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Quantitative Paper-based Detection of Male Fertility Biomarkers

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ABSTRACT

Male infertility is a reproductive disorder culminating from hormonal imbalances which may be caused by stress and use of health supplements. Invasive diagnostic tests and privacy issues have relatively deterred men from seeking infertility treatment. A rapid and reliable test using non-invasive samples is sought to observe the reproductive effects of subchronic thymoquinone administration and prolonged artificial light exposure to rats. Detection of testosterone as the male fertility biomarker from non-invasive samples was employed using adult male Sprague-Dawley rats treated with thymoquinone or exposed to 24-hours artificial light. All interventions were given within a 56 days period and sampling of blood, saliva and urine was performed at day 0 and day 56. Testosterone and corticosterone levels were determined using enzyme-linked immunosorbent assay (ELISA). Sperm analysis parameters subsequent to testes harvest at day 56 were measured and followed by a histological assessment. Paper-based lateral flow assay (PLFA) strip was developed based on the colour change from the antigen-antibody reaction on paper reflecting testosterone levels from urine samples. The colour change was recorded using a smartphone camera by an application that captures the RGB colour value. This study demonstrated that 30 mg/kg subchronic thymoquinone supplementation can reduce testosterone levels thus possibly affecting fertility. Meanwhile, the 24-hour light exposure showed significant effects on testosterone but not corticosterone levels compared to controls. The testosterone level assessment using the PLFA produced comparable data with ELISA results. This sensor holds potential to increase patient compliance with sampling by using non-invasive samples to test infertility in men.

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INTRODUCTION

According to the World Health Organisation (WHO), infertility is defined as the failure of couples to achieve a successful pregnancy after 12 months or more of regular unprotected sexual intercourse. Perturbation of normal hormonal pathways have been the mainstay of fertility disorders and incidences have occurred following exposure to environmental agents and individual lifestyle (Sharpe, 2000).

Consuming a variety of health supplement products without the supervision of a physician has become a trend that could have detrimental effects on health (Wilcock *et al.,* 2004). Thymoquinone (TQ),

is an active component in *Nigella sativa*, a popular traditional herbal medicine that has been widely used around the world for its many pharmacological properties. The anti-oxidative and anti-inflammatory attributes of the natural product was shown to be useful in managing diseases such as diabetes (Haseena *et al.,* 2015), respiratory tract disorders (Isik *et al.,* 2005), fibrosis (Bai *et al.,* 2014), bone formation (Kara *et al.,* 2012), kidney (Ragheb *et al.,* 2009) and arthritis (Umar *et al.,* 2012) . However, the optimal dose and its long-term supplementation effects on male fertility have not been comprehensively elucidated (Mahdavi *et al.,* 2015).

Modern lifestyle has also exposed people to continuous light that has been proven to be linked with cancer, disruption of endogenous circadian rhythm and sleep disorders (Stevens *et al.,* 2014). Consequently, it can affect the hormonal production and regulation of hormones such as the stress hormone, cortisol, and also testosterone which could cause infertility problems (Jung *et al.,* 2010).

The diagnosis of male infertility usually involved semen examination in observing sperm quality and hormonal level assay. This sampling method has hindered men from attending infertility consultation due to the embarrassment and also the fear of needle prick (Bennett *et al.,* 2012). This current method of testing requires well-trained staff and specialized equipment hence is time-consuming, laborious and expensive.

Paper-based lateral flow assay (PLFA) is gaining public interest as a compact, portable and simple method for detection of biological molecules. PLFA is based on immune-chromatographic assay where the antibody-antigen binding of the target would produce a colour change reaction that will determine the results. Pregnancy and ovulation test kits are popular examples of PLFA available in the market. However, most of the current PLFA demonstrate qualitative detection instead of quantitative results which would be advantageous and vital especially when determining analyte concentrations (Akiyama *et al.,* 2006). The smartphone could be the answer as it is equipped with a camera, data storage and rapid development of applications that could assist in achieving quantitative outcomes. In the PLFA assays, the camera can capture digital images to extract colourimetric quantitative information and subsequently analyse the image in terms of colour code value such as grayscale or RGB colours for the pixel of interest. The RGB colour model makes use of primary colours of red (R), green (G) and blue (B) with different intensities to produce millions of colours. Each parameter of R, G and B has an integer value ranging from 0 to 255 where different number combinations of these three parameters would result in different colour combinations.

This study aimed to evaluate the effects of sub-chronic TQ administration and 24-hours artificial light exposure on testosterone levels and sperm quality. The use of PLFA to measure testosterone concentration and smartphone-based colour application as compared to conventional procedures were also included to achieve the study objectives.

MATERIAL AND METHODS

Animal Work

Animal care and the study protocol was approved by the committee of research ethics of the International Islamic University of Malaysia [IIUM/IACUC Approval/2016/(9)(46)]. The animal experimentations were conducted on adult male Sprague-Dawley rats aged between 15-17 weeks old. The rats were acclimatized to laboratory conditions at 12/12 hour dark and light cycles with a constant temperature of 24˚C one week prior to the commencement of experiments.

