THE PROPHYLACTIC EFFECTS OF *NIGELLA SATIVA* AND THYMOQUINONE AGAINST CYCLOPHOSPHAMIDE TOXICITY ON REPRODUCTIVE CAPACITY AND EMBRYO DEVELOPMENT IN MICE

BY

SAHEERA BINTI KAMARZAMAN

A thesis submitted in fulfilment of the requirement for Doctor of Philosophy (Health Sciences)

Kulliyyah of Allied Health Sciences
International Islamic University Malaysia

AUGUST 2014

ABSTRACT

Advances in the treatment of cancer have allowed adolescent patients to become long-term survivors able to lead normal lives. However, the concern about the effects of anticancer drugs on fertility has led to many efforts to preserve germ cells of these patients. This study focuses on ways to assess the effectiveness of *Nigella sativa* and its active compound, thymoquinone, in lowering chemotherapeutic-associated toxicity of cyclophosphamide on the ovaries and testes of Balb/c mice. Histological and morphological effects on the germ cells were examined via microscopy techniques and possible fragmentations of sperm DNA was assessed using the COMET Assay. The RNA expression of OGG1 and FGF2 in the testicular cells was quantitatively analysed using the real-time PCR followed by quantification of the stages of fertilisation and embryo division using the inverted microscope. Supplementation of *N. Sativa* oil and thymoquinone exhibited noticeable protective effects on the histology and morphometry of the ovaries and seminiferous tubules as well as being effective in reducing the total DNA fragmentation in spermatozoa. *N. Sativa* extract reduced the percentage of abnormal sperm head post to cyclophosphamide treatment and preserved the normal chromatin condensation indicative of protection against sperm DNA alteration. The expressions of DNA repair and fibroblast growth factor genes were also shown to increase suggestive of possible reduction in mutagenic modifications. Thymoquinone supplementation increased the implantation and fertilisation rates, pregnancy outcome as well as preserved fair quality embryos following paternal and maternal exposures to cyclophosphamide. *N. Sativa* and thymoquinone are both–suitable exogenous agents that offer viable chemoprotective potential against toxicity induced by cyclophosphamide. This study is part of an effort towards improving interventions to preserve fertility and to assist in the development of techniques in achieving favourable reproductive outcomes for adults who survived childhood cancer following chemotherapy.

ملخص البحث

ان التقدم في علاج السرطان قد سمح للمرضي البالغين ان يصبحوا قادرين علي ممارسة حياة طبيعية على المدى الطويل.ومع ذلك، أدى القلق بشأن آثار الأدوية المضادة للسرطان على الخصوبة إلى الكثير من الجهود للحفاظ على الخلايا الجنسيه لهؤلاء المرضى.وتركز هذه الدراسة على طرق لتقييم فعالية العلاج بالأعشاب التقليدية مثل الحبه السوداء ومركبها الفعال الثايموكينون في خفض سمية العلاج الكيميائي المرتبط بالسيكلوفوسفاميد على المبيضين والخصيتين للفئران.تم فحص الآثار النسيجية والمورفولوجيه على الخلايا المنتشة عبر تقنيات الفحص المجهري وجرى تقييم الشظايا الممكنة من الحمض النووي للحيوانات المنوية باستخدام مقايسة الCOMET وقد تم التحليل الكمي لتعبير ال RNA الخاص ب OGG١ و FGF٢ لخلايا الخصيه باستخدام ال PCR ثم تلاه تحليل كمي لمراحل الأخصاب وانقسام الجنين باستخدام المجهر المقلوب.ولقد ثبت ان مكملات زيت الحبه السوداء ومركبها الفعال الثايموكينون أشارت الي آثار وقائية ملحوظه علي التحليل النسيجي والشكلي للمبيضين والأنابيب المنوية بالأضافه لفاعليتها في الحد من تجزئة الحمض النووي في الحيوانات المنوية.مستخرج الحبه السوداء خفض نسبة رؤس الحيوانات المنويه الغير طبيعيه بعد العلاج الكيمائى بالسايكلوفوسفامايد وحافظ علي التكثيف الطبيعي للكروماتين مما يدل علي الحمايه ضد تغيير الحمض النووي للحيوانات المنويه وكذلك إصلاح الحمض النووي وجينات الأورمه الليفيه لتقليل مخاطر التشوهات الخلقية.هذا بالضافه الي ان مستخرج الثايموكينون زاد معدل انغراس البويضه الملقحه ونتائج الحمل بالأضافه للحفاظ علي جودة الأجنه عن طريق تخفيض نسب عيوب الخلايا البلاستوليه ونسب تشدف الأجنه بعد تعرض الأب أو الأم للعلاج الكيمائي بالسايكلوفوسفامايد.الحبه السوداء ومركبها الفعال الثايموكينون لها خصائص كيميائيه وقائيه ضد السمية الناجمة عن العلاج الكيميائي.هذه الدراسة هي جزء من جهد من أجل تحسين التدخلات للحفاظ على الخصوبة والمساعدة في تطويرالتقنيات لتحقيق نتائج انجابيه إيجابية للبالغين الذين نجوا من لسرطان في مرحلة الطفولة بعد العلاج

