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GENOTOXICITY EFFECTS OF NEWCASTLE DISEASE VIRUS STRAIN AF 2240 IN VITRO AND IN VIVO

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By

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Newcastle disease virus (NDV) is a member of the Paramyxoviridae that causes severe economic losses in the poultry industry worldwide. Several strains of NDV were reported to induce cytolysis to cancerous cell lines. Newcastle disease virus is a potential oncolytic as it can replicate up to 10,000 times better in cancerous cells than in most normal human cells. In this study, a local strain of NDV AF2240 was evaluated for its genotoxicity properties against breast cancer cell line (4T1 cancer cell lines) and normal cell lines (3T3 fibroblast cell lines). The cytolysis effects of NDV AF2240 were determined using Microtetrazolium (MTT) assay. Further studies were carried out to observe the genotoxicity potential of NDV AF2240 using comet assay. In the assay, individual cell was screened for DNA damage after treatment with NDV AF2240. The clastogenetic effect of NDV AF2240 was also observed using bone marrow micronucleus assay. The safety of the virus was investigated *in vivo* using 9 New Zealand white albino rabbit. The irritation effects of the virus were observed on rabbit

eyes. The inhibition concentration (IC_{50}) for NDV AF2240 to inhibit 50 % of 4T1 cancer cells population were 32 HA unit/ml and 64 HA unit/ml for co-culture and monolayer methods, respectively. No significant cytolytic effect was observed on normal 3T3 fibroblast cell lines at the same virus titer used in breast cancer cell lines. The proliferation rates of treated breast cancer cell was reduced significantly with time and titration of virus compared to the untreated control. It was noticed that 4T1 breast cancer cell line treated with NDV AF2240 gave a strong genotoxic response as the formation of comet tail were significantly longer than 3T3 fibroblast cell line treated with NDV AF2240. The study indicated that NDV AF2240 did not damage the DNA of normal cells but caused damage to breast cancer cells exclusively. It was noticed that the proliferation rate of normal erythrocytes to polychromatic erythrocytes of mice after treatment with NDV AF2240 were normal as compared to positive control. There was no significant increase in the induction of micronucleus formation in young erythrocytes which indicates that the virus has no clastogenic effect, thus will not result in chromosomal damage in mitotic apparatus of reproductive system in mice model. It was noticed that NDV AF2240 did not cause any irreversible effects to rabbit eyes. It only caused mild conjunctival redness as observed in group treated with 2048 HA unit of NDV and disappeared 72 hours post treatment. Scanning electron microscopy (SEM) evaluation of rabbit epithelial cornea surface revealed that the distribution of light, medium and dark reflex cells, the size, morphology of cell boundaries, structure and the distribution of microvilli of cornea treated with NDV AF2240 at 64 HAU, 512HAU and 2048 HAU, were similar to the control cornea. It was observed that NDV AF2240 did not cause any ocular disease to the corneal surface. Therefore, it is possible to use NDV strain AF2240 in clinical trials

since it is proven to be an effective anti-cancer agent against breast tumor, and NDV AF2240 exhibited none or minimal side effects to animal model used in this study. However, further study need to be carried out to learn more about the effects of NDV AF2240 on human in terms of understanding the viral replication and localization in preclinical studies before it can be used extensively in clinical trial phase.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Master Sains

KESAN GENOTOKSISITI OLEH VIRUS NEWCASTLE DISEASE JENIS AF2240 *IN VITRO* DAN *IN VIVO*

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Virus penyakit Newcastle (NDV) merupakan virus dari kumpulan Paramyoxviridea yang telah menyebabkan kerugian ekomoni dalam industri penternakan ayam dan itik seluruh dunia. Beberapa jenis NDV dilaporkan mampu menyebabkan sitolisis pada sel kanser. NDV mempunyai potesi onkolitik di mana ia mampu mengganda dalam sel kanser sehingga 10, 000 kali lebih baik berbanding dalam sel biasa. Dalam kajian ini, NDV jenis tempatan AF2240 telah dinilai akan potensi genotoksisiti virus ini pada sel kanser payudara mencit (4T1) dan sel normal mencit (3T3). Kesan sitolisis ditentukan melalui keadah Microtetrazolium (MTT). Kepekatan NDV jenis AF2240 yang diperlukan untuk merencat 50% populasi sel kanser 4T1 adalah 32 HA unit/ml dan 64 HA unit/ml untuk teknik “co-culture” dan “monolayer”. Tiada kesan sitolisis yang signifikasi diperhatikan pada sel biasa fibroblast 3T3 pada kepekatan virus yang sama digunakan pada sel kanser 4T1. Kadar proliferasi sel kanser 4T1 yang dirawat didapati menurun

dengan amat signifikan bergantung pada masa dan titrasi virus berbanding dengan sel kawalan yang tidak dirawat.