36 adult male Sprague-Dawley rats were equally divided into six groups (n=6). Group I is the negative control. Group II consists of positive control group administered with subcutaneous (s.c.) 10 mg/kg corticosterone for 10 successive days and intraperitoneal (i.p.) 10 mg/kg cisplatin five days prior to the day of sacrifice. Groups III, IV, V consists of rats treated with TQ via intraperitoneal [i.p.] the route at doses of 5 mg/kg, 10 mg/kg and 30 mg/kg respectively, twice a week for 56 days. Rats in group VI were exposed to 24-hours artificial fluorescent light continuously for 56 days.

Sample Collection

Samples of urine, blood and saliva were collected at the beginning of the experiment and day 56 before sacrifice. The samples were centrifuged and the supernatant aliquoted and stored at -80°C until further assay. All groups were euthanized at the end of intervention to harvest the testes and caudal epididymis. The weight of the testes was measured and testes were then fixed in 10% formalin solution for histological processes. The caudal epididymis was prepared for sperm suspension.

Analysis of Sample

All blood, urine and saliva were tested using Enzyme-Linked Immunosorbent Assay (ELISA) kit (Enzo) to determine the levels of testosterone. For corticosterone assay, only samples from the 24-hours light group were tested using ELISA kit. Sperm count and motility assessments were performed along with haematoxylin and eosin (H&E) staining of sections from the testes.

Paper Strip Preparation

The test pad was prepared using nitrocellulose membrane (NC) incubated with 17 µl of Testosterone Monoclonal Antibody (mAB) for 90 minutes at room temperature then kept dried at 4°C overnight. Chromatography paper was cut out as sample and waste pads, then attached to the top of the pre-treated NC membrane. Antigen-antibody formation and colour reaction took place on the NC membrane. The strip was then attached to glass slides as support. The dimensions of the assembled PLFA is illustrated in Figure 1.

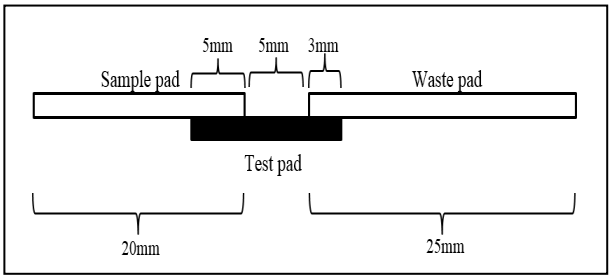

Figure 1: Schematic diagram of the PLFA strip dimensions

Standard Curve

The standard curve for testosterone was generated by preparing six standard solution in buffer at concentrations of 0 pg/ml, 1000 pg/ml, 1200 pg/ml, 1400 pg/ml, 1600 pg/ml, 1800 pg/ml and 2000 pg/ml. A volume of 34 µl of each standard solution was pre-mixed with 17 µl of Testosterone-conjugated Alkaline Phosphatase (T-AP) before being applied onto the sample pad. The solution later progressed towards the test pad where the target analyte, for example, testosterone in the standard solution competed with T-AP to bind to the antibody mAB on the test line to form an antigen-antibody complex. The reaction was left to take place for 12 minutes before the addition of 30 µl of p-nitrophenyl phosphate (pNpp) substrate which reacted with the T-AP. The yellow chromogenic product, p-nitrophenol, on the test line is formed when the substrate is attached to the antibody. RGB reading was taken at 25 minutes following this colour change reaction. The RGB image was captured using a smartphone camera, Xiaomi Redmi 3S (Android system) and a free application known as Color Grab by Loomatix Version 3.6.1 from Google Play store. In this first instance, readings were taken in a dark room with the camera placed 5cm above the test strip with flash auto mode.

The procedures were repeated where the standard solution was replaced with six rat urine samples selected from the previous ELISA experiments where testosterone levels have been quantified. The intensity of the yellow colour from the antigen-antibody interaction at the test pad from the different RGB quantifications was then used to determine the concentrations of testosterone. The concentrations measured by the two methods were later compared.

Statistical Analysis

The testosterone and corticosterone levels in the blood, urine and saliva samples following pre- and post-treatment with TQ and light exposure were calculated for each group and compared with controls. The parameters from sperm analyses were also evaluated across the groups. The difference between testosterone concentration using PLFA strip and ELISA method was also observed. One-way analysis of variance (ANOVA) and Tukey's test was performed in all data where a *p*-value of <0.05 was considered statistically significant. All analyses were performed using the Statistical Package for the Social Sciences (SPSS) version 21.

RESULTS

Thymoquinone (TQ) Treated Group

Testosterone Levels

Subchronic treatment with TQ showed decreased levels of testosterone in blood, saliva and urine at the end of the treatment compared to baseline before the treatment as shown in Figure 2. There were no significant differences in testosterone concentration between the TQ-treated group with controls (*p>0.05*) except for the 30 mg/kg TQ group for the urine sample (*p<0.05*).

