ESTABLISHING A STANDARD FIBROBLAST CELL LINE DERIVED FROM KIDNEY TISSUE OF SUMATRAN RHINOCEROS (PUNTUNG)

BY

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ABSTRACT

Establishment and cryopreservation of cell cultures have been applied in biodiversity conservation, essential to critically endangered wildlife such as the Sumatran rhinoceros. It serves as the foundation for advanced cellular techniques. Obtaining desired tissues from living organism as a source for primary culture is not feasible. As alternative, preserved tissues from Puntung which was euthanized due to terminal skin cancer was utilized. The aim of this study is to establish, characterize and authenticate fibroblast cells derived from kidney tissue of Sumatran rhinoceros carcass. Primary cultures were isolated from kidney tissue using mixed enzymatic-explant method, supplemented with complete media (DMEM + 10% FBS + 1% antibiotic) and kept at 37ºC with 5% CO_2 incubator. Following routine trypsinization, viability and growth curves were obtained by Trypan blue counting method. Frozen stocks were preserved in media containing 90% FBS and 10% DMSO. Cellular senescence was quantified by Sa-β-gal staining assay, while chromosome spreads were stained with Giemsa solution. Mycoplasma contaminations were detected by PCR method. Derivation of fibroblast cells from the kidney tissues generates a total of 81 frozen stocks. The cell viability maintained over 80% during serial passages and after 3 months of cryopreservation, but only cells at P5 and P10 show reasonable recovery after 6 months. From the growth curves, the cell population doubling time at P5 was 20.45h while 22.35h at both P10 and P15. The senescence level significantly increase from P5 to P10, and especially significant at P15. Genetic stabilities were considered stable at P5 and P10. All cultures were free from mycoplasma contamination. The results of this study have concluded that cells cultured up to P10 were suitable for the development of Sumatran rhinoceros cell banking system, which are applicable for advanced research. This also provides simple reference in the development of cell bank for other endangered wildlife in Malaysia.

