# THE EFFECTS OF THYMOQUINONE AND ARTIFICIAL LIGHT EXPOSURE ON RAT FERTILITY

BY

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# ABSTRACT

Infertility is a common reproductive disorder in both men and women. Urban lifestyle has compelled people to consume excessive and unnecessary supplements and also caused disruption of sleep cycle which resulted in unhealthy lifestyle that can cause the society to be infertile. Moreover, the battery of invasive diagnostic tests has relatively deterred men from seeking assistance and treatment for their infertility issues. A rapid and reliable test making use of non-invasive samples will be sought by both patients and clinicians alike. The purpose of the study was to observe the effects of subchronic thymoquinone administration and prolonged artificial light exposure to Sprague-Dawley rats. This also includes detection of testosterone as male fertility biomarker from non-invasive samples using paper-based lateral flow assay (PLFA) strip with smartphone usage. Adult male Sprague-Dawley rats were equally divided into six groups (n=6). One group was used as a negative control with no intervention. The positive control group was treated with corticosterone (10 mg/kg) for 10 successive days, followed by a single dose of cisplatin (10 mg/kg), all prior to the day of sacrifice. The next three groups were given thymoquinone via intraperitoneal route at doses of 5 mg/kg, 10 mg/kg and 30 mg/kg, twice a week. The final group was exposed to a 24 hours cycle of artificial fluorescent light to induce stress. All the interventions were given within a 56 days period including sampling of blood, saliva and urine at day 0 and day 56 respectively All the animals were sacrificed at day 56 to harvest the testes. Testosterone and corticosterone hormone levels in the blood, saliva and urine were measured using enzyme-linked immunosorbent assay (ELISA) kit. Sperm analysis parameters were measured followed by a histological assessment of the testes using haematoxylin and eosin (H&E) stain. Paper-based lateral flow assay strip was then developed based on the colour change from antigen-antibody reaction on paper which reflects the testosterone levels from urine samples. The colour change was captured using a smartphone camera and an applications that captured the RGB colour value. The results were then compared with ELISA test data. The results showed that the thymoquinone supplementation at 30 mg/kg subchronically can reduce the testosterone level and thus affecting fertility ($p<0.05$). Meanwhile, in the 24-hour light exposure group, no significant effect of reduced fertility or increasing stress were detected compared to controls. The B value from RGB colour was chosen to measure the testosterone as it have higher correlation ($r2=0.98$) than R and G value. This testosterone level assessment using the paper-based colourimetric test produced comparable data with ELISA result. Therefore, this sensor holds the potential of testing infertility in men more efficiently using non-invasive samples while at the same time increasing compliance in the sampling approach.