Testicular Weight and Sperm Parameters

The results displayed in Figure 3 demonstrated that TQ treatment led to lower testicular weights, sperm concentrations and sperm motility compared to controls, although not significantly different across all groups (*p>0.05*). There was only a significant difference seen in sperm motility in the 10 mg/kg TQ-treated group compared to positive control (*p<0.05*).

Artificial Light Group

Testosterone Level

Effects of subchronic 24-hours light exposure on testosterone levels exhibited in Figure 4 showed that the test group had a significant increase of testosterone levels in blood and saliva samples compared to both controls (*p<0.05*). Urine samples showed a significant increase when compared to positive control only (*p<0.05*).

Corticosterone Level

The effect of subchronic 24-hours light-exposure on corticosterone levels are shown in Figure 5.

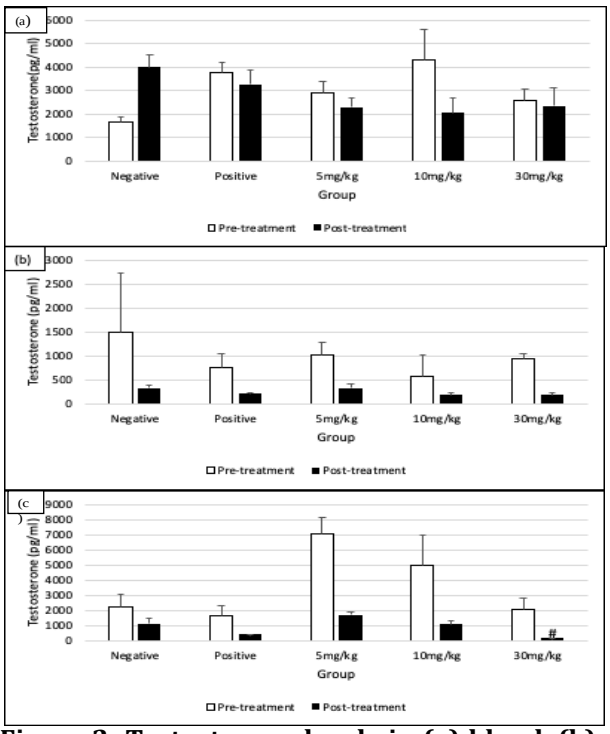

Figure 2: Testosterone levels in (a) blood, (b) saliva, and (c) urine at pre- and post- 56 days treatment of thymoquinone (TQ). Values are expressed as Mean ± SD. # significantly different to the positive control group; * significantly different to the negative control group; Positive control – 10 mg/kg cisplatin (i.p.).

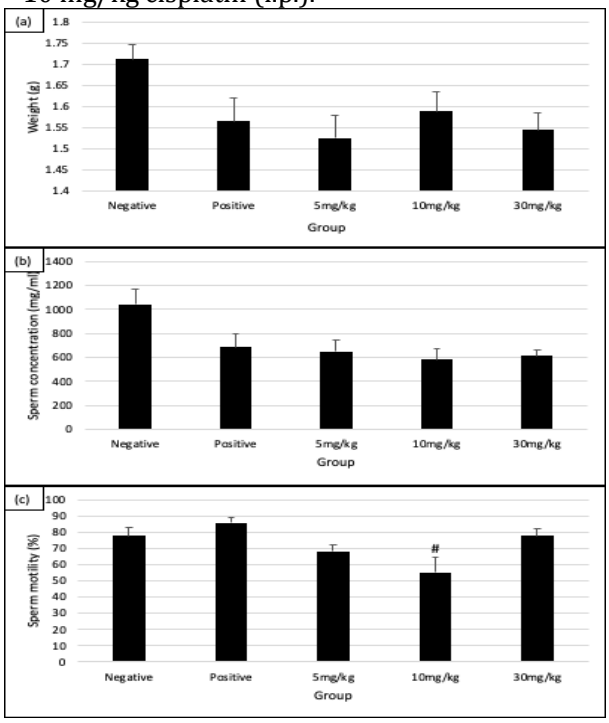

Figure 3: Parameters indicate (a) weight of testis (g), (b) sperm concentration (mg/ml), and (c) sperm motility (%) for the controls and TQ-treated group. Values are expressed as Mean ± SD. # significantly different to the positive control group; * significantly different to the negative control group; Positive control – 10 mg/kg cisplatin (i.p.)