مخلص البحث

إن تَطْبِيقْ إِنْشَاءْ وَحِفْظْ الخَلَايَا بِالتَبْرِيدْ فِي التَنَوُعْ البَيُولُوجِي أَمْرٌ ضَرُورِي لِلْحَيَاةِ البَرِيّةِ المُهَدّدَةِ بِالإِنْقِرَاضْ مِثْلْ وَحِيدُ القَرْنِ السُومَطْرِي. إِنّهَا بِمَثَابَةِ الأَسَاسْ لِتِقْنِيَاتْ خُلَوِيّة مُتَقَدِمَة. للحُصُولْ عَلَى الأَنْسِجَة المَرْغُوبَة مِنْ الكَائِنَاتِ الحَيّة كَمَصْدَرْ لِلْثَقَافَةِ الأَوّلِي غَيْرُ مُمْكِنْ. كَبَدِيلْ، تَمّ اِسْتِخْدَامْ أَنْسِجَة مَحْفُوظَةْ مِنْ وَحِيدْ القَرْنِ الذِيْ تَمّ قَتْلُهُ بِسَبَبِ سَرَطَانِ الجِلْدِ في مَرَاحِلِهِ الأَخِيرَة. الهَدَفْ مِنْ هَذِهِ الدِرَاسَة هُو تَحْدِيدْ وَتَمْيِيزْ وَتَوْثِيقْ الخَلاَيَا اللِيفِيّة المُشْتَقّة مِنْ أَنْسِجَةْ الكِلَى في جَسَدْ وَحِيدْ القَرْنْ السُومَطْرِي. تَمّ عَزْلْ الأَنْسِجَة الأَوَلِيةْ مِنْ أَنْسِجَةِ الكِلَى بِاِسْتِخْدَامِ طَرِيقَةْ زَرْعِ الإِنْزِيمْ المُخْتَلِطْ، تُسْتَكْمَلْ بَأَوْسَاطٍ كَامِلَة (وَسَطْ النَسَر المُعَدّلْ مِنْ قِبَلْ دُولْبِيكُو + 10% مَصِلْ بَقَرِي جَنِينِي + 1% مُضَادْ حَيَوِيْ) تم حْفَظْها عِنْدَ 37 دَرَجَة مِئَوِيَة مَعَ حَاضِنَه 5% مِنْ ثَانِي أُكْسِيْدِ الكَرْبُونْ. بَعْدَ التِرِبْسِينْ الرُوتِينِي، تَمّ الحُصُولْ عَلَى مُنْحَنَيَاتْ قَابِلِيّةْ الحَيَاةْ وَالنُمُو مِنْ خِلَالِ طَرِيقَةِ عَدّ زُرْقَةِ التِرْيبَانْ. تَمّ الإِحْتِفَاظْ بِالأَسْهُمْ المُجَمّدَة في وَسَائِطْ تَحْتَوِي عَلَى (90% مِنْ مَصِلْ بَقَرِي جَنِينِي وَ10% مِنْ ثُنَائِي مِيثِيلْ سُلْفُوكْسِيدْ) تَمّ تَحْدِيدْ الشَيْخُوخَةْ الخَلَوِيّة بِوَاسِطَةْ مُقَايَسَةْ تَلْطِيخْ إِنْ بِيتَا جَالَاكْتُوزِيدَازْ المُرْتَبِطْ بِالسِنِيسِنْس. بَيْنَمَا الكُرُومُوسُومَاتْ المُنْتَشِرَة مَصْبُوغَة بِمَحْلُولْ غِيْمْزَا. يُوَلّدْ اِشْتِقَاقْ خَلَايَا الأَرُومَة اللِيفِيّة مِنْ أَنْسِجَة الكِلَى مَا مَجْمُوعُه 81 مِنْ الأَسْهُم المُجَمّدَة. حَافَظَتْ قَابِلِيّة الخَلِيّة عَلَى البَقَاءِ لِأَكْثَرِ مِنْ 80٪ خِلَالِ القَنَوَاتِ التَسَلْسُلِيّة وَبَعْد 3 أَشْهُرْ مِنْ الحِفِظْ بِالتَبْرِيدْ، وَلَكِنْ فَقَطْ الخَلايَا فِي ب5 وَ ب10 تُظْهِرْ شِفَاءً مَعْقُولًا بَعْدْ 6 أَشْهُرْ. مِنْ مُنْحَنَيَاتِ النُمُو، كَانَ عَدَدْ الخَلَايَا المُضَاعَفَة فِي ب5 هُوَ 20.45 سَاعَة بَيْنَمَا 22.35 سَاعَة فِي كُلّ مِنْ ب10 وَ ب15. يَزْدَادْ مُسْتَوَى الشَيْخُوخَة بِشَكِلْ مَلْحُوظْ مِنْ ب5 إِلى ب10 ، وَخَاصَةً بِشَكْلٍ مَلْحُوظْ عِنْدَ ب15. أُعْتُبِرَتْ الثَبَاتَاتْ الجِينِيّة مُسْتَقِرّه عِنْدَ ب5 وَ ب10. كَانَتْ جَمِيعْ الثَقَافَاتْ خَالِيَة مِنْ تَلَوُّثْ المِيكُوبَلَازْمَا.

APPROVAL PAGE

I certify that I have supervised and read this study and that in my opinion, it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a thesis for the degree of Master of Science (Biotechnology).

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DECLARATION

I hereby declare that this thesis is the result of my own investigations, except where otherwise stated. I also declare that it has not been previously or concurrently submitted as a whole for any other degrees at IIUM or other institutions.

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This thesis is dedicated to my supervisor, co-supervisor, colleagues and family

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LIST OF SYMBOLS

CO_2	Carbon dioxide
P	Passage
cm	Centimetre
kg	Kilogram
km^2	Square kilometre
^oC	Degree Celsius
%	Percent
s	Seconds
min	Minute
h	Hour
rpm	Rotation per minute
µl	Microlitre
ml	Millilitre
O_2	Oxygen

