Middle-East Journal of Scientific Research 17 (7): 955-964, 2013
ISSN 1990-9233

DOI: 10.5829/idosi.mejsr.2013.17.07.7673

Protective Effect of Black Seed *Nigella sativa* (L.) Against Cyclophosphamide-Induced Toxicity on Reproductive and Acrosomal Function in Mice

Suzanah Abdul Rahman, Noor Faridah Shaik Dawood, Shaik Sadak Basha and Saheera Kamarzaman

Department of Biomedical Science, Faculty of Allied Health Sciences, International Islamic University Malaysia (IIUM), Malaysia

Abstract: To determine the effect of ethanol extract of *Nigella sativa on* cyclophosphamide reproductive toxicity and acrosome function of sperm. Male Balb/c mice, aged between 5 to 7 weeks were divided into four groups, each of six animals. Cyclophosphamide was administered via intraperitoneal injection at 200 mg/kg body weight and ethanol extraction of *Nigella sativa* was similarly injected at a dose of 10 mg/kg and continued on alternate days for the duration of 32 days. All mice were sacrificed on day 33 and testes and epididymides were excised. Group I served as control while group II was treated with cyclophosphamide and group III with 10 mg/kg of *Nigella sativa* and group IV with a combination of cyclophosphamide and *Nigella sativa* at the same concentrations. Effects of cyclophosphamide include reduction of weights of testes, epididymides and sperm count. A higher value of sperm count was observed in the cyclophosphamide plus *Nigella sativa* group when compared to the cyclophosphamide alone group, perhaps indicating the medicinal plant antioxidant properties in the germ cell line. Treatment with cyclophosphamide led to a high percentage of acrosome reacted sperm possibly to spontaneous acrosomal reaction caused by free radicals. Although the combination treatment seemed to show similar histology to the effects of cyclophosphamide alone, a protective outcome was indicated with the higher number of sperm count with the addition of *Nigella sativa*. Paradoxical effects of *Nigella sativa* were observed on the cells in the testis and acrosome reaction including in the protection against oxidative agents and alkylating effects of cyclophosphamide. Further investigation is recommended to determine outcomes in the presence of oocytes, at the time when sperm capacitation and acrosome reaction usually occur.

Key words: Acrosome · Cyclophosphamide · Male Mice · *Nigella sativa* · Testis

INTRODUCTION

Despite the use of modern medicine, markets for complementary and alternative medicine have become very demanding especially in India, China, Korea, Japan and Vietnam. These countries have all developed their own unique versions of traditional medicine [1]. Plants are natural factories for the production of chemical compounds, many of which are used to promote health and fight diseases while some are marketed as well known food or herbal medicines [2]. Herbal medicines have long been viewed as a source of curative remedy based on religious and cultural traditions [3, 4]. The use of indigenous plant medicines in developing countries became a World Health Organization policy since 1970. Of the 520 new drugs approved in the period between 1983-1994 by either the US Food and Drug Administration or comparable entities in other countries, 30 drugs came directly from natural product sources, 173 were either semi-synthetics or synthetics originally modeled on a natural parent product [5].

Cyclophosphamide (CPA) is an alkylating agent used commonly to treat cancers worldwide [6]. It is derived from the group of oxazophorines. Similar to nitrogen mustards, it causes integration of the alkyl group to the

Corresponding Author: Suzanah Abdul Rahman, Department of Biomedical Science, Faculty of Allied Health Sciences, International Islamic University Malaysia (IIUM), Jalan Sultan Ahmad Shah, Bandar Indera Mahkota, 25200 Kuantan, Pahang, Malaysia. Tel: +609-5705203/5204,

N7 position of the guanine base of DNA which then causes DNA-DNA cross-links, inhibiting DNA synthesis, causing a break in the single strand and ultimately cell death [7]. CPA is administered in its dormant form and undergoes transformation in the liver via oxidases enzyme into few active metabolites (aldophosphamide, phosphoramide mustard). It is widely used in the treatment of prostate cancer, testicular cancer, breast cancer, ovarian cancer, chronic lymphoid leukaemia, chronic myeloid leukaemia and many other cancers [8].