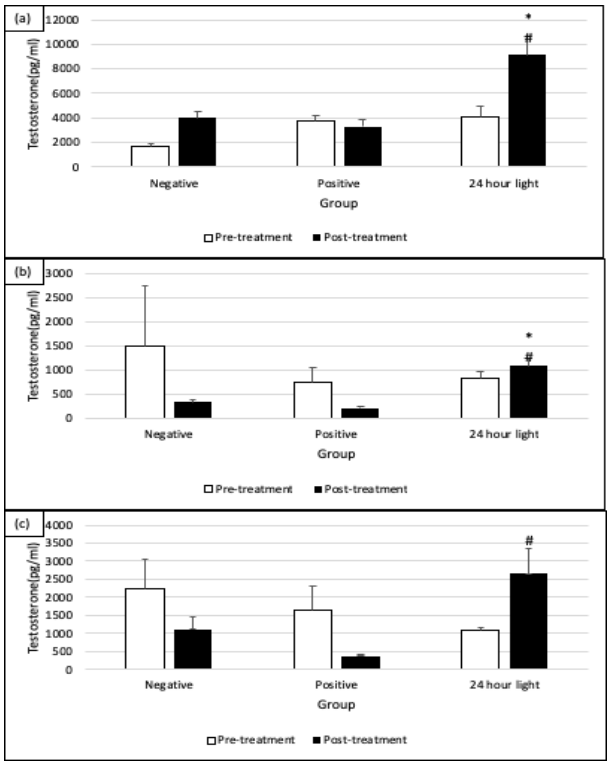

Figure 4: Testosterone levels in (a) blood, (b) saliva, and (c) urine at pre- and post- 56 days exposure to 24-hours artificial light. Values are expressed as Mean ± SD # significantly different to the positive control group; * significantly different to the negative control group; Positive control – 10 mg/kg corticosterone (s.c.).

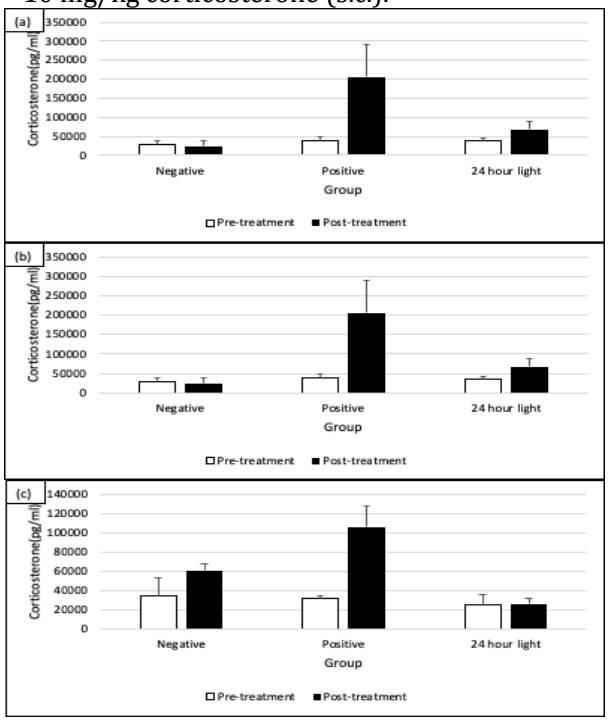

Figure 5: Corticosterone levels in (a) blood, (b) saliva, and (c) urine at pre- and post- 56 days exposure to 24-hours artificial light. Values are expressed as Mean ± SD # significantly different to the positive control group; * significantly different to the negative control group; Positive control – 10 mg/kg corticosterone (s.c.).

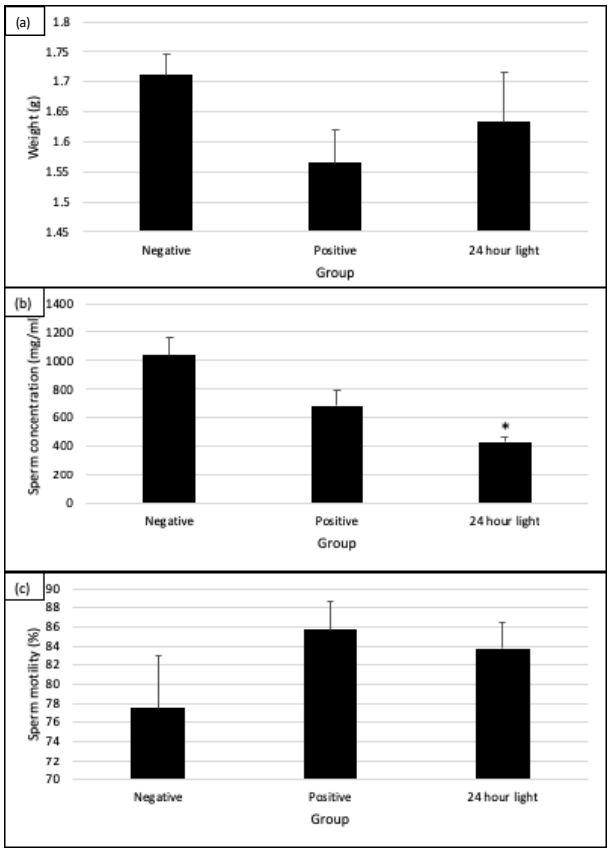

Figure 6: Parameters indicate (a) weight of testis (g), (b) sperm concentration (mg/ml), and (c) sperm motility (%) for the controls and 24-hours artificial light-exposure group. Values are expressed as Mean ± SD # significantly different to the positive control group; * significantly different to the negative control group; Positive control – 10 mg/kg corticosterone (s.c.).

The test group showed increased corticosterone levels after the exposure, but no significant difference was observed when compared to controls (*p>0.05*).