LIST OF ABBREVIATIONS

IUCN	International Union for Conservation of Nature
D.s	*Dicerorhinus sumatrensis*
WWF	World Wildlife Fund
iPSCs	Induced pluripotent stem cells
ESCs	Embryonic stem cells
OSKM	Oct3/4, Sox2, Klf4 and c-Myc
iSCNT	Interspecies somatic nuclear transfer
IVF	*In vitro* fertilization
MCB	Master cell bank
WCB	Working cell bank
WHO	World Health Organization
Sa-β-gal	Senescence-associated-β-galactosidase
FACS	Fluorescence activated cell sorting
MACS	Magnetic activated cell sorting
WJMSCs	Wharton's jelly mesenchymal stem cells
BHK-21	Baby hamster kidney cell
MDCK	Madin-darby canine kidney cell
MDBK	Madin-darby bovine kidney cell
VERO	African green monkey kidney cell
J3K	Japanese macaque cell line
PKC	Porcine kidney fibroblast cell
PFF	Porcine fetal fibroblast cell
PEF	Porcine ear fibroblast
HEp-2	Human epithelial type-2
DMEM	Dulbecco's Modified Eagle Medium
FBS	Fetal Bovine Serum
PBS	Phosphate Buffer Saline
EDTA	Edetic acid; Ethylenediaminetetraacetic acid
DMSO	Dimethyl sulfoxide
HBSS	Hank's balanced salt solution

CHAPTER ONE

INTRODUCTION

1.1 BACKGROUND OF STUDY

Sumatran rhinoceros (*Dicerorhinus sumatrensis*) is justified as critically endangered wildlife by the International Union for Conservation of Nature (IUCN). As of 2015, the species has been assumed to be extinct in the wild of Malaysia (Havmoller *et al.*, 2016); last Malaysia's rhino (Iman) died on November 2019 at Tabin Wildlife Reserve Sabah. Despite historical distribution in several countries in Asia, presently, it is estimated that fewer than 80 individuals survive in Indonesia. Apart from the conventional conservation efforts, the application of cell culture technology has emerged as a new tool for ex-situ conservation (Ryder *et al.*, 2016).

Establishment of cell lines have been widely used to preserve the genetic materials of many rare and endangered animals such as Bengal tiger and Jaguar (Guan *et al.*, 2010; Mestre-Citrinovitz *et al.*, 2016). This is further enhanced by the cryopreservation technique where it extends the period of storage of the established cell line, ensures its indefinite supply and distribution, preventing genetic and phenotypic instability, delay cell senescence and transformation, reduce the risk of contamination as well as for economic purpose. Several considerations must be taken into account before the development of cell bank; provenance of the primary culture, quality, characterization and authentication of the cell line (Freshney, 2010).

1.2 PROBLEM STATEMENT

Development of a standard fibroblast cell line involves several stages which includes primary cell derivation from the desired tissue of an organism, followed by subculture techniques. The collection of tissue from the living organism may minimally or sufficiently affect their health. Obtaining tissue sources for primary culture from living wildlife raises the concern of possible injury and even death. As alternative, tissues harvesting and preservation was done on deceased individual.

On June 4th 2017, a female Sumatran rhino (Puntung) was euthanized due to terminal cell skin cancer. Tissue samples have been distributed to several institutions in Malaysia to be preserved and provide sources in generating the primary cell culture for the species. In this research, standard fibroblast cell lines derived from kidney tissue of Puntung will be established. Following cell line establishment, characterization and authentication analysis will be carried out to prevent misidentification and contamination of the cells.

1.3 OBJECTIVES OF RESEARCH

The aim of this research is to establish a standard fibroblast cell line derived from kidney tissue of Sumatran rhinoceros. It is carried out with the following objectives:

i. To derive primary fibroblast cell from kidney tissue of Sumatran rhino.
ii. To evaluate the characteristics of the established fibroblast cell lines.
iii. To analyse the authenticity of the established fibroblast cell lines.

1.4 RESEARCH QUESTIONS

i. How can primary fibroblast cells be derived from the kidney tissue of Sumatran rhino?
ii. Do the characteristics of the established fibroblast cell line remain stable over passages?
iii. Is the established fibroblast cell lines free from cross-contamination and misidentification?

1.5 HYPOTHESIS

The hypothesis of this research is as follows:

i. Cell derivation using enzymatic-explant method of the Sumatran rhinoceros kidney tissue result in successful primary fibroblast cells establishment.
ii. The characteristics of the established cell line are altered during serial passages.
iii. The identities of the established cell line are altered during serial passages.

1.6 SIGNIFICANCE OF STUDY

At the end of this research, the establishment of standard fibroblast cell lines derived from kidney tissue of Sumatran rhino will provide an opportunity in the development of cell banking system of the species. This ensures the continuous distribution of the characterized and authentic rhino genetic resources for advanced research such as the induced pluripotent stem cells (iPSCs) and interspecies somatic nuclear transfer (iSCNT) technologies. Apart from that, this study can also serves as a guide in the establishment of cell line from other rare and endangered wildlife species in Malaysia.