A study was conducted by Meriggiola *et al.* [9] which proved that CPA caused fertility related side effects such as azoospermia, oligospermia and infertility in patients who were treated for childhood sarcomas with CPA as part of VAC regime (Vincristine, Actinomycin and CPA). Other side effect observed includes heritable DNA mutations [10]. These side effects occur with treatment of 1-2 mg/kg of CPA for more than 4 months which led to altered gene expression in the male germ cells [7]. Researchers has proved that CPA causes oxidative stress [11, 12] damaging the plasma membrane, motility and penetrating ability of sperm [13].

Nigella sativa is ascribed to have many medicinal properties. Black seed *Nigella sativa* (L.) is from the family of Ranunculaceae [14] and it is commonly known as '*Habbatus Saudah'* in the Middle East, '*Karum Cheerakam'* in South India and '*Chernushka*' in Russia. The multiple uses of *Nigella sativa* in folk medicine encouraged many scientists and researchers over the world to isolate its active components. *Nigella sativa* is known to be one of the most extensively studied plants, both phytochemically and pharmacologically. It was reported that the active component of *Nigella sativa* known as thymoquinone (TQ) was discovered in 1956 by a researcher named Chopra. Mahfouz and El-Dakhakhny (1960) then isolated 'Nigellone' from the oil of *Nigella sativa* seed, a dimer of TQ, which was later, named dithymoquinone (DTQ) [15]. *Nigella sativa* possesses main components known as thymol and quinones (thymoquinone, thymohydroquinone, dithymoquinone) [16]. Ashraf *et al*. [17], in determining the antioxidant level of *Nigella sativa* found that TQ, the active agent of *Nigella sativa*, has a strong antioxidant activity. TQ and the other active components of the herbal plant have also been shown to have cytotoxicity effects against cancer cells and a multitude of other effects [18, 19]. The protective effects against oxidation were also shown by TQ and other components in an *in vitro* study on MCF-7 breast cancer cell lines induced by the oxidative stressor H_2O_2 treated with aqueous and alcohol extraction of *Nigella sativa* in a study by Farah and Begum [20]. The antitumor activity of *Nigella sativa* has also been proven by Musa *et al*., (2004) and Zaher *et al*., (2008) in their research where they proved that there was a significant reduction in cell proliferation, DNA synthesis, mitotic percentage and prolongation of life span of the mice due to the antiangiogenic and antioxidative activity of the herbal plant [21, 22]. *Nigella sativa* has also been shown to have antigenotoxicity and antibiotic properties as experimented on a variety of biological species [23, 24].

Due to the low degree of toxicity of the seeds of *Nigella sativa* [19], the present study was designed to investigate its extract in the evaluation of acrosomal reaction following exposure to CPA using animal models.

MATERIALS AND METHODS

Materials

Experimental Animals: The experiment was performed on adult male Balb/c mice of 5-7 weeks of age weighing 15-20 grams each. All of the animals were allowed to acclimatize in metal cages inside a well-ventilated room for 2 weeks prior to the experiment. They were housed in polycarbonate cages under standard laboratory conditions at a room temperature of 22 ± 4°C, humidity of 30-70% with 12 h light/dark cycle. The animals were fed with standard diet and tap water was given *ad libitum* until treatment or time of sacrifice. The study was performed according to the Guidelines for Animal Study and was approved by the Faculty of Allied Health Sciences Research Committee of the institution.