Testicular Weight and Sperm Parameters

The bar graph in Figure 6 showed that the weights of testes in the test group were lower compared to negative controls but no significant difference (*p>0.05*). While the sperm motilities were lower than the positive control, no significant differences across the groups were observed (*p>0.05*). For sperm concentration, test group indicated the lowest sperm concentration compared to both controls and significantly different to the negative controls (*p<0.05*).

Testicular Morphology

As shown in Figure 7, cross-sections of testes in negative controls demonstrated normal morphology where seminiferous tubules have abundant spermatocytes and spermatids in different stages of development. Leydig cells can also be seen. Compared to negative controls, positive controls have a lesser amount of spermatogonia cells and structure while groups exposed to 5 mg/kg and 10 mg/kg of TQ have almost similar asymmetrical morphology of testes. The group exposed to 30 mg/kg of TQ demonstrated obvious disruption of seminiferous tubules with a diminished amount of cells. For 24-hours light-exposure group, the spermatogonia and interstitial cells were reduced.

Paper-based Lateral Flow Assay for Testosterone

The standard curve graph was constructed based on the RGB values against six different standard concentrations (Figure 8). As the concentration increased, R and G values were decreased as opposed to the B values. When analysed, the R^2 for B has a higher value than the rest (R^2= 0.9801) in the RGB trendline. Thus, the B value was chosen to construct the calibration curve and to predict the unknown concentration of testosterone. Six rat urine samples from the previous experiment were used to determine the testosterone concentrations using the paper strip prepared. The concentrations of testosterone using the strip was interpolated from B signal standard curve. The results were compared with ELISA measurements and which showed no significant differences in testosterone concentration between the two methods as displayed in Figure 9.

DISCUSSION

Thymoquinone (TQ) Toxicity

Subchronic TQ administration was found to decrease testosterone levels. Although no significant differences in testosterone levels were observed compared to negative controls, the declining trend was consistent in all samples of blood, saliva and urine. Cisplatin was given to rats as positive controls to lower the testosterone levels. Cisplatin is a chemotherapeutic drug which poses side effects that damage male fertility by reducing testosterone production (Dasari and Bernard Tchounwou, 2014). The results showed that 30 mg/kg TQ dose consistently lowered the testosterone level compared to the positive control group in all samples especially in urine where the decrease was significant. This may indicate the toxic effects of high concentration of TQ which appeared to be more adverse than that of cisplatin. As the Maximum Tolerable Dose (MTD) of TQ for i.p. injections in male rats were determined to be 22.5 mg/ kg, the higher dose of 30 mg/kg may have caused the damaging effects (Al-Ali *et al.,* 2008). TQ was reported to exhibit the beneficial effects, i.e. anti-inflammatory, anticancer, antioxidant and cytoprotective effects only at doses between 5–12.5

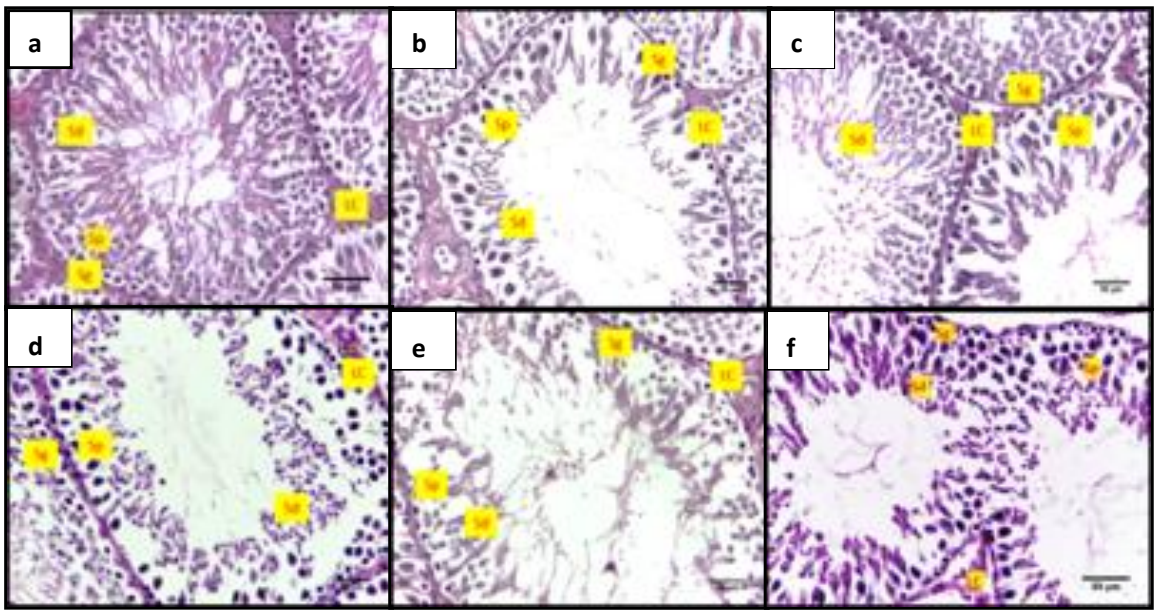

Figure 7: Cross sections of testes for (a) negative control, (b) positive control (c) 5mg/kg TQ, (d) 10mg/kg TQ, (e) 30mg/kg, (f) 24-hours artificial light-exposure. (H&E; 40X) Sd-Spermatid, Sg-Spermatogonia, Sp-Spermatocytes and LC-Leydig cells

