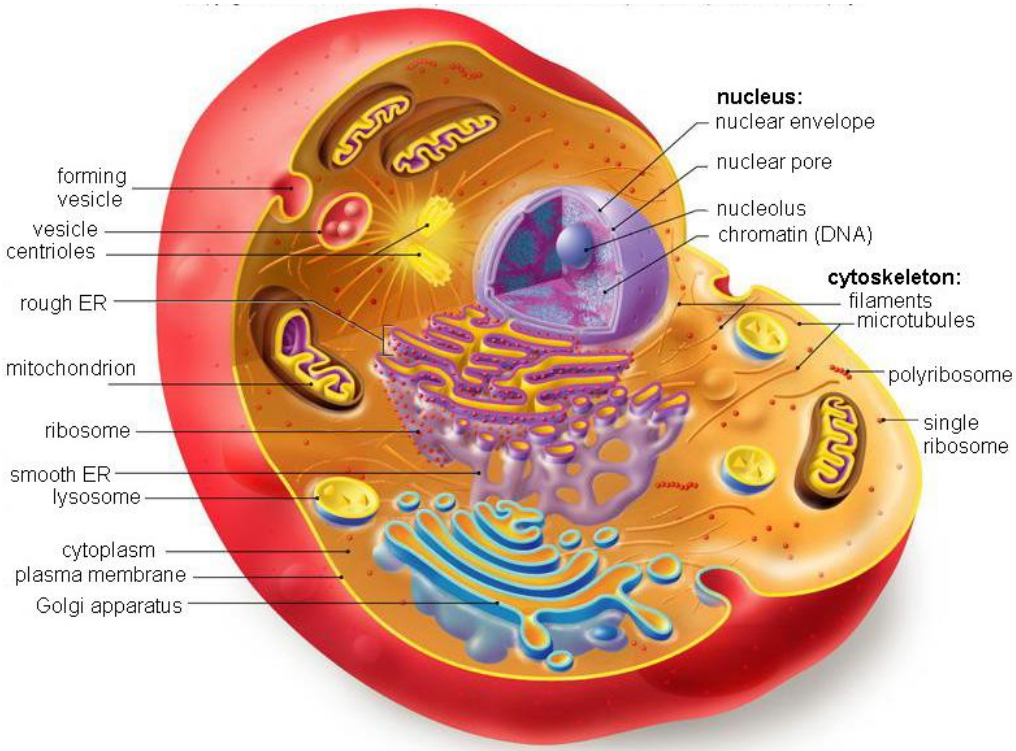

Figure. 2.2 Eukaryotic cell

(http://legacy.hopkinsville.kctcs.edu/instructors/Jason-Arnold/VLI/Module%202/m2cellstructure/m2cellstructure4.html and http://7marshscience.blogspot.com/2015/09/)

2.3. HeLa Cells (Human Cervical Cancer Cells)

HeLa cells are one of the examples of eukaryotic cell type. It is derived from human cervical cancer cells. HeLa is named based on cancer patient name Henrietta Lacks. This cell was taken from Henrietta Lacks who passed away on 4 October 1951 after fighting for eight months from her cervical cancer disease. HeLa cells were first cultured in 1951 in a laboratory (J. Zhang et al., 2015). It has been continuously grown as first human cells known. HeLa cells have a high capability of division and growing indefinitely and continuously as long as the environment of the cell is still

suitable for HeLa cells to grow and adapt. This characteristic made HeLa cells easily immortalize literally.

For a decade in cancer research area, many scientists and researchers explored and studied the HeLa cells characteristics due to its availability and easiness to find because it is cultured continuously in the laboratory. HeLa cells are very tough and adaptable cell. It can survive in any ambient where other cells cannot and die easily. HeLa cell is also a very potent cell that is able to contaminate another cell. Figure 2.3 showed the structure of HeLa cell taken during experiment.

Figure. 2.3 Human Cervical Cancer Cell (HeLa Cell) scale bar 50µm

2.4. Electroporation

Electroporation is the method of external application of electric field to the cell membrane to increase the permeability of the cell membrane. This method is widely used in medicines, food processing and pharmaceutics (M. Yarmush et al., 2014). Electroporation also is a method to create pores on cell membrane to allow any substances to enter the cell easily (N. J. Yang & M. J. Hinner, 2014).

2.4.1. Membrane Electroporation

R. Stampfli (1958), stated that our bodies are built from trillions of biological cells enveloped by the plasma membrane. The plasma membrane is largely composed of a double layer (bilayer) of lipids. Its size is about 5nm. The inside of the membrane is partly gel and partly liquid. It functions as a protector and separator of the cell from its surroundings environment. It is very stable and reliable. The lipid bilayer contains different types of proteins which act as pumps and channel for transporting purpose. It can transport specific molecules that want to across the plasma membrane. Plasma membrane can be electrically be viewed as a sheet of thin insulator surrounded by electrolyte aqueous solutions on both sides.

Based on A. J. H. Sale & W. A. Hamilton (1967), when strong electroporation is exposed to the membrane, it will undergo electrical breakdown that allows it to be permeable to other molecules to freely cross it. This process is called the electroporation of the membrane. If the exposure of electric field is sufficiently short, then the membrane can effectively undergo rapid recovery for the cell to remain viable. It is called reversible electroporation. Otherwise, it is called irreversible electroporation (E. Neumann & K. Rosenheck, 1972).

Electroporation becomes a useful tool in biotechnology and medicine area. As for irreversible electroporation, it becomes popular for electrochemotherapy where the method is by introducing drugs into tumor cells (G. Sersa et al., 2008). Another

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