

Nigella sativa AND ITS DERIVATIVE
THYMOQUINONE AMELIORATES THE NEGATIVE
IMPACT OF CYCLOPHOSPHAMIDE ON MOUSE
MALE GERM CELLS AND EMBRYO

BY

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

Kulliyyah of Allied Health Sciences
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JANUARY 2020

ABSTRACT

Chemotherapy drugs like cyclophosphamide (CPA) is an alkylating agent that can cause damage to the male germ cells and follicles of the gonads resulting in adverse reproductive problems and infertility. This study aims to investigate any potential to salvage the embryo from the toxic paternal exposure with the use of *Nigella sativa* extract (NSE) and thymoquinone (TQ). The survival and damage of embryos following fertilization from sperm exposed to CPA were studied in order to investigate the protective effects of NSE and TQ on male mice at 8 to 10 weeks of age according to the groupings as follows: i) control, ii) CPA only, iii) NSE only, iv) TQ only, v) CPA+NSE and vi) CPA+TQ, as well as supplementation of NSE (5mg/ml, 10mg/ml, and 15mg/ml) and TQ (1 μ M, 10 μ M and 100 μ M) in the culture media of developing embryo. Non-invasive embryo examination was based on simple methods of observation focused on morphology and dynamics of embryo development under the inverted microscope without fixation and staining. The level of embryo glucose uptake was then determined to evaluate the quality of the preimplantation embryos while quantitative polymerase chain reaction (Q-PCR) and immunofluorescence methods were employed to investigate the tendency of the embryos whether to survive or undergo apoptosis. In addition, the effects of NSE and TQ supplementation in the *in vitro* fertilization (IVF) culture media were also investigated. The results obtained from the preliminary study involving Balb/c has shown that the introduction to NSE and TQ have potential protective effects on the development of the embryo *in vitro* with a significant ($p < 0.05$) in the percentage of motile sperm, number of fragmented DNA, sperm morphology, sperm head abnormalities and fertilization rate. NSE and TQ were also seen to improve the embryo quality in terms of embryo grading based on the fragmentation and structure of blastomere. The experiment using Swiss bred mice strain (ICR) has proved that treatment with NSE and TQ has a significant positive impact ($p < 0.05$) on sperm motility, reduction in the number of abnormal sperm and percentage of sperm head abnormalities. Study on the embryo metabolites, indicates that there is no significant difference ($p > 0.05$) in the level of glucose uptake in the culture media between the groups. In addition, molecular study which investigated the presence of five genes important in embryo development (*Bcl-xL*, *BAX*, *SOX2*, *Oct4*, and *CD29*), revealed that there are no significant differences ($p > 0.05$) in the levels of gene expression. The molecular findings were confirmed with immunofluorescence staining of the embryo for the detection of *Bcl-xL*, *BAX*, and *Oct4*. NSE and TQ supplementation in the culture media showed no effect on the embryo quality. Results from the *in vivo* experiments demonstrated the potential of NSE and TQ in the paternal protection from the alkylating effects of CPA to allow for the normal development of the embryo.