CHAPTER TWO

LITERATURE REVIEW

2.1 SUMATRAN RHINOCEROS

Scientifically known as *Dicerorhinus sumatrensis*, Sumatran rhino is one of the five remaining species of rhinoceros. Apart from Sumatran rhino, there are also the Javan rhino (*Rhinoceros sondaicus*), Indian rhino (*Rhinoceros unicornis*), Black rhino (*Diceros bicornis*) and White rhino (*Ceratotherium simum*). Of these five species, the Sumatran alongside the Javan and Indian rhinos are found in Asia while the Black and White rhinos are found in Africa. Paleogenomic study by Shapiro and Hofreiter (2014) suggests that Sumatran rhinos evolutionary diverged from the extinct Woolly rhino (*Coelodonta antiquitatis*), once inhabited northern Europe and eastern Asian.

According to Foose *et al.* (1997), Sumatran rhinos were once distributed in parts of the South-Central and South-East Asia. This includes Malaysia (Peninsular and Borneo), Indonesia (Kalimantan and Sumatra), Myanmar, Bangladesh, Bhutan, Brunei Darussalam, Cambodia, India, Laos, Thailand and Vietnam. Few years back, however, the living Sumatran rhinos can only be found in Indonesia and Sabah (Malaysia). With the recent assumption of the species extinction in the wild of Malaysia as of 2015 (Havmoller *et al.*, 2016) followed by the death of Malaysia's last rhino (Iman) in captivity, it is believed that only fewer than 80 still survives in Indonesia.

There are three known subspecies of Sumatran rhinos which are based on their skull and teeth measurement as well as the area they roamed about. Rhinos that inhabit India, Bhutan, Bangladesh and Myanmar are of the subspecies *D. s. lasiotis*, characterized to have

large skull and teeth. Those occupying the island of Borneo (Sabah, Sarawak, Brunei, Kalimantan), *D. s. harrissoni*, have small skull and teeth. In Peninsular Malaysia, Thailand and Sumatra, *D. s. sumatrensis* is characterized to have large skull and medium teeth. Of these three subspecies, *D. s. sumatrensis* constitutes the largest population of Sumatran rhinos whereas *D. s. lasiotis* has been officially declared as extinct (van Strien, 1974; Brattstrom *et al.*, 2016).

2.1.1 Puntung

Puntung, a female Sumatran rhino was captured from the wild and transported to Tabin Wildlife Reserve Sabah in 2011. It is believed that she had escaped poaching likely using snare due to the missing front left foot. Known to have developed by almost half of the female Sumatran rhino in captivity, the reproductive pathology with cysts in the lining of the uterus may have caused failure in the captive breeding programme between Puntung and Tam (last male rhino in Malaysia; died of old age on 27th May 2019). What was thought to be jaw swelling due to dental infection was later diagnosed as terminal squamous cell cancer. Two infected molars and a premolar were able to be extracted on April, but the cancer spread rapidly within the following month. As of 4th June 2017, she was euthanized as to end her suffering (Stephanie Pappas, 2017).

2.1.2 Physical Appearance of Sumatran Rhinoceros

Sumatran rhino can be shown in figure 2.1. It is the smallest among rhinoceros species. According to the World Wildlife Fund (WWF), it stands at an average height of around 130 cm from toe to shoulder measurement. This is supported by the list of measurements reported by van Strien (1974), with the shortest being 121 cm to the tallest height of 144 cm. In terms of weight, it can reach as much as 600 to 950 kg. As there are two horns present on their skull

along with the African rhinos, they are also known as the Asian two-horned rhinos. The other two Asian rhinos have only one horn situated on their skull. The most distinguishable characteristics of the Sumatran rhinos in contrast with the other rhino species is the appearance of hairy and brownish skin.

Figure 2.1: Sumatran Rhinoceros (Puntung).
(Source: Borneo Rhino Alliance)

2.1.3 Life Cycle

Sumatran rhinos have an average lifespan between 35 to 40 years in captivity, with no reported distinction between male and female or in the wild. Sexual maturity is reached at least around 6 years in male and 5 years in female (Roth *et al.*, 2013). Female rhinos were found to be an induced ovulator, where ovulations are stimulated by means of mechanical mating and seminal fluid introduction rather than natural cycle (Roth *et al.*, 2001). If fertilization occurs, gestation period proceeds for up to 475 days with one calf for an interval of 3 to 4 years of pregnancy. The calf will live on with their mother for about 2 to 3 years before wander off to find its own territory.