# خلاصة البحث

العقم هو اضطراب تناسلي مشترك في كل من الرجال والنساء. وقد أجبر نمط الحياة في المناطق الحضرية الناس على تناول المكملات المفرطة وغير الضرورية، كما تسبب في اضطراب دورة النوم مما أدى إلى نمط حياة غير صحي يمكن أن يتسبب بأن يكون المجتمع عقيما. وعلاوة على ذلك، فإن كثرة الاختبارات التشخيصية المجتاحة قد ردع الرجال نسبيا من طلب المساعدة والعلاج لقضايا العقم. وسيتم طلب اختبار سريع وموثوق به عن طريق استخدام عينات غير مجتاحة من قبل كل من المرضى والأطباء على حد سواء. وكان الغرض من هذه الدراسة هو مراقبة آثار الثيموكينون تحت المزمن والتعرض للضوء الاصطناعي لفترات طويلة لجرذان سبراغ-داولي. ويشمل هذا أيضا الكشف عن هرمون تستوستيرون كعلامة بيولوجية للخصوبة عند الذكور من عينات غير مجتاحة باستخدام شريط فحص التدفق الجانبي الورقي (بلفا) مع استخدام الهاتف الذكي. تم تقسيم ذكور فئران سبراغ-داولي البالغين إلى ست مجموعات (ن = 6). واستخدمت إحدى المجموعات كسيطرة سلبية دون تدخل. تم التعامل مع مجموعة السيطرة الإيجابية مع الكورتيزون 10ملغم/كغ لمدة 10 أيام متتالية، تليها جرعة واحدة من سيسبلاتين 10ملغم/كغ ، وكلها كانت قبل يوم التضحية. أعطيت المجموعات الثلاث التالية التيموكينون عن طريق اداخاله في الصفاق بجرعات من 5 ملغم/كغ ، 10ملغم/كغ و30 ملغم/كغ ، مرتين في الأسبوع. تعرضت المجموعة النهائية لدورة 24 ساعة من الضوء الفلورسنت الاصطناعي للحث على الإجهاد. وقد أعطيت جميع التدخلات في غضون 56 يوما بما في ذلك أخذ عينات من الدم واللعاب والبول في اليوم 0 واليوم 56 على التوالي. تم تضحية جميع الحيوانات في اليوم 56 للحصول على الخصيتين. تم قياس هرمون التستوستيرون ومستويات هرمون الكورتيزون في الدم واللعاب والبول باستخدام المقايسة المناعية المرتبطة بالانزيم (إليسا). تم قياس التحاليل للحيوانات المنوية يليه تقييم النسيج للخصيتين باستخدام صبغة هيماتوكسيلين ويوزين(H & E) . بعد ذلك تم تطوير شريط فحص التدفق الجانبي الورقي على أساس تغير اللون من تفاعل مستضد للأجسام المضادة على الورق الذي يعكس مستويات هرمون التستوستيرون من عينات البول. تم التقاط تغيير اللون باستخدام كاميرا الهاتف الذكي وتطبيق التي التقظ قيمة RGB للون. ثم تمت مقارنة النتائج مع بيانات اختبار إليسا. وأظهرت النتائج أن مكملات الثيموكينون في 30 ملغم/كغ دون تزامن يمكن أن تقلل من مستوى هرمون التستوستيرون وبالتالي تؤثر على الخصوبة. وفي الوقت نفسه، في مجموعة التعرض للضوء لمدة 24 ساعة، لم يتم الكشف عن أي تأثير كبير من انخفاض بالخصوبة أو زيادة بالتوتر مقارنة مع الضوابط. وأدى تقييم مستوى هرمون التستوستيرون باستخدام اختبار اللونية الورقية إلى بيانات قابلة للمقارنة مع نتائج إليسا. لذلك، هذا الاستشعار يحمل القدرة على اختبار العقم لدى الرجال أكثر كفاءة باستخدام عينات غير المجتاحة وفي نفس الوقت زيادة الامتثال في نهج أخذ العيناتخ.

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# 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 Health Sciences

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# DECLARATION

I hereby declare that this dissertation 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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# LIST OF ABBREVIATIONS

| | |
|---|---|
| ANOVA | Analysis of Variance |
| BTB | Blood Testis Barrier |
| EDC | Endocrine Disruptors Chemical |
| ES | Ectoplasmic Specialization |
| ELISA | Enzyme-Linked Immunoassay |
| FSH | Follicle-stimulating Hormone |
| GC-MS | Gas Chromatography Mass Spectrometry |
| GnRH | Gonadotrophin-releasing Hormone |
| HPA` | Hypothalamus-pituitary-adrenal |
| HPG | Hypothalamus-pituitary-gonadal |
| HPLC | High Performance Liquid Chromatography |
| IP | Intraperitoneal |
| LFA | Lateral Flow Assay |
| LH | Luteinizing Hormone |
| mAB | Monoclonal Antibody |
| NC | Negative Control |
| PC | Positive Control |
| PLFA | Paper-based Lateral Flow Assay |
| pNPP | P-nitrophenylphosphate |
| POC | Point-of-Care |
| PTM | Peritubular myoid |
| p-value | Probability value |
| RGB | Red Green Blue |
| ROS | Reactive Oxygen Species |

| | |
|---|---|
| SD | Standard Deviation |
| SC | Subcutaneous |
| T-AP | Testosterone-conjugated Alkaline Phosphatase |
| TQ | Thymoquinone |
| WHO | World Health Organisations |