Kajian selanjutnya dilakukan untuk memerhati potensi genotoksisiti NDV jenis AF2240 mengunakan keadah “Comet”. Melalui keadah ini, sel diperhatikan setiap satu untuk kerosakan selepas dirawat dengan NDV jenis AF2240. Adalah didapati bahawa sel kanser payudara mencit 4T1 memberi respon genotoksik yang kuat di mana pembentukan ekor comet didapati lebih panjang berbanding sel normal 3T3. Kajian ini menunjukkan bahawa NDV jenis AF2240 tidak merosakkan sel biasa tetapi menyebabkan kerosakan yang teruk pada sel kanser payudara.

Kesan toksisiti pada mitotic apparatus mencit juga diperhatikan menggunakan keadah “Bone Marrow Micronucleus”. Didapati kadar proliferasi ertythrocytes matang berbanding erythrocytes muda mencit selepas dirawat dengan NDV jenis AF2240 adalah sama seperti mencit biasa yang tidak dirawat. Tetapi mempunyai perbezaan yang signifikasi berbanding kadar proliferasi kumpulan mencit yang dirawat dengan kawalan positif. Tiada kenaikan yang signifikasi pada insiden pembentukan micronuclei di dalam erythrocytes muda. Ini menunjukkan bahawa virus ini tidak mempunyai kesan clastogenik, oleh itu ia tidak akan menyebabkan kerosakan pada kromosom dalam mitotic apparatus dalam sistem pembiakan mencit.

Keselamatan penggunaan virus ini juga disiasat secara *in vivo* mengunakan arnab albino New Zealand putih. Kesan iritasi oleh virus diperhatikan pada mata arnab. Didapati NDV jenis AF2240 tidak memberi kesan yang kekal. Ia hanya menyebabkan “mild redness conjunctivitis” seperti yang diperhatikan pada kumpulan 2048 HA unit,

akan tetapi radang itu hilang selepas 72 jam rawatan. Evaluasi microskop scanning electron (SEM) terhadap permukaan Kornea epitelium arnab menunjukkan bahawa taburan sel terang, sederhana, dan sel reflex gelap, saiz, bentuk pembahagi dinding and taburan microvilli adalah sama seperti cornea kawalan yang tidak dirawat. Didapati bahawa NDV jenis AF2240 tidak menyebabkan sebarang penyakit ocular pada permukaan kornea arnab. Oleh kerana itu, adalah berkemungkinan untuk menggunakan NDV jenis AF2240 dalam percubaan klinikal kerana telah diperhatikan virus ini mampu menjadi agent anti-kanser terhadap kanser payudara dan ia juga tidak merosakkan atau hanya menyebabkan kesan yang kecil pada medel haiwan yang digunakan dalam kajian ini. Walaubagaimanapun, kajian selanjutnya perlu dijalankan untuk mengetahui secara lebih mendalam tentang kesan NDV jenis AF2240 pada manusia, seperti replikasi virus dan lokalisasi dalam kajian pre-klinikal sebelum ia digunakan secara meluas pada fasa klinikal.

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I certify that an Examination Committee has met on _____ to conduct the final examination of Zaid Mohamed Shahrin on his Master of Science thesis entitle "Evaluation of Genotoxicity Effects of Newcastle Disease Virus Strain AF2240 *In Vitro* and *In Vivo*" in accordance with the Universiti and Universiti Colleges Act 1971 and the constitution of the Universiti Putra Malaysia [P.U.(A) 106] 15 March 1998. The Committee recommends that the student be awarded the degree of Master of Science.

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DECLARATION

I declare that the thesis is my original work except for quotations and citations which have been duly acknowledged. I also declare that it has not been previously, and is not concurrently submitted for any other degree at Universiti Putra Malaysia or at any other institution.