خلاصة البحث

تعتبر ادوية العلاج الكيميائي مثل سيكلوفوسفاميد CPA ذو البيئة القلوية ضارا لخلايا المنسل (ذكرية او وبصيلات الغدد التناسلية مما يؤدي إلى مشاكل التكاثر الضار والعقم. تهدف هذه الدراسة إلى التحقيق في إمكانية إنقاذ الجنين من التعرض الأبوي السام من خلال إستخدام مستخلص نيجيلا ساتيفا (NSE) وثيموكينون (TQ). بقاء وتلف الأجنة بعد الإخصاب من الحيوانات المنوية تمت دراستها من أجل تفصي الآثار الوقائية ل NSE و TQ على الفئران الذكور التي تبلغ 8 إلى 10 أسابيع من العمر وفقا للتجمعات على النحو التالي: 1) السيطرة ، 2) CPA ، 3) NSE فقط ، 4) TQ فقط ، 5) CPA+NSE ، 6) CPA+TQ بالإضافة الى مكملات من NSE (ملغ / مل ، 10 ملغ / مل و 15 ملغ / مل) و TQ (1 μ M ، 10 μ M و 100 μ M) في الثقافة وسائل الإعلام لتطوير الجنين. واستند الفحص الجيني غير الغازي اعتمدت على طرق بسيطة في الملاحظة التي ركزت على التشكل وديناميات تطور الجنين تحت المجهر المقلوب دون تثبيت وتلطبخ. مستوى امتصاص الجلوكوز في الأجنة تم تحديده لتقييم جودة الأجنة قبل الزرع وكانت سلسلة تفاعل البلمرة الكمي (Q-PCR) وطرق المناعي التي تستخدم للتحقيق في ميل الأجنة سواء للبقاء على قيد الحياة وموت الخلايا المبرمج. بالإضافة إلى ذلك ، آثار مكملات NSE و TQ في الإخصاب في المختبر تم التحقيق أيضا (IVF) وسائل الإعلام الثقافة. النتائج التي تم الحصول عليها من الدراسة الأولية التي أجريت على أساس البالـب / ج قد أظهرت أن NSE و TQ لها آثار وقائية محتملة على تطور الجنين في المختبر مع وجود ($p < 0.05$) نسبة مئوية كبيرة في الحيوانات المنوية المتحركة وعدد الحمض النووي المجزأ ومورفولوجيا الحيوانات المنوية وتشوهات رأس الحيوانات المنوية ومعدل الإخصاب. كما لوحظ أن NSE و TQ عمل على تحسين جودة الجنين من حيث تصنيف الجنين بناءً على التجزئة و الهيكل. أثبتت التجربة التي أجريت على سلالة الفئران السويسرية المرباة (ICR) أن العلاج باستخدام NSE و TQ له تأثير إيجابي ($p < 0.05$) كبير على حركية الحيوانات المنوية ، وتخفيض عدد الحيوانات المنوية غير الطبيعية والنسبة المئوية لتشوهات رأس الحيوان المنوي. تشير الدراسة التي أجريت على مستقلبات الأجنة إلى أنه لا يوجد فرق كبير ($p > 0.05$) في مستوى امتصاص الجلوكوز في أوساط الثقافة بين المجموعتين. بالإضافة إلى ذلك ، الدراسة الجزيئية التي تحققت من وجود خمسة جينات مهمة في تطور الجنين (*Bcl-xL*, *BAX*, *SOX2*, *Oct4* and *CD29*) كشفت أنه لا توجد اختلافات كبيرة ($p > 0.05$) في مستويات التعبير الجيني. تم تأكيد النتائج الجزيئية بتلوين مناعي للجنين للكشف عن *Bcl-xL* و *BAX* و *Oct4* وأظهرت مكملات NSE و TQ عدم وجود أي تأثير على جودة الجنين. وأظهرت النتائج من التجارب في الجسم الحي إمكانات NSE و TQ في حماية الأب من الآثار المؤلدة لل CPA للسماح للتطور الطبيعي للجنين.

APPROVAL PAGE

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DECLARATION

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ACKNOWLEDGEMENTS

In the name of Allah, The Most Beneficent, The Most Merciful. Alhamdulillah, with His blessings, finally, I manage to complete my thesis after the hard work, blood, sweat and tears spent during the whole study period.

First and foremost, I would like to express my deep and sincere gratitude to my parents, Hj Abdul Samat and Hjh Salihah, my lovely husband, Nabil Buhler, for their infinity supports throughout the study. Secondly, I would like to present my humble appreciation to my supervisors, Assoc. Prof. Dr. Suzanah Abdul Rahman and Asst. Prof. Dr. Lokman Md Isa for their great effort to guide me and sharing their wonderful knowledge trough out the research.

I take this opportunity to express my gratitude to the people who have been instrumental in the successful completion of this project. My best colleagues; Amalina, Syazana, Raihan and Dr. Azantee Yazmie for assisting the labwork, Dr. Norazsida Ramli who always give her hand in doing statistical analysis.

I wish to thank my wonderful friends, and all my postgraduate friends who always be my bread and butter, be together with me with laughter and tears. Allah's bless will always with you guys and in sha allah you will success here and hereafter. Last, but not least, thank you to my wonderful siblings; Najwa Hanis, Nabila Hanis and Nabihah Hanis, not forgotten, to in laws, my cute niece and nephew, for their encouragement and moral support during the production of this dissertation.