2.1.4 Territorial Preferences

The rhinos are territorial animals with home range of up to 40 km^2 which accounts for the foraging, wallowing and salt licking area. It is mostly influenced by the food availability within the area. They depend mainly on leaves and woody sapling, seasonally fruits. These food sources are found most abundant on lowland area, and then decreased with increasing topography on slope and eventually ridges. As a result, rhinos are usually found inhabiting the lowland. Their home range expands up to 80 to 120 km^2 when food sources are scarce, and some might even ventures into plantation area (Mohd Momin Khan, 2014).

2.1.5 Threats toward Extinction of Sumatran Rhinos

The dwindling number of Sumatran rhinos is mainly associated with the poaching activities by local hunters. The continuous killings of the rhinos occur throughout the 19^{th} century, at the time where the consumer market for rhino horn increased tremendously in Southeast Asia particularly China, Japan, Korea and Taiwan. Body parts of rhinos especially the horn was believed to possess medicinal properties of which is used in the Chinese traditional medicine (Lindenmayer *et al.*, 2005).

Deforestation typically for urbanisation, industrial and agricultural purposes further diminished the number of rhinos. Palm oil plantations are the primary drive for the massive clearance of trees, which leads to the destruction of wildlife habitat. This, in turns, creates a fragmented population of rhinos (Mohd Momin Khan, 2014). As Sumatran rhinos are territorial, interference of their natural habitat forces them to wander in search for a new territory as well as for food and water. Several individuals of rhinos have even been found in plantation area. This increased the chance of encounter between rhinos and poachers.

With the implementation of anti-poaching laws and establishment of protective zones, the rhinos likely faced a decline threats from illegal poaching and habitat loss. In Malaysia, particularly Sabah, all species of wildlife and plants are protected under the Wildlife Conservation Enactment 1997. Any breach of regulations is penalized with fine or imprisonment. In addition, the global Rhino Protection Units physically guard the protective zones from potential poachers. Back in the 1980s, extensive captive breeding program have also been initiated, but no calf was ever produced in Malaysia (Lindenmayer *et al.*, 2005).

The major problem now shifts towards the reproductive isolation of surviving rhinos in the wild. With very low number of individuals that are segregated due to habitat fragmentation, the chances of encounter between male and female rhinos for mating is highly unlikely (Jani Actman, 2015). The chances are further diminished by the fact that the rhinos are solitary animals. Moreover, female rhinos are prone to cancer development of the reproductive organs with long non-reproductive periods. This reduces the ability of a female rhino to produce calf (Fiuza *et al.*, 2017).

2.2 SIGNIFICANCE OF CELL CULTURE IN WILDLIFE CONSERVATION

2.2.1 Induced Pluripotent Stem Cells (iPSCs)

Pluripotency is the ability of cells to differentiate into all somatic cell lineages developed from ectoderm, mesoderm or endoderm germ line which is only displayed by embryonic stem cells (ESCs). Due to the ethical issues and limited availability for some interested organism, somatic cells have been programmed into iPSCs to mimic the properties of ESCs. Back in 2006, Takahashi *et al.* successfully generate ESCs-like cells following the introduction of Oct3/4, Sox2, Klf4 and c-Myc (OSKM) by retroviral transfection into mouse tail-tip fibroblast cells and then Fbx15-selection. Modification was then made where these genes were transfected into the fibroblast cells with Nanog-selection few days post-transfection. It was suggested that OSKM factors are essential in the initial step in reprogramming while Nanog is responsible in maintaining cell pluripotency (Okita *et al.*, 2007).

The iPSCs of northern white rhinoceros (*Ceratotherium simum cottoni*) have been successfully generated by the introduction of human OSKM factors using retro-VSV.G viruses into fibroblast cell lines. The iPSCs of drill monkey (*Madrillus leucophaeus*) was also generated with the same system using retroviral vectors. Both inductions were driven by the same objective; obtaining iPSCs to develop primordial germ cells and eventually into ovum or sperm, to be applied in *in vitro* fertilization. The *in vitro* generation of germ cells from these embryoid bodies is still in progress (Ben-Nun *et al.*, 2011; Pessôa *et al.*, 2019).