ZAID MOHAMED SHAHRIN

Date: 18 June 2012

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LIST OF ABBREVIATIONS

aa	Amino acid
AJCC	American Joint Committee on Cancer
ANOVA	Analysis of variance
ATCC	American Type Culture Collection
BSC	Breast conserving surgery
cm	Centimeter
CO_2	Carbon dioxide
DCIS	Ductal carcinoma *in situ*
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleotide acid
EDTA	Ethylenediamine-tetra-acetic acid
ER	Estrogen receptor
F	Fusion
FBS	Fetal bovine serum
g	Gram
hrs	Hours
HSV	Herpes simplex virus
H_2O_2	Hydrogen peroxide
HA	Heamaglutination
HAU	Heamaglutination unit
HN	Heamagglutinin-neuraminidase
IC_{50}	Inhibition concentration for 50 % cell population

IDC	Invasive ductal carcinoma
ILC	Invasive lobular carcinoma
IFN	Interferon
IL	Interleukin
LIVES	Laboratory of Immunotherapeutic and Vaccine Study
KCL	Potassium chloride
KH_2PO_4	Potassium dihydrogen phosphate
M	Molar
mg	Milligram
ml	Milliliter
mM	milimolar
min	Minutes
MnPCE	Micronucleated polychromatic erythrocyte
MTT	Microculture tetrazolium
MV	Measles virus
NA	Neuraminidase
nm	Nanometer
NaCl	Sodium chloride
$NaHCO_3$	Sodium bicarbonate
Na_2PO_4	Sodium phosphate
NCE	Normochromic erythrocyte
NCR	National cancer registry
ND	Newcastle disease
NDV	Newcastle disease virus

NK	Natural killer
NP	Nucleocapsid protein
NTE	NaCl, Tris-HCl and EDTA
OD	Optical density
PBS	Phosphate buffer saline
PCE	Polychromatic erythrocyte
RBC	Red blood cell
rpm	Rotation per minutes
s	Second
RNA	Ribonucleotide acid
SCGE	Single cell gel electrophoresis
SEM	Scanning electron microscope
TBS	Tris- base saline
UV	Ultra violet
VSV	Vascular stomatisis virus
V	Volt
v/v	Volume/ volume
WHO	World Health Organization
w/v	Wight/ volume
°C	Degree Celsius
µl	Microlitre
µm	Micrometer

CHAPTER I

INTRODUCTION

Today, cancer is a major health problem worldwide. Globally, out of the 50 million deaths that occur annually, 7.4 million is attributed to cancer. The World Health Organization estimated that by the year 2030 the death due to cancer will increase to 21 million whereby 5-25% of this number is due to breast cancer (Landau, 2010). Breast cancer is the most common cancer among women. It is the leading cause of cancer mortality in women worldwide. The rate of cancer incidences differ between different countries. The incidence of breast cancer is the highest in the United States of America. The American Cancer Society (2010) reported that about 192,370 new cases of invasive breast cancer occur throughout 2009 and 20% of them die of the disease. In addition, according to Baum *et al.* (1994) about one in every twelve women in the United Kingdom will eventually develop this disease. Breast cancer is also one of the most common types of malignancy among women in Malaysia. About 1500 new cases are reported annually. Statistically data from National Cancer Registry (NCR) showed that breast cancer accounted about 31% and 16% of the total reported cancer and the total female cancer cases respectively (NCR, 2006).

Basically, death as a result of breast cancer is due to the distant spreading or metastasis of malignant tumor cells from the breast to other vital organs of the body like the liver, lungs, bone and brain (Rosai, 1989). Progression of cancer can clinicopathologically be divided into 5 stages, namely stage 0, stage I, stage II, stage III and stage IV (Chandrasoma and Taylor, 1991). Metastasis starts to occur once the cancer cells break away from a tumor and spread to other parts of the body through the blood or lymph system, settle in new places and form new tumors (Sukumar *et al.,* 1995). Patient at stage IV cancer where distant metastasis has occurred has only 10-20 % of the 5 year relative survival rate. Therefore, early detection of breast cancer is very important because the smaller the lesion the greater is the likelihood of cure. The currently available treatments for breast cancer are surgery, chemotherapy, hormone therapy, and radiation therapy. Although known as the standard for cancer treatment, they often has unpleasant side effects where patient will experience short term side effects of fatigue, nausea and vomiting, loss of appetite, hair loss, mouth sores, change in menstrual cycle, high risk of infection, easily bruising or bleeding and some irreversible effects of early menopause, pregnancy problems, birth defect, heart damage, chemobrain and the risk of leukemia. This has given the scientist and research group to find an alternative way for treatment of cancer.

Over the last 10 years, with the development of advanced molecular biology techniques, viruses of animal origin have been tested for viral therapy

(virotherapy) which has attracted many researchers, causing this field to be expended to a new dimension ever since. Scientists found that viruses have a unique oncolytic characteristic in which it can recognize cancerous cells and can kill the cells without destroying the normal mammalian cells (Vile *et al.,* 2002).