I dedicate this thesis for my beloved children Muhammed, Noor and Hassan, my husband, Nabil Buhler and my parents whose affection, love, encouragement and prays of day and night make me able to get such success and honor.

Thank you Allah for Your wonderful gift,

Wassalam

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LIST OF ABBREVIATIONS

ART	Assisted Reproductive Technology
BAX	BCL2 Associated X
<i>Bcl-xL</i>	B-cell lymphoma-extra large
<i>CD29</i>	Integrin beta-1
COC	Cumulus Oocyte Complex
CPA	Cyclophosphamide
CTCF	Corrected total cell fluorescence
DNA	Deoxyribonucleic Acid
ICSI	intracytoplasmic sperm injection
IF	Immunofluorescence
IVF	<i>In vitro</i> Fertilization
NSE	<i>Nigella sativa</i> extract
<i>OCT4</i>	octamer-binding transcription factor 4
PBUH	Peace Be Upon Him
RNA	ribonucleic acid
ROS	Reactive Oxygen Species
RT-qPCR	Real-time quantitative polymerase chain reaction
<i>SOX2</i>	SRY (sex determining region Y)-box 2
TQ	Thymoquinone

CHAPTER ONE

INTRODUCTION

1.1 RESEARCH BACKGROUND

Statistics has reported that cancer cases in Malaysia as well as worldwide has been increasing with time. Ministry of Health has reported in Malaysian National Cancer Registry Report 2007 to 2011 that 103,507 number of new cancer cases recorded in Malaysia for the period of 2007 to 2011 (Azizah, Nor Saleha, Noor Hashimah, Asmah, & Mastulu, 2016). It is mentioned in the report that the most common cancer among Malaysians were breast cancer, followed by colon and lung cancer and the cases is high in the patients with an age range 20–60 years old. There were 3,829 childhood cancer cases (0–18 years old) reported in 2007–2011 where 56% were happened in male while the rest 44% in female. The highest number of cancer is leukemia where 47% cases were reported in males and 45.5% in females (Azizah et al., 2016).

Advanced technology in medical science resulted with different types of cancer treatment, including chemotherapy using anticancer drugs. Studies have demonstrated that chemotherapeutic agents cause injury and destruction to germ cells and follicles of the gonads resulting in adverse reproductive consequences including premature menopause and sterility (Barekati, Gourabi, Valojerd, & Yazdi, 2008). A large number of patients survived cancer treatment and many of them are still of reproductive age. Concerns on fertility effects of anticancer drugs have led to many efforts of germ cell preservation. Our goal is to assess the survival and damage of embryos following fertilization from sperm produced from germ cells, which have

been exposed to the damaging alkylating effects of the anticancer agent cyclophosphamide (CPA).

There are several conditions that can lead to DNA damage such as chemotherapy, exposure to chemicals, cigarette smoke as well as environment employed during *in vitro* fertilization (IVF) (Sakkas & Alvarez, 2010). Drugs used in chemotherapy have been proven to produce reactive oxygen species (ROS) and induce oxidative stress thus lead to DNA fragmentation. ROS can cause direct oxidation with DNA molecules and lead to structural and functional changes as well as deleterious effects on the reproductive system (Mousavi, Tayarani-Najaran, Asghari, & Sadeghnia, 2010).

Chemotherapy drugs usually come with adverse effect as it also causes damage to other normal growing cells other than cancer cells. CPA is one of the common drug used in chemotherapy treatment and it is a bio activated metabolite and an alkylating agent. CPA cause damage to the DNA by the alkylation at N7 position on guanine and the formation of DNA-protein crosslink, DNA-DNA crosslink and also single-strand breaks (Kim W., Kim S., Park, & Chang, 2012). Besides its therapeutic effects, CPA was proven with a wide range of adverse effect, including causing reproductive toxicity either in human or experimental animals (Nadia Hanis, Nur Amalina, & Raihan, 2017; Alenzi, El-Bolkiny, & Salem, 2010; Saheera Kamarzaman, Azzanti Yazmie, & Suzanah, 2014).