# LIST OF SYMBOLS

| | |
|---|---|
| & | And |
| ºC | Degree Celcius |
| < | Less than |
| > | More than |
| µg | Microgram |
| µl | Microliter |
| et al., | (et alia): and others |
| g | Gram |
| i.e. | That is |
| Kg | Kilogram |
| M | Meter |
| Mg | Milligram |
| mg/kg | Milligram per kilogram |
| ml | Milliliter |
| ml/kg | Milliliter per kilogram |
| vs. | Versus |
| pg | Picogram |

# CHAPTER ONE

# INTRODUCTION

## 1.1 BACKGROUND OF THE STUDY

Fertility problems in male, female or both partners can cause the couple to have difficulty in conceiving a child. The couple who were involuntarily childless, would have suffered psychologically as the results of infertility (Cousineau & Domar, 2007). The countries with low birth rate will also have an economy impact in the future. Thus, identifying the causes of infertility and finding solutions to the problems were essentials.

Infertility is a complex disorder as many factors that could contribute to the problems including abnormal reproductive structure, hormonal and genetic problems. Environmental exposure and lifestyle factor also has greatly raised concerns in recent years. People became more aware about their health status, and now opted to consume herbal products as supplements for health (Adimoelja, 2000). There were now a variety of health supplement products and alternative medicines in the market to fulfil this demand. However, most of these product have been taken without consultation and recommendation from the physician, thus consumers were not well-informed about the side effects which could affect individuals (Wilcock, Pun, Khanona, & Aung, 2004).

Thymoquinone (TQ), is an active component in *Nigella sativa*, which is one of a popular traditional herbal medicine widely used around the world. It has many pharmacological properties such as anti-oxidative and anti-inflammatory characteristics which is useful in managing diseases like diabetes (Aithal, Haseena, Das, & Saheb, 2016), respiratory tract disorders (Isik, Kati, Bayram, & Ozbek, 2005), fibrosis (Bai et al., 2014; Kara et al., 2012), bone formation (Kara et al., 2012), kidney (Ragheb et al.,

2009), arthritis and many more. The optimal dose and its long-term supplementation effect on male fertility have not been comprehensively investigated (Mahdavi, Heshmati, & Namazi, 2015).

Besides that, modern lifestyle has also exposed people to continuous light that was proven to be detrimental to health. It has been linked to cancer, disruption of endogenous circadian rhythm and sleeps disorders (R. G. Stevens, Brainard, Blask, Lockley, & Motta, 2014). Consequently, it can affect the hormonal production and regulation of hormones such as cortisol, a stress hormone and also testosterone which could cause infertility problems (Jung et al., 2010; Razavi & Janfaza, 2015).

The diagnosis of male infertility usually involved semen examination to observe sperm quality and hormonal level assay to measure the testosterone level when needed. This would require men to masturbate to get the sperm sample and a painful procedure to obtain the blood sample. Due to this sampling method, it hinders men from attending infertility consultation as they feel ashamed and afraid of the needle prick (Bennett, Wiweko, Hinting, Adnyana, & Pangestu, 2012). Besides that, this type of test also needs well-trained staff and specialized equipment to be done hence is time consuming, laborious and expensive. Thus, the poor-resources area have limited access to the test. Therefore, there were needs for portable sensing devices that was simple, cheap and user-friendly tools to replace the existing method.

Paper-based lateral flow assay (PLFA) is popular in the market nowadays as it is compact, portable, and simple to use. The combination of components such as the structure, formats, detector molecules, labels, detection systems and applications is important determinant in building PLFA (Muller, 2015). PLFA is based on immunochromatographic assay where the antibody-antigen binding of the target would produce colour change reaction that will determine the results. In the market, pregnancy

test kit and also ovulation test kit are popular examples of PLFA (Batool et al., 2014). However, most of the PLFA was only for qualitative detection of analyte and not quantitative result which would be an advantages and vital especially when determining analyte concentration (Akiyama et al., 2006). Smartphone could be the answer as it was equipped with a camera, data storage and rapid development of apps that could help fill the gap.