APPROVAL PAGE

The thesis of Saheera Kamarzaman has been approved by the following:

_____
Suzanah Abdul Rahman
Supervisor

_____
Munirah Sha'ban
Co-Supervisor

_____
Muhammad Taher Bakhtiar
Internal Examiner

_____
Siti Amrah Sulaiman
External Examiner

_____
El-Fatih Abdullahi Abdelsalam
Chairman

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.

Saheera Kamarzaman

Signature..... Date.....

INTERNATIONAL ISLAMIC UNIVERSITY MALAYSIA

DECLARATION OF COPYRIGHT AND AFFIRMATION OF FAIR USE OF UNPUBLISHED RESEARCH

THE PROPHYLACTIC EFFECTS OF *NIGELLA SATIVA* AND THYMOQUINONE AGAINST CYCLOPHOSPHAMIDE TOXICITY ON REPRODUCTIVE CAPACITY AND EMBRYO DEVELOPMENT IN MICE

Affirmed by Saheera Binti Kamarzaman.

…… Signature

……. Date

In dedication to my loving father and mother whose affection, love, encouragement and prays of day and night make me able to get such success and honor.

ACKNOWLEDGEMENT

I would like to express my deepest appreciation to my supervisor, Associate Professor Dr. Suzanah Abdul Rahman, Deputy Dean of Academic Affairs, Kulliyyah of Allied Health Sciences (KAHS), International Islamic University Malaysia (IIUM) for her great guidance from the initial step of my study. Thank you for encouraging my research and for allowing me to grow as a research scientist. Your advice and brilliant comments have been priceless. You have been a tremendous mentor to me. My appreciation also goes to my co-supervisor, Assistant Professor Dr. Munirah Sha'ban, Deputy Dean of Postgraduate and Research, KAHS, whose contribution in stimulating suggestions and encouragement has helped me to coordinate my project wisely.

I take this opportunity to express my gratitude to the people who have been instrumental in the successful completion of this project. My utmost gratitude for the generous help of Dr. Norlelawati A. Talib from Kulliyyah of Medicine (KOM), IIUM, and Mr. Shashi Kumar from Roche Diagnostics (M) Sdn Bhd, for their kind assistance in RT-qPCR analysis, Madam Sri Viorwarti Noerdin from Kulliyyah of Pharmacy (KOP), IIUM, for the histology technical assistance and Madam Norhafizah Ab Manan from Faculty of Medicine (FOM), Cyberjaya University College of Medical Sciences (CUCMS), for her assistance with statistical analysis. I also would like to extend my thanks to the technical staff of KAHS for their constant support and help.

My sincere thanks to the International Islamic University Malaysia for the financial support granted through the research endowment funds.

Many thanks to my fellow friends at KAHS, KOM and KOP of IIUM, as well as my supportive colleagues at CUCMS for a very pleasant, happy and memorable time that we share together during the last three years.

I take this moment to express my never ending love and respect to my beloved parents for their prayers and blessings. I express my sincere appreciation to my brothers and sisters for all their affection showered on me. To my dear husband, Dr Hazem Foda, thank you for the unfailing love, support and continuous encouragement throughout the process of writing this thesis. I will be forever grateful for your love.

Above all, I owe it all to the Almighty Allah for granting me the wisdom, health and strength to undertake this research work and enabling me to its completion.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATION

ACTB	Actin, beta
AIF	Apoptosis Inducing Factor
ALDH	Aldehyde dehydrogenase
ANOVA	One-way Analysis of Variance
AO	Acridine orange
APE1	AP endonuclease 1
ART	Assisted Reproductive Techniques
ARE	Antioxidant Response Element
ATP	Adenosine Triphosphate
BER	Base Excision Repair
BHA	Butylated Hydroxyanisole
BM	Basement Membrane
CAT	Catalase
CCl_4	Carbon tetrachloride
cDNA	Complementary DNA
CHEK1	Checkpoint Kinase 1 Homolog
COC	Cumulus-Oocyte-Complex
COS31	Osteosarcoma Cancer Cells
CPA	Cyclophosphamide Monohydrate
CP	Crossing Point
CT	Cycle Threshold
CYTOXAN®	Cyclophosphamide Monohydrate
DEHP	Di(2-ethylhexyl)phthalate
DNA	Deoxyribonucleic Acid
E2	17β-extradiol
EDTA	Ethylenediaminetetraacetic Acid
ERK	Extracellular Signal-Regulated Kinases
FGF2	Fibroblast growth factor 2
FGFRs	Fgf receptors
FSH	Follicle-Stimulating Hormone
G	Guanine
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GM	Gentamicin
GPx	Glutathione Peroxidase
GSH	Glutathione
hCG	Human Chorionic Gonadotrophin
HEPES	Hydroxyethyl piperazineethanesulfonic acid
HepG2	Hepatocellular carcinoma
H&E	Haematoxylin and Eosin
ICR	Imprinting Control Region
ICSI	Intra-Cytoplasmic Sperm Injection
IP	Intraperitoneal
IUI	Intrauterine insemination
IVF	*In Vitro* Fertilisation