Nigella sativa or *habbatus sauda'* is a known spice used widely, especially in the Middle East in food preparation as well as alternative treatment for various types of disease. Previous studies have proven that the high content of antioxidant is one of the reasons behind its benefits and its active compound, thymoquinone (TQ) shows the similar bioactivity with the extract itself. Therefore, the effects of *N. sativa* extract

(NSE) and TQ, in the cultivation of the embryo as potential nurturing agents will also be investigated.

The noninvasive embryo examination was initially be based on simple methods of observation focused on morphology and dynamics of embryo development under the inverted microscope without fixation and staining. The level of embryo glucose uptake is then determined to evaluate the quality of the pre-implantation embryos while fluorescence methods was employed to investigate the tendency of the embryos whether to survive or undergo apoptosis.

The output derived from this research is expected to provide a mechanistic understanding of alteration to the normal embryo development following paternal chemical exposure. Novel adjustments to the techniques and methods of evaluation such as these would provide an edge to conventional methods of *in vitro* and *in vivo* fertilization possibly leading to significantly enhanced birth rates and pregnancy success. Cancer patients would therefore have an alternative solution to alleviate fertility setbacks towards an improved quality of life.

CHAPTER TWO

PROBLEM STATEMENT, HYPOTHESIS, OBJECTIVES, AND EXPERIMENTAL DESIGN

2.1 PROBLEM STATEMENTS

One of the negative effects of chemotherapy drug which was widely used for cancer treatment is induction of infertility as it causes damage in the germ cell. Statistic reported that a large number of patients that survived from cancer with chemotherapy are still under reproductive age (Ab Manan et al., 2016). Therefore, our concern is to preserve the germ cells in order to prevent infertility, thus increase the chance for the cancer survivor to produce offspring.

The list of benefits of NSE and TQ as treatment for various morbidities has become one of the reason for this study being conducted as it is very interesting to know if this prophetic herb has the potential to significantly improve the embryo quality and repair the damage caused by the chemotherapy drug, CPA. High antioxidant level in both *N. sativa* and TQ might be the cause of positive effects by improving the quality of sperm and embryo as well as preventing damage from happen.

IVF is one of the advanced technologies applied in order to assist couple who cannot conceive naturally due to several reproductive problems. The quality of embryo produced by IVF is crucial in order to make the procedure successful and results in a healthy baby. There are many factors that might disturb the process of quality embryo production including the sperm and oocyte itself and the environment of the culture. NSE and TQ are proven to contain a significant high antioxidant level and this making them as free radicals scavenger and protect cellular damage due to

oxidative stress. This will help to increase production of good quality embryos in IVF procedures as well as increase the chance to produce offspring among chemotherapy survivor.

Animal study was adopted in this research by using mouse sperm, oocyte and embryo in order to understand the mechanism of how NSE and TQ might improve the quality of the embryo prior to the toxic paternal exposure to the alkylating agent, CPA.

2.2 HYPOTHESIS

- i. Paternal supplementation with *N. sativa* and TQ might protect the embryo from damage by the alkylating agent and improve the quality of the embryo.
- ii. NSE or its active component TQ incorporated in the culture medium may enhance the viability of the embryo in culture in tandem with increased embryo glucose uptake level and improved embryo development following paternal exposure to the alkylating anticancer agent CPA.

2.3 GENERAL OBJECTIVE

This study was conducted in order to determine the potential of *N. sativa* and its derivative, TQ, in protecting the male germ cells and the embryo following the paternal exposure of CPA in animal model.

2.4 SPECIFIC OBJECTIVES

- i. To analyse the damage caused by CPA on sperm quality and to determine the potential of NSE and TQ in protecting the sperm from the oxidative-stress effect.
- ii. To assess the effects of NSE and TQ on fertilization rates and the quality of mouse embryos *in vitro* following paternal exposure to alkylation injury.
- iii. To determine the quality of embryo and their ability to survive by the embryo's morphology, glucose intake evaluation and specific genes and proteins expression.
- iv. To investigate the effect of NSE and TQ supplementation in the embryo culture media on the embryo quality and development following paternal-mediated CPA exposure by the evaluation of embryo's glucose uptake and specific genes and proteins expression.