Experimental Design: Male mice were divided randomly into four groups consisting of six animals each. Group I served as controls (Normal saline 0.9% NaCl), while Group II was treated with cyclophosphamide (CPA) at 200 mg/kg body weight. Group III mice received treatment of *Nigella sativa* at 10 mg/kg body weight and those in Group IV were treated with a single dose of CPA 6 hours prior to treatment with *Nigella sativa.* CPA (SIGMA c0768, USA) was injected intraperitoneally (i.p) with a single toxic dose of 200 mg/kg and *Nigella sativa* extract (NSE) was given also via the peritoneum at a concentration of 10 mg/kg. Supplementation with NSE dissolved in normal saline was additionally continued on alternate days throughout the length of the study. All mice were weighed before the commencement of the

experiment and prior to euthanization at day 33 via cervical dislocation and epididymis and testis excised for record and observation.

Methods: The *Nigella sativa* seeds (Syria) were purchased from a local herb certified store. Seeds of *Nigella sativa* were powdered in a mixer and 400g of air-dried powder was extracted with ethanol (40-60 degree Celsius) in a Soxhlet Extractor for 16 hours. Solution was then evaporated to dryness under reduced pressure and controlled temperature by using a rotary evaporator. The extract was stored in a refrigerator at 4°C in an air-tight bottle until further use [21].

Sperm Suspension: Immediately following euthanasia, mice sperm was collected from the epididymis with the aid of scissors and thorough internal rinsing with 4.0 ml of sperm preparation medium (Quinns Advantage®- Sperm Washing Medium) in a conical tube (Eppendorf Research, Eppendorf Corp, Germany). The medium was incubated at 37°C in a 5% CO_2 incubator (490-ICE, Thermo-Scientific, USA). Tubes were kept at a 45° angle while partially covered with the cap to allow the most motile sperm to swim up into the medium via the swim-up procedure [25].

Sperm Count: Sperm count was determined by counting the number of cells using a haemocytometer (Hirschmann® Laborgerate). The number of cells in at least two of the corner larger squares (1 mm^2) was counted, ignoring the cells on the upper and right boundaries of each small square and the mean value was calculated. The number of sperm cells/ml was calculated by multiplying the mean value by 10^4.

Capacitation Period: Capacitation of mice sperm was induced by using the same sperm preparation medium. The dissected epididymis was placed in an Eppendorf tube containing 4 ml of sperm preparation medium. Suspension specimen was then incubated for 1, 3, 6 and 24 hours to obtain different capacitation period at 37°C in a 5% CO_2 incubator (490-ICE, Thermo-Scientific, USA). These conditions are known to induce sperm capacitation and acrosome reaction [26].

Evaluation of Acrosomal Function: Status of acrosome was accessed using a modified method as described by Elangovan *et al*. [25]. 1 ml of mice epididymal sperm suspension exposed to different treatments was centrifuged together with 1 ml of normal saline (0.9% NaCl) (Opticare, USA) in a microcentrifuge tube (Eppendorf Research, Eppendorf Corp, Germany) at 1200 RPM/min for 5 minutes. The supernatant was removed and 10 µl of the cell pellets was smeared on a glass microscope slide and later air-dried. Smeared slides were stained with methanol (Fisher Scientific, Malaysia) for 2 minutes and then washed with 1X PBS (Fisher Scientific, Malaysia) for three times. Cells were stained for 20 minutes at room temperature with 0.22% Coomassie Brilliant Blue (CBB) (Merck, Germany) solution prepared freshly in 40% methanol (Fisher Scientific, Malaysia) and 60% distilled water. Stained cells were washed with PBS (Fisher Scientific, Malaysia) three times, air dried and covered with a cover slip over a drop of DPX (SIGMA-Aldrich, USA) and later observed under a bright field microscope (Thermo Scientific, Zess, USA) at a 100X magnification. Spermatozoa with intact acrosome exhibited intense staining on the anterior region of the sperm head but remained unstained in acrosome reacted sperm. For every experiment, 300 spermatozoa were observed and classified as expressing one of the two staining patterns of Fig. 3A and Fig. 3B showing intact acrosome with uniform head staining and acrosome reacted with no head staining respectively.