KFU	King Faisal University
LD50	Lethal Dosage
LDL	Low-Density Lipoprotein
MAPK	Mitogen-Activated Protein Kinases
MDA	Malondialdehyde
MKP3	MAPK Phosphatase 3
MRM	Multiple Reaction Monitoring
MOH	Ministry of Health
NaCl	Sodium Chloride
NaOH	Sodium Hydroxide
NAPDH	Nicotinamide adenine dinucleotide
NCR	National Cancer Registry
NO	Nitric Oxide
NOx	Nitrate/ Nitrite
NRF2	Nuclear Factor Erythroid-derived 2
NSE	*Nigella sativa* Extract
NSO	*Nigella sativa* Oil
NTC	No-Template Control
OD	Optical Density
OGG1	8-oxoguanine DNA-glycosylase 1
PASMC	Pulmonary Arterial Smooth Muscle Cells
PBS	Phosphate Buffered Saline
PBUH	Peace Be upon Him
PCR	Polymerase Chain Reaction
PGE2	Prostaglandin E2
PMF	Primordial Follicle
PPM	Parts Per Million
RFU	Relative Fluorescence Units
RNA	Ribonucleic Acid
ROS	Reactive Oxygen Species
RPM	Revolutions Per Minute
RT-PCR	Real Time Polymerase Chain Reaction
SCSA	Sperm Chromatin Structure Assay
SD	Standard Deviation
SER	Smooth Endoplasmic Reticulum
SOD	Superoxide dismutase
TBARS	Thiobarbituric acid-reactive substances
TE	Tris-EDTA
THQ	Thymohydroquinone
TL	Theca layer
TUNNEL	Terminal deoxynucleotidyl transferase (TdT) dUTP Nick-End Labelling
TQ	Thymoquinone
TQ_2	Dithymoquinone
UHPLC	Ultra High Performance Liquid Chromatography
WHO	World Health Organization
ZP	Zona Pellucida
8-oxoG	8-oxoguanine
β-ME	β-mercaptoethanol

l-NAME	N (omega)-nitro-l-arginine methyl esters
P815	Mouse lymphoblast-like mastocytoma cell

CHAPTER ONE

INTRODUCTION

1.1 BACKGROUND

The advancement in the field of oncology and the increase in treatment modalities improved the survival rates of both adult and childhood cancers. A report from the National Cancer Registry (NCR) Malaysia and Ministry of Health Malaysia (MOH) showed a total of 18,219 cancer cases diagnosed among Malaysians in Peninsular Malaysia in the year of 2007; 44.6% of males and 55.4% of females were under age 60 years with breast cancer being the most common cancer among women accounting for 32.1% and cancer of the trachea, bronchus and lung in men with 16.3%. A total of 319 and 220 of childhood cancer cases in boys and girls, respectively were diagnosed in 2007 and registered with NCR. Leukaemia is reported as the most frequent cancer in children between the age of 0 to 14 years old in Malaysia; 48% in boys and 44.5% in girls (Ministry of Health, 2011). In a National Childhood Cancer Survey conducted by Lin (1999), the crude incidence rate of paediatric malignancies in Malaysia was 77.4 per million children aged less than 15 years old, with leukaemia (35%) being the most common childhood tumours. The prevalence of the incidence has increased to more than 10% in eight years. The impact of cancer treatments on the quality of life especially fertility is greater than generally perceived. It is increasingly noted that a high number of adult survivors of childhood cancer malignancies were not aware of the risk of infertility and relevant late effects (Hess et al., 2011). Based on the types of cancers seen in these young age groups, many of

them are still of reproductive age and approximately half of them may have received cancer treatment that would have a major impact on their future reproductive capacity.