For this study, we used male Sprague Dawley rats as subjects of the experiment where three different doses of TQ were given for twice a week in a duration of 56 days, which follows the period of rat spermatogenesis cycle completion to discover the effect until the mature sperm formed. Another group was given exposure to 24 hours artificial light with same duration. Blood, saliva and urine were collected to measure testosterone and corticosterone levels respectively. PLFA strip was then fabricated to detect testosterone and then incorporated with usage of the smartphone camera and an application called Color Grab to measure the level of hormones.

## 1.2 RESEARCH OBJECTIVE

The study aimed to achieve the following objectives:

- To evaluate the effects of subchronic thymoquinone administration and 24 hours artificial light exposure on testosterone levels and sperm quality.
- To determine the relationship of testosterone levels in saliva, urine and blood sample and the effects following thymoquinone and 24 hours artificial light exposure.
- To conduct preliminary investigation on the use of paper-based lateral flow assay to measure testosterone and smartphone-based colour application.

## 1.3 RESEARCH HYPOTHESIS

The subchronic thymoquinone supplementation would lower the testosterone levels in blood, saliva and urine samples of male Sprague Dawley rats. The 24 hour artificial light exposure will increase the corticosterone level and lower the testosterone level in all the samples. Consequently, it will cause a decrease in sperm quality with abnormal structure in the histology of the testes. It is postulated that a paper-based lateral flow assay could quantify the testosterone level from the samples in conjunction with the use of a colour application that can easily be downloaded into a smartphone.

## 1.4 SIGNIFICANCE OF THE STUDY

The study could give further insight on the subchronic toxicity of the TQ in male fertility study so that people would be more conscious of the safety in the supplements they took that could affect their overall health, especially fertility. Besides that, it will also give an input on the effect of the prolong exposure to the artificial light on the stress level and male fertility. This would be beneficial to the people who were affected from this light pollution such as shift worker and people living in urban cities. Lastly, this study could further elucidate on the potential of the paper-based lateral flow assay in detecting testosterone levels with smartphone detection. It would be useful in improving the simplicity, affordability and portability of the male infertility diagnosis and thus would be more accessible to rural area and useful as point-of-care diagnosis.

# CHAPTER TWO

# 2LITERATURE REVIEW

## 2.1 INFERTILITY

### 2.1.1 Epidemiology of Infertility

According to World Health Organisations (WHO), infertility is defined as the failure of couples to achieve a successful pregnancy after 12 months or more of regular unprotected intercourse. In 2010, an estimated 48.5 million couples worldwide were unable to have a child after five years based on ability to become pregnant. The global prevalence of infertility was estimated to be 3.5–16.7% in developing countries and 6.9–9.3% in developed countries (Bahamondes & Makuch, 2014). According to WHO, 15-10% of couples have experienced some forms of infertility problems and the trends were unchanged for two decades since 1990 (G. Stevens, Mascarenhas, & Mathers, 2009).

### 2.1.2 Impact of Infertility

Fertility trends across the globe were disturbing especially to developed countries such as Japan and other Western Europeans countries, as the total fertility rate has a direct impact on economic growth and cultural stability. The population ratio in future would have more geriatric people than younger generations and that could pose a bigger challenge to the future generations (Eslami, 2016).

For married couples who were involuntary childless, their inability to conceive a child could cause depression, social stigma and consequently isolation (Cousineau & Domar, 2007). This psychological misery was more prominent to the women, who will have feelings of alienation and shame when comparing themselves to another

fertile woman. Couples affected will feel that they have failed to meet life expectations. Frequently, these couple would feel alone without support to deal with the incidence of infertility (Patel et al., 2016).

### 2.1.3 Male Factors of Infertility

Since ancient times, many couples who struggle to conceive a child would only think of the woman's condition, but through the years it was proven that the male contributed to the condition too. Male factors are estimated to account for approximately 20% of couple infertility (Wu & Eisenberg, 2012). 10 - 15% of infertile men have azoospermia condition with the complete absence of sperm in the semen while 50% of men dealing with infertility have low sperm counts. It was reported that every 1 in 5 men between the ages of 18 and 25 produced low sperm counts (Agarwal, Mulgund, Hamada, & Chyatte, 2015; Louis et al., 2013).