Histopathology: Testis was preserved in 10% of neutral buffered formalin immediately after their removal from the animal for 1 hour and tissues were dehydrated in an ascending grade of alcohol and later 3 changes of 100% alcohol for 1.5 hours each. Dehydration followed with immersion in equal parts of alcohol and toluene for 30 minutes and toluene alone overnight. The dehydrated tissues were then embedded in paraffin blocks using wax of the same grade. The paraffin blocks were mounted and sections were later cut using a rotary microtome at a 3 µm thickness and mounted on labelled slides.

Following floatation process and adequate drying, the paraffin was first melted in an incubator at 60°C for 10 minutes and sections were allowed to cool before staining. Sections were later deparaffinized by immersion in two changes of xylene for 5 minutes followed by washing in 100% alcohol and descending solutions of alcohol each for 3 minutes. Staining commenced with immersion in Ehrlich's hematoxylin for 15 minutes followed by counter-staining in 1% aqueous eosin for 1 minute. Excess of both stains were removed by washing with tap water following the normal procedures and sections were dehydrated with ascending grades of alcohol ending with two washes with xylene. When the

sections were cooled, they were mounted in DPX and dried overnight. The architecture of the sectioned tissues was later observed using a low-power objective under a microscope.

Statistical Analysis: All data are expressed using Mean ± S.D. Statistical differences between the groups were analysed using one-way ANOVA (for more than two groups), followed by Tukey's Post Hoc test and using the SPSS version 12.0. The differences were considered statistically significant at $p<0.05$.

RESULTS

Body Weight: The body weights in Table 1 measured at day 0 and at day 33 to in means ± S.D. did not indicate any significant changes between the treatments.

Weight of Testis and Epididymis: The weight of testes and epidydimides were measured after treatment at day 33 after the mice were euthanized to determine direct effects on targeted organs. The means ± S.D. values of the weights of the testes (Table 2) showed no significant difference between all other treatment groups and the control group. Only the values for weights of epididymides between the group receiving NSE on its own and the combination group showed significant difference in results ($p<0.05$).

Sperm Count: The means ± S.D. values (Table 3) of the group of mice treated with cyclophosphamide alone showed significant difference ($p<0.05$) from the sperm count of the control group. All other groups did not indicate any significant difference in the count of sperm.

Histology: Fig. 1 showed normal morphology of the control mouse testis (A) and abnormal spermatogenic epithelium when exposed to CPA alone (B). The administration of NSE alone (C) and in the group receiving combination treatment of CPA and NSE (D) comparable spermatogenic epithelium and normal seminiferous tubules were observed as that shown in the control group.

Acrosomal Status: Figure 3 showed representation of either intact acrosome of sperm or acrosome-reacted sperm of mice in the various treatment groups. The mean percentages of acrosome-reacted sperm is shown in Figure 4 and results indicated a higher percentage of acrosome reaction in the group treated with CPA alone as compared to the control group and also the group of mice treated with NSE alone. However, no significant difference of values at $p<0.05$ was determined between all groups.

DISCUSSION

Overall body weights were determined as a function of increasing age [27] and from the results observed, the control mice showed a 7% increase in body weight from the initial weight. A 5% increment was observed in CPA treated mice which contradicted the findings by Elangovan *et al*. [25] whereby 200 mg/kg of CPA was shown to cause a reduction in body weight. A significant reduction was expected in mice injected with CPA for a period of 5 weeks compared to the single dose that had been used in this research. Elangovan *et al*., (2006) also showed that there was a significant reduction of weights of testes and epididymides in the CPA alone treated group as compared to controls. This trend, however, was not seen in this study and the group treated with the combination of both CPA and NSE seemed to show a reduced average of weights for both testes and epididymides possibly indicating a reduced compliance by the mice due to i.p. injections of the NSE on alternate days for the length of the study. There was also a significant decrease in the weight of epididymides of mice treated with both CPA and NSE as compared to that of mice administered with NSE alone.