Many environmental conditions can cause DNA fragmentation such as chemotherapy, radiation, drugs prescription, chemicals, smoking and Assisted Reproductive Techniques (ART) preparation protocols (Fossa et al., 1997; Rignell-Hydbom et al., 2005; Rubes et al., 2005). Chemotherapeutic agents have been shown to cause significant systemic toxicity due to the overproduction of reactive oxygen species (ROS) that cause oxidative stress (Pryor et al. 2000; Meirow and Nugent 2001b; Mitchell et al., 2003, Alenzi et al., 2010). ROS are oxygen-derived free radicals that are formed during the intermediate steps of oxygen reduction (Agarwal and Allamaneni, 2004a). They can cause direct oxidation by combining with other molecules which can lead to structural and functional changes, and result in cellular damage (Guerin et al., 2001; Agarwal et al., 2005b). In the event of excessive ROS production exceeding the antioxidant defence mechanism of the cells, it results in oxidative stress accompanied by other adverse effects (Park et al., 2010). ROS activity plays a major role in DNA strand breakage which can be attributed to significant deleterious effects on the reproductive outcome.

Anticancer drugs, in general, are mutagenic as they can interfere with DNA metabolism (John and Timothy, 2007). There is a strong indication that DNA damage could play an important role in male fertility and reproduction (Zitzmann et al., 2003). The commonly used anticancer drug, cyclophosphamide (CPA), is one of the most damaging alkylating agents that affect the DNA of replicating cells and rapidly multiplying cells especially in the gonads and pituitary which results in miscoding, cross-linking and DNA breakage. It acts by the transfer of alkyl groups to the guanine compound of the DNA (Becker and Schoneich, 1982). The genotoxic effects of

cyclophosphamide on male germ cells showed that it can reach the spermatogonia in significant quantities. Germ cells are specifically sensitive to cyclophosphamide treatment due to its high proliferating activity (Jarrell et al., 1991).

Spermatogenesis is one of the most productive self-renewing systems of which four to sixty million spermatozoa are produced daily per gram of testis tissue in mammalian species (Hess and De Franca, 2008). However, cell death during the process reduces the final production of sperm substantially (Russell and Clermont, 1977). Stem cells persist throughout the reproductive life and are constantly under attack from DNA-damaging agents produced by endogenous and exogenous agents (Lindahl, 1993). They can accumulate a large amount of chemical exposure and may result in cell death if not repaired. Cells that survived radiation or chemicals continue to divide and differentiate (Oakberg, 1974) and further result in mutations and transmitted to the offspring if not repaired by the time of replication. Once the unrepaired lesions in male germ cells are transmitted to the zygote, it may lead to fetal death (pre- or post-implantation loss). New mutations that developed in the paternal genome also will not be eliminated in the fertilised egg.

Administration of cyclophosphamide has been demonstrated to cause oligospermia, azoospermia, testicular damage and germ cell toxicity in male rodents (Elangovan et al., 2006; Tripathi and Jena, 2008a). It induces defects in mice foetuses (Khaksary et al., 2012) and increases the incidence of pre-implantation loss (Trasler et al., 1987). Recent studies have investigated the deleterious effects of cyclophosphamide on chromosomal aneuploidy (Barton et al., 2003) and chromatin condensation (Codrington et al., 2007). Previous studies have reported significant correlations between chromatin abnormalities and morphological alterations (Sailer et al., 1996; Ferrari et al., 1998; Ostermeier et al., 2001). Mammalian sperm heads

consist almost totally of chromatin. Oxidative stress is associated with DNA strand breaks (Manicardi et al., 1998). Therefore, the loosely condensed sperm chromatin that suffers DNA damage will lead to the weakening of the sperm chromatin condensation following alterations in the relative proportion of protamines (Brewer et al., 1999). DNA denaturation has important implications on fertility outcomes. Some sperms with chromatin abnormalities are able to fertilise oocytes *in vivo* and *in vitro*, but the DNA damage can persist throughout the embryonic period which will induce apoptosis and embryo fragmentation that can ultimately lead to abortion (Ellington et al., 1998; Twigg et al., 1998b).

In the females, cyclophosphamide can induce ovarian damage by destructing the ovarian follicles (Meirow et al., 1999; Meirow and Nugent, 2001b) that leads to depletion in the primordial follicular (PMF) reserve. It affects all age groups with older females appears to be more affected as they have a smaller ovarian follicular reserve. Since PMF pool is non-renewable, older women treated with chemotherapy have a higher incidence of ovarian failure when the chemotherapeutic agent destructs an already low follicular reserve needed to sustain ovarian function (Kumar et al., 1972; Gosden and Faddy, 1994).

Cyclophosphamide exposure was previously reported to alter the expression of stress response genes in male germ cells such as DNA repair, antioxidant defense and heat shock protein, which are regulated during germ cell development (Aguilar-Mahecha et al., 2001a). Paternal exposure to cyclophosphamide was also found to decrease the expression of DNA repair genes of the base excision repair (BER) pathway in rat pre-implantation embryo (Harrouk et al., 2000a) and alter the expression profile of specific gene in embryo (Harrouk et al., 2000b; 2000c). Alkylating agents and oxygen radicals commonly caused lesions in the DNA double