## 2.2 MALE REPRODUCTIVE SYSTEM

### 2.2.1 Spermatogenesis

Male fertility depends on the proper function of a complex system of organs and regulation of hormones. Testes have tunica albuginea that covered the outer surface and filled with numerous seminiferous tubules. In each tubule is where the spermatogenesis took place. Spermatogenesis is a complex, multi-step process involving proliferation and differentiation of spermatogonia into mature sperm (Cooke & Saunders, 2002). Each of the seminiferous tubules are composed of peritubular myoid (PTM) cells, Sertoli cells and germ cells. PTM cells covered the outer wall of the tubule and can contract to push sperm along the tubule. Sertoli cells functions was to convey external signals and provide necessary factor required for the spermatogenesis of germ cells. It

also contribute to renewal of spermatogonial stem cells so that germ cells can be produced continuously (Holdcraft & Braun, 2004). Leydig cells, situated in the interstitial space between the tubules, produce testosterone that diffuses into the seminiferous tubules and blood vessels (Smith & Walker, 2014).

Spermatogenesis is a process that undergoing mitotic and meiotic cycles to form haploid germ cells. It stars with spermatogonia cell which has diploid number (2n) that turns into two secondary spermatocytes during meiosis I. In meiosis II, these cells then in turn are converted into spermatids that contain halves of the chromosome (n) that moves towards the lumen of the testes (Walker, 2011).

Structurally, the tip of the sperm head has acrosome that contains enzymes that enables sperm penetration of the female egg while the main sperm head carries the nuclear DNA. The middle piece contains mitochondria, which supplies energy to the tail which moves with whip-like movements. The sperm has to reach the uterus and the fallopian tube in order to fertilize the egg (Wu & Eisenberg, 2012).

The spermatogenesis requires the production of gonadotropin-releasing hormone (GnRH) in the hypothalamus giving signals to the pituitary gland so it can manufacture follicle-stimulating hormone (FSH) and luteinizing hormone (LH) through hypothalamus-pituitary axis (Fujisawa, Yamasaki, Okada, & Kamidono, 2002).

### 2.2.2 Importance of Testosterone

Testosterone is the most important of male androgens and it is mainly produced by the Leydig cells, although small quantities are also produced by the adrenal glands in both sexes. Luteinizing hormone (LH), main regulator hormone of spermatogenesis in mammals induced Leydig cells to produce testosterone and follicle-stimulating hormone (FSH) (Petersen et al., 2011). Testosterone diffuses into the seminiferous

tubules activates androgen receptor (AR) at cytoplasm and nucleus of Sertoli cells, Leydig cells, PTM cells, arteriole smooth muscle and vascular endothelial cells then trigger the functional responses required to support spermatogenesis. Testosterone is required for at least four critical processes during spermatogenesis. First, maintenance of the blood testis barrier (BTB) whereby it helps in gathering components to form the new BTB on the basal side of the transiting spermatocyte (Meng, Holdcraft, Shima, Griswold, & Braun, 2005). Second, proteins and processes in meiosis of germ cells was also regulated by testosterone. Next, this androgen also responsible for the sertoli–spermatid adhesion connections called ectoplasmic specialization adhesion complex where it prevents the germ cells from being released prematurely. Lastly, sperm release also happen due to the testosterone activation at the last stage of spermatogenesis.(Smith & Walker, 2014).

Besides that, androgens are crucial to develop secondary male characteristics such as hair growth pattern, sebaceous gland activity and libido. Low testosterone levels can lead to underdeveloped genitalia, delays in skeletal and muscle development, and diminished masculinity (Walker, 2011).

## 2.3 CAUSES OF INFERTILITY

About 50% of the male infertility aetiology is unknown as it is a multifactorial syndrome with a wide variety of disorders and could be congenital or acquired. A male could become infertile if there were any disruption of testicular or ejaculatory function. It could result from a number of environmental, behavioural, genotoxic and genetic factors to impairment of spermatogenesis at various stages.