Katoh *et al*., (2002) mentioned that diminished production of sperm is highly related with decreased testicular weight as the mass depended on differentiated spermatogenic cells [28]. This, nevertheless, was not obvious in this study as there seemed to be no relation in the decreased sperm count in the CPA alone treated group to its average weight of testes. Similarly, the sperm count in the group treated with the combination of CPA and NSE did not seem to be affected by the reduced testes mass as indicated by the lower average weight of testes in the group. No significant difference was detected between sperm count values between all groups but a higher value of sperm count was observed in the CPA + NSE group when compared to the CPA alone group perhaps indicating the positive antioxidant properties of the herbal product in the development of the germ cell line in the testis.

Normal seminiferous tubules and spermatogenic cells at various stages of development were observed in the testis of the control mouse. The histological sections of testis treated with CPA alone seemed to show similar morphology but vacuolizations and irregular sperm distribution were observed in the seminiferous tubules. Normal spermatogenic cell development was shown by

Body Weights and Reproductive Tissue Analysis

Table 1: Effects of CPA and NSE on the body weight of Balb/c mice before and after 33 days post-treatment

Group	Body weight (before) (g)	Body weight (after) (g)
CTRL	18.00 ± 1.67	19.33 ± 1.53
CPA	19.00 ± 1.41	20.00 ± 0.00
NSE	18.17 ± 2.40	18.17 ± 2.32
CPA + NSE	18.67 ± 1.75	15.25 ± 1.71

Values are in Means ± S.D. CTRL – Control; CPA- Cylophosphamide; NSE – *Nigella sativa* extract.

Table 2: Effects of CPA and NSE on weight of testis and epididymis of Balb/c mice at 33 days post-treatment.

Group	Testis weight (mg)	Epididymis weight (mg)
CTRL	94.17 ± 3.82	27.17 ± 2.02
CPA	93.17 ± 6.45	26.67 ± 0.76
NSE	93.42 ± 7.36	26.42 ± 3.88
CPA + NSE	78.38 ± 17.09	19.88 ± 3.75*

Values are in Means ± S.D. CTRL – Control; CPA- Cylophosphamide; NSE – *Nigella sativa* extract. * $p<0.05$ between the combination treatment group and that treated with NSE alone.

Table 3: Effects of CPA and NSE on the sperm count of Balb/c mice at 33 days post-treatment.

Sperm Concentration = Σ [Box 1 to Box 5] x 10 x 5 x 10 000	
Group	Sperm Count (million/ml)
CTRL	$2.23 \times 10^7 \pm 2.93 \times 10^6$
CPA	$0.95 \times 10^7 \pm 5.22 \times 10^6$
NSE	$2.06 \times 10^7 \pm 8.95 \times 10^6$
CPA + NSE	$2.95 \times 10^7 \pm 1.92 \times 10^7$

Values are in Means ± S.D. CTRL – Control; CPA- Cylophosphamide; NSE – *Nigella sativa* extract.

Histology of Testis:

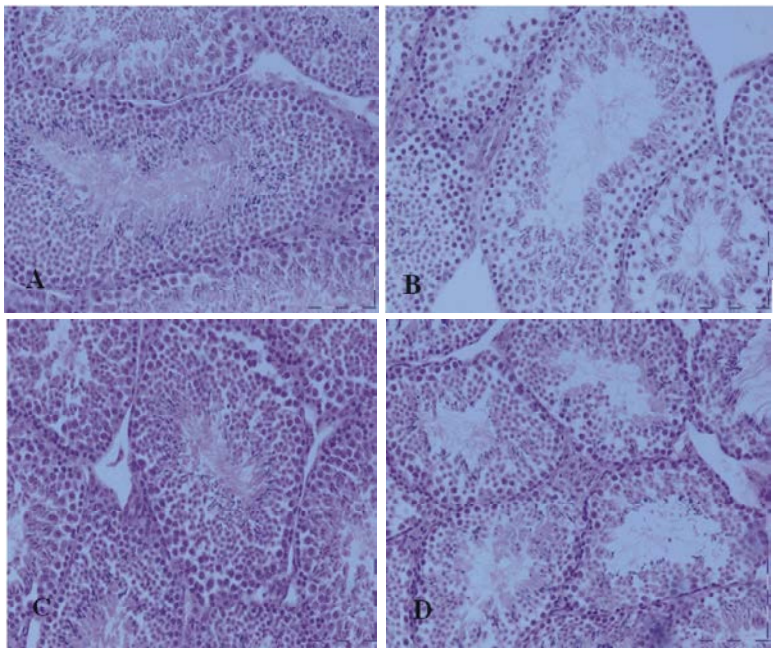

Fig. 1: Histological effects on the testes of Balb/c mice 33 days following administration of CPA and NSE (Haematoxylin and Eosin staining, original magnification X20). Morphology shows (A) Control testis (B) Testis of mouse treated with 200 mg/kg CPA (C) Testis of mouse treated with 10 mg/kg NSE and (D) 200 mg/kg CPA + 10 mg/kg NSE treated mouse testis

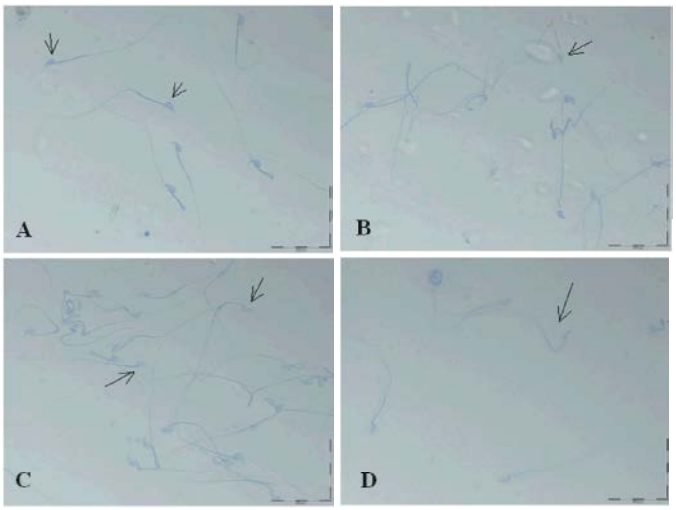

Fig. 2: Effects of 200 mg/kg CPA and 10 mg/kg NSE on the spermatozoa of Balb/c mice at 33 days post-treatment (Commassie Bright Blue (CBB) staining, original magnification X40). (A) Control group. (B) CPA treated group. (C) NSE treated group. (D) CPA + NSE treated group

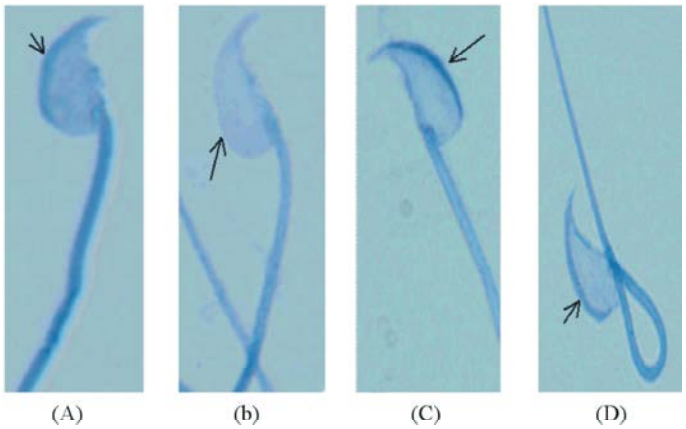

Fig. 3: Staining patterns of Balb/c mice spermatozoa in evaluation of acrosomal status during capacitation following treatment with 200 mg/kg CPA and 10 mg/kg NSE (Commassie Bright Blue (CBB) staining, original magnification X100). (A) Intact acrosome of control group. (B) Acrosome-reacted sperm of CPA treated group. (C) Intact acrosome in NSE treated group. (D) Intact acrosome of CPA + NSE group