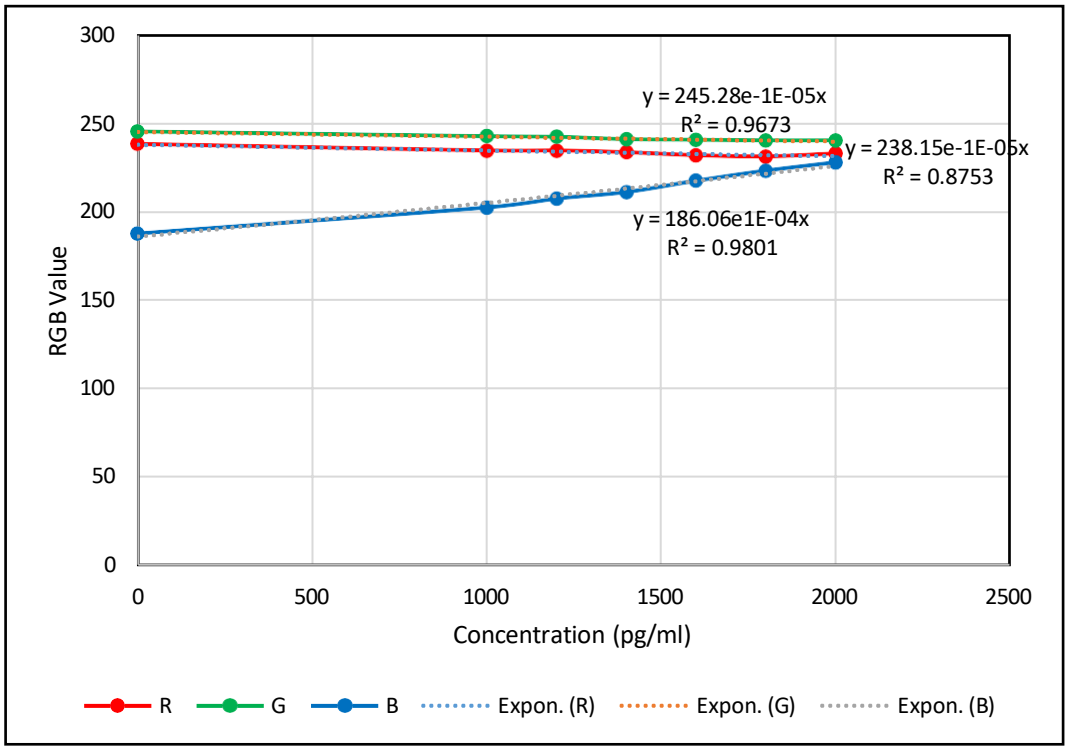

Figure 8: Scatterplot of RGB value against standard testosterone solution

mg/kg injected intraperitoneally without significant deleterious effect (Ragheb *et al.,* 2009). However, this study showed that at 5 mg/kg and 10 mg/kg of TQ, deleterious effects in the testes and decreased steroid hormones levels were detected as what might have been indicated by previous works in another rodent species by Abdul Rahman *et al.,* (2014).

This study was in accordance with the work done by Rahmani and Aly (2015) whereby TQ given for 5 weeks at low doses decrease the levels of testosterone in normal rats.

Besides its benefit as a potent antioxidant, TQ is also believed to act as a pro-oxidant. Through redox cycle, TQ can produce reactive oxygen species (ROS) such as semiquinone and quinone dianion which can lead to apoptosis of cancerous cells such as lung cancer and prostate cancer (Wajid *et al.,* 2014). The high dose of 30 mg/kg TQ given to normal rats for 56 days could have multiplied the production of ROS that may have an effect on fertility. However, the effect of TQ as a pro-oxidant on other normal cells have not been extensively investigated.