2.5 EXPERIMENTAL DESIGN

This study was conducted in three main divisions; i) Preliminary study: The effects of NSE and TQ in the Balb/c mouse sperm and the embryo quality prior to the CPA exposure, ii) Part One: The potential of NSE and TQ in salvaging the ICR mouse embryo from toxic paternal exposure to CPA, and iii) Part Two: The effect of NSE and TQ supplementation in culture media on the mouse embryo quality. Brief note about the study phases can be obtained from Figure 2.1.

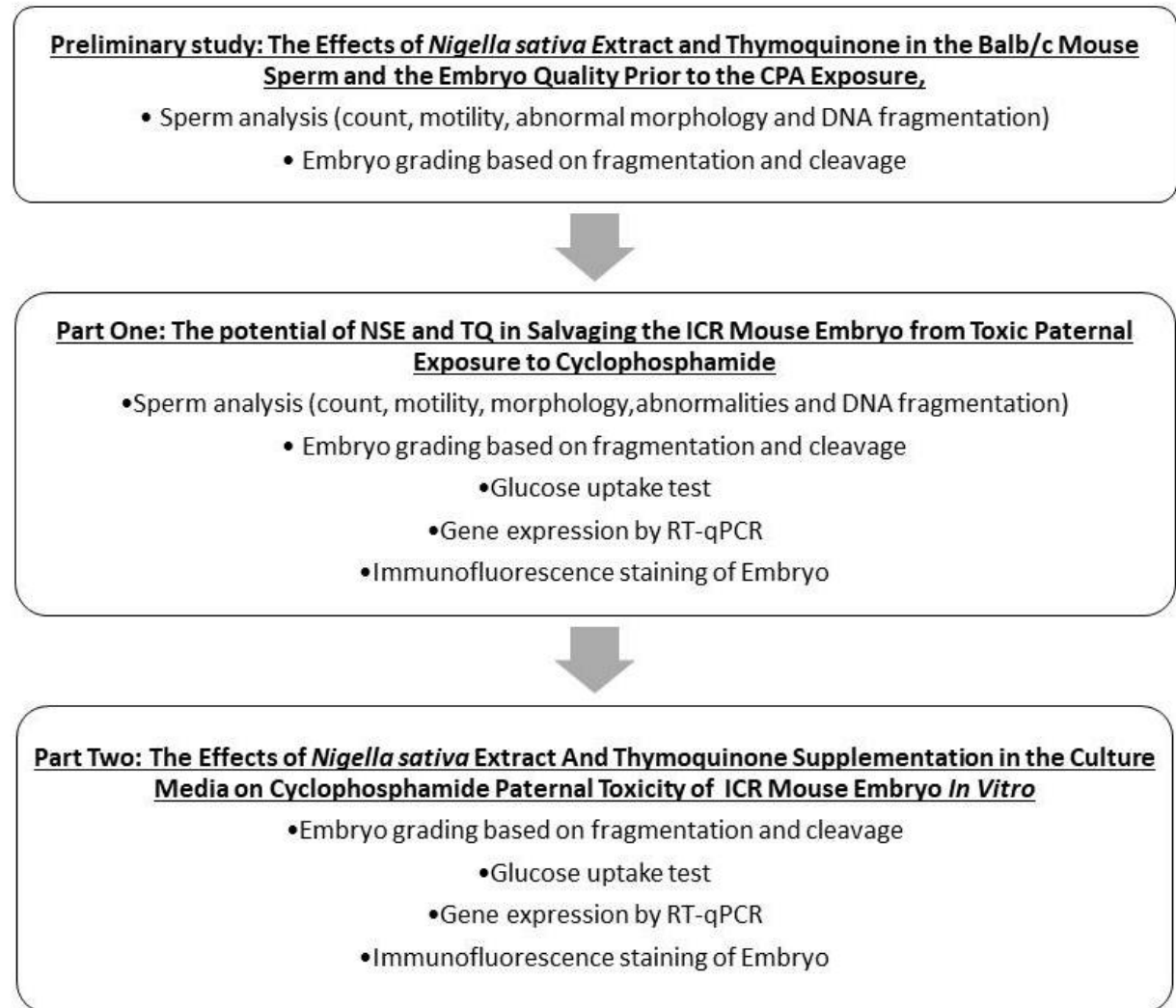


Figure 2.1 Flowchart of the experimental design