Newcastle disease virus (NDV) is an economically important avian paramyxovirus which causes a highly contagious and fatal disease in poultry, known as Newcastle disease (ND). It is a member of the family *Paramyxoviridae* and subfamily *Paramyxovirinae* (Seal *et al.,* 2002). It has a non-segmented, negative-sense single stranded RNA genome. The virus is pleomorphic and enveloped with projections of the hemaglutinin-neuraminidase (HN) and fusion (F) glycoproteins on its surface. In addition to these proteins, the virus also encodes other four structural proteins, nucleocapsid protein (NP), phosphoprotein (P), large protein (L), and the matrix protein (M) (Yusoff and Tan, 2001). NDV has been shown to exhibit outstanding anti-cancer effect on various cancers. The viral oncolytic properties play an important role in destroying cancerous cells than normal cells. Like all replication competent viruses, oncolytic viruses seek to infect host cells that express the appropriate cell-receptor, replicate in them and release the infectious progeny (Vidal *et al.,* 2006).

The intense interest in NDV as cancer treatment was originated in the 1960s. This interest was then branched in two directions. Csatary, (1971) was the first to

realize the oncolytic character of NDV where he noted complete remission of metastatic gastric cancer in chicken farmer during a severe outbreak of Newcastle disease within the chicken population and Dr Cassel who first was using the live virus then developed tumor oncolysate which contained killed viruses and worked on oncolysate ever since (Cassel and Garrett, 1965). Some NDV strains have been proven to kill and destroy cancer cells. Such NDV oncolysate strains, NDV strain 73-T, NDV strain PV 701, NDV strain *MTH-68/H*, NDV Strain *La Sota,* NDV strain Italian and NDV strain Ulster have already been tested on various cancers on human and were reported in the clinical literature describing tumor regression or complete remission in cancer patients during viral infection or immunization phase (Lorence *et al.,* 1994; Cassel and Garrett, 1965; Cassel and Murry, 1988; Russell, 2002; Csatary *et al.,* 2004; Schirrmacher *et al.,* 2007).

A Malaysian isolate Newcastle disease virus strain AF 2240 which is a very highly virulent strain in chickens have shown to be a potential candidate as anti-cancer agent for treatment of breast cancer (Narayani *et al.,* 2003). Many studies have been done to fully understand the characteristic of the virus as the virus recognize the sialic acid receptor on cancerous cell and will infect and kill the cell through apoptosis (Elankumaran *et al.,* 2008). The strong affinity of the virus towards cancer cells rather then normal mammalian cells makes the NDV a very effective oncolytic virus with high possibility to be used as anticancer agent for breast cancer treatment.

As results showed the potential of NDV AF 2240 in breast cancer treatment, the study on the virus have reached the pre-clinical stage where the study has covered most of the *in vitro* and *in vivo* parts (Narayani *et al.*, 2003: Mahani, 2006: Zolkapli, 2006). In order for the virus to be used in clinical trial phase, several requirements need to be fulfilled as required by FDA/ISO 10993, whereas one of the requirements is the biological evaluation of the agent particularly the genotoxicity of the virus. Although it is known that NDV is relatively safe for mammals including human (Kenney and Pagano, 1994, Narayani *et al.,* 2003), a complete study need to be done in order to confirm this hypothesis, where there is no direct relationship or minor effects between NDV AF2240 and the toxicity to normal mammalian cells. Evaluation on the genotoxicity effects of NDV AF2240 may reveal minute differences in mammalian cells tested and it is possible to correlate these genotoxicity effects to the virotherapy using NDV AF2240. It is hypothesized that NDV AF2240 does not cause any adverse effects *in vitro and in vivo.*

Therefore, the main objective of this study is to evaluate the genotoxicity effects of Newcastle disease virus strain AF 2240 in *vitro* and *in vivo.*

The specific objectives of this study are:

1) To determine the inhibitive concentration (IC_{50}) value of NDV AF2240 on 4T1 breast cancer cell line as well as normal mammalian cell line through Methylthiazol tetrazolium (MTT) assay.

2) To determine the effects of NDV AF2240 on DNA damage on mammalian cell *in vitro* using normal mammalian cell lines and breast cancer cell line via Comet assay.

3) To study the effect of NDV AF2240 on the reproductive toxicity on mammalian model *in vivo* using the ICR mice as animal model through bone marrow micronucleus assay.

4) To analyze the irritability effect of NDV AF2240 on mammalian eyes *in vivo* using albino rabbit as animal model via eye irritation test.

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