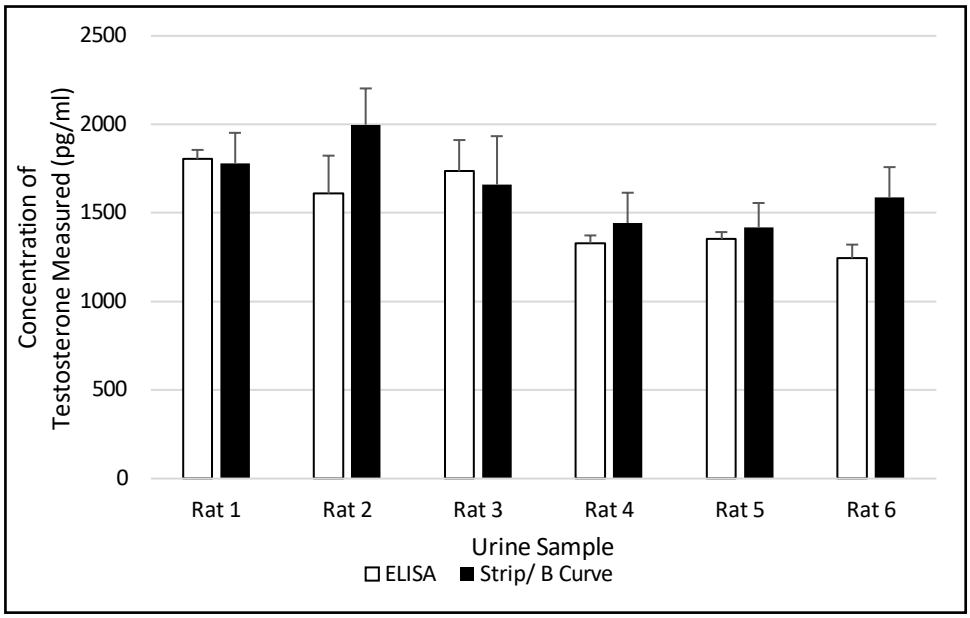

Figure 9: Bar graph showing a comparison of the concentration of testosterone in urine sample measured using ELISA and PLFA strip. Values are expressed as Mean ± SD

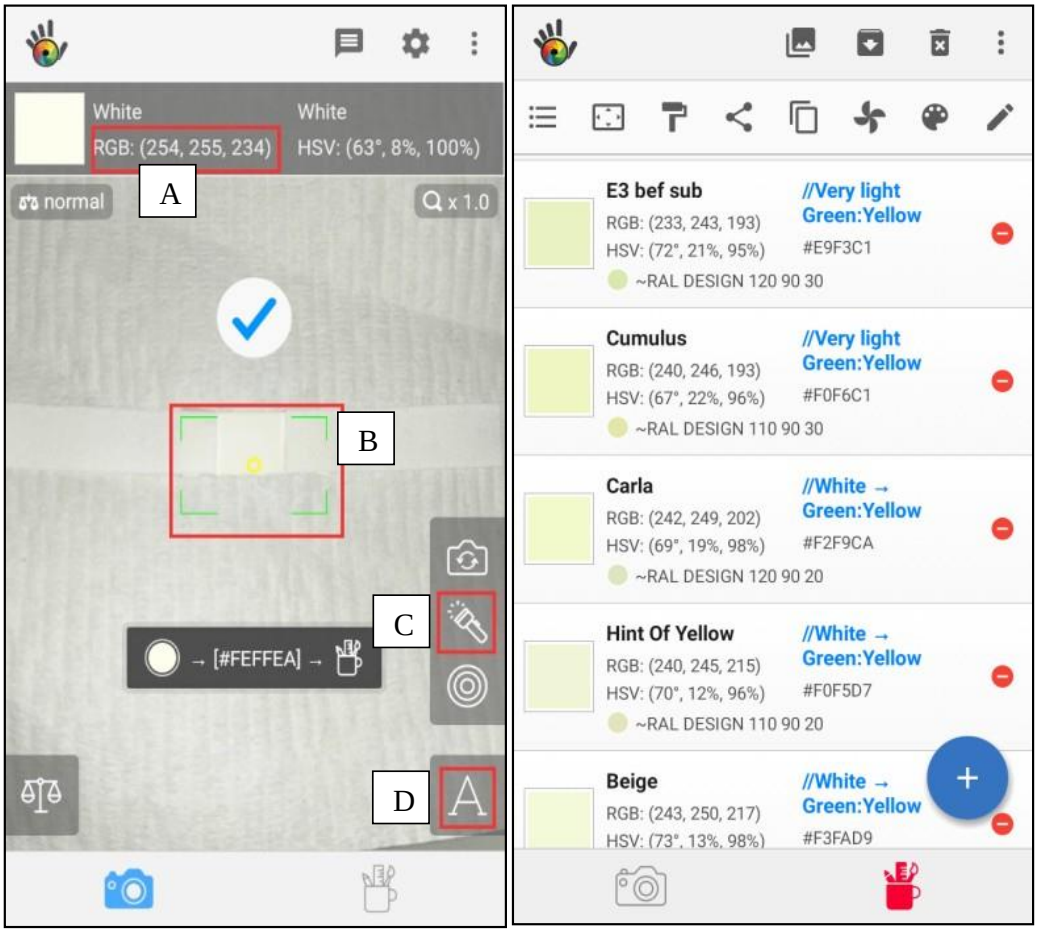

Figure 10: Image on the left showing (A) RGB Value, (B) Area of detection, (C) Flashlight, and (D) Auto camera mode. Image on the right shows the colour that had been captured

In this study, the sperm analysis demonstrated that the weight of testes, sperm concentration and sperm motility were decreased in all TQ-treated groups compared to the normal control group.

The histology of testes further illustrated the TQ toxic effects on spermatogenesis as a loss of spermatogenic cells inside the tubules and disrupted morphology. The low level of testosterone as a result of TQ treatment may have caused the decrease in germ cells and consequently low testes weight,

as testosterone has an important role in the development of spermatogenesis (Al-Ali *et al.,* 2008).

Light Exposure

Following light exposure, overall results showed that the 24-hours artificial light exposure group had increased testosterone and corticosterone levels at the end of the experiment. This contradicts with the initial expectation that testosterone level would be decreased when corticosterone is increased. A previous study by Dauchy et al. (2010) showed prolonged light exposure induced stress as indicated by an increase in plasma levels of corticosterone. As corticosterone levels increased, testosterone concentrations are expected to be decreased via the inhibitory effect on the hypothalamus-pituitary-gonadal axis (Retana-Márquez *et al.,* 2014; Viau, 2002). This is due to the decline in corticotrophin-releasing hormones (CRTH) that will, in turn, cause the production of an adrenocorticotrophic-releasing hormone (ACTH) in the hypothalamus hence the inhibitory effect and decreased testosterone levels. This study also showed that the 24-hours light exposure resulted in the lower average weight of testis and sperm concentration compared to the normal controls. When compared to the positive controls, the test group showed significantly lower sperm concentration and motility. The loss in the testes weight and sperm concentration can also be seen in the histology of the testes. The damage could be related to the high corticosterone levels which have been demonstrated in male rats exposed to stress through immobilization and forced swimming. These rats have substantial loss of germinal cells and damage to the spermatogenic function (Nirupama *et al.,* 2013).

In this study, the increasing corticosterone levels observed may have been caused by the disrupted circadian rhythmicity of the hormone due to light exposure and intensity, duration, and circadian phase (Bedrosian *et al.,* 2016). The increase in testosterone levels could be due to production from other sources such as the adrenal gland. The light-stimulation may also change other gonadal steroid pathways that indirectly increase the testosterone levels through stimulation of follicle-stimulating hormone (FSH) (Hardy *et al.,* 2005) Additionally, testosterone and corticosterone hormones have different circadian variation where testosterone tends to have a sleep-dependent factor in terms of its duration and quality for hormone production (Wittert, 2014).

Fabrication of Paper-based Lateral Flow Assay

The PLFA strip in this study was fabricated to measure testosterone levels by determining the RGB colour with the help of a smartphone camera and an application known as Color Grab. The test strip demonstrated reliability when compared to ELISA test as no significant difference were observed between the two methods. However, the test strips used have limited detection range and are, at present, less sensitive as it can detect analyte concentration only between 1000 pg/ml to 2000 pg/ml, while ELISA has a lower limit of detection (LOD) between 0 pg/ml to 2000 pg/ml. Comparing the methods, the PLFA strip however required only a small volume of sample (approximately 17 µl per sample). ELISA test, however, needed larger volumes between 50 µl to 100 µl per sample. Besides that, the assay for the strip involved simple steps and shorter time for analysis that is 25 minutes compared to ELISA, which takes several hours. In addition, the strip is also economically effective as the volume of the reagents needed and the materials to build the assay are relatively cheaper. With smartphone usage, it further adds to the portability aspect of the assay which would be beneficial especially in remote areas with limited resources. Smartphone, as a portable sensing device, has been used in various areas for environmental testing purposes such as water quality sensing to determine pH and chlorine levels (Lane *et al.,* 2010). In the area of fertility, an automated smartphone-based semen analyser for quantitative measurement of sperm concentration and motility have been developed with 98% accuracy using clinical semen specimen (Kanakasabapathy *et al.,* 2017). Therefore, this research presents an alternative potential in male infertility diagnosis by using testosterone levels as the non-invasive sample parameter and utilizing the smartphone application as a point-of-care device.

The PLFA strip is in the preliminary stage of development whereby many factors still need to be considered. For example, there is no instrumentation built for this strip hence images have been taken in the darkroom where the illumination only comes from the camera flashlight. Maintaining short and constant distance between the camera and the strip, and the usage of the same phone and settings have helped in getting a consistent reading. Besides that, in a typical test strip, there are control and conjugate lines which have not been placed on this strip. The control line is needed to ensure that the samples flow properly to reach the waste pad while the conjugate line functions as an attachment for the conjugate. In this strip, the conjugate or labelled molecules have been added together with the samples before being applied to the test strip. Therefore, no conjugate lines were needed. Moreover, the conjugate reagent also has a hint of blue colour, and thus we could see that the sample flow together with the conjugate until it reaches the waste pad consequently eliminating the use of the control line. Further improvements to the strip for

development into an effective point-of-care device in detecting male infertility are hopefully imminent.

CONCLUSIONS

This study has proved that in normal conditions, i.e. with no medical conditions, subchronic supplementation of TQ, the active ingredient of *N. sativa* could have negative effects on male fertility. Concentrations of 5 mg/kg and 10 mg/kg of TQ showed slight toxic effects on the testes of the Sprague-Dawley rats, while at 30 mg/kg dose, the adverse toxic effect on testosterone levels, sperm concentration and quality and the testes, seemed to be even more severe than the common anticancer agent cisplatin.

The study has discovered that the 24-hour light-exposure promotes increased corticosterone and testosterone levels with lower sperm quality and quantity and affecting the testes. This study is also a preliminary experiment combining PLFA with a smartphone application showing potential in quantifying the testosterone levels by using RGB values which produce comparable results with ELISA.

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