Acrosomal status evaluation

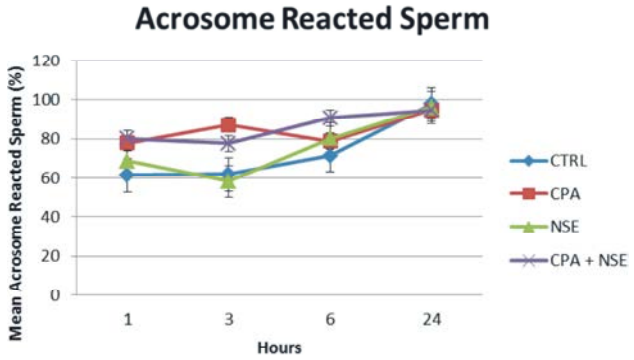

Fig. 4: Mean percentage of acrosome-reacted sperm of control Balb/c mice and that of mice treated with 200 mg/kg CPA alone or in combination with 10 mg/kg NSE at 1, 3, 6 and 24 hours following sacrifice at 33 days post-treatment

the morphology of testis of mouse treated with NSE alone substantiated with mature spermatozoa in the lumen. This is similarly seen in the testis of the CPA + NSE treatment group. The histology of this group seemed similar to that of the control group and the presence of a considerable number of spermatogonia cells including appropriate distribution of sperm in this group is seen to be higher compared to the CPA alone group.

Elangovan *et al.*, (2006) previously showed that the testis was severely damaged when mice were administered with 200 mg/kg of CPA for one week [25], coinciding with the morphological alteration to the testis following the single exposure to CPA in this study. CPA is an alkylating agent which is used as an anticancer drug due to its capability in affecting rapidly dividing cells thus the effect on the number of sperm has an indication of possible modification to the proliferative spermatogonia cells 33 days earlier. A restoration of the distribution of sperm in the tubules can be seen in the group that received CPA and NSE as compared to that receiving CPA alone, a phenomenon probably attributed to the protective properties of NSE [29].

Observation of the spermatozoa (Fig. 2B) of mouse treated with CPA alone showed distortion of sperm structure including observation of the detachment of a sperm head from its tail, an occurrence which was not seen in the other treatment groups. The smeared spermatozoa for the NSE alone and the combination treatment of CPA and NSE groups seemed to show no tangible modification of the morphology seen in the control group.

In an intact acrosome (Fig. 3A), the acrosome part located at the posterior head of the sperm is stained an intense blue colour. The same intense blue color was however not seen for the acrosome reacted sperm (Fig. 3B) as it has already undergone acrosomal reaction. The blue color stain is Commassie Bright Blue (CBB) which is known to be very stable and convenient in evaluating acrosome function [26]. Intact acrosome is an indication of sperm that are unable to release acrosome content although the sperm had been induced to undergo acrosome reaction *in vitro*. Studies by Obembe and Raji (2012) proved that intact acrosome is due to incapacitated sperm [26]. Aguilar-Mahecha *et al*., (2002) showed that CPA injected mice expressed the ornithine decarboxylase (ODC) gene which is responsible for polyamine synthesis and formation of putrescine [7]. Putrescine forms spermidine and spermine and these polyamines which originated from amino groups help in cell growth and differentiation. Over expression of the ODC gene has become a main concern among researchers as it could lead to male infertility. Spermine, a byproduct of putrescine is initiated by this gene and it was postulated that spermine acts as an inhibitory factor in sperm capacitation. In the early days of research into sperm capacitation, its definition overlaps with acrosome reaction but later in 1984, Chang, M. C. suggested all processes leading up to acrosome reaction should be considered as the first part of sperm capacitation, a period in which sperm uncover receptors that may recognize chemicals in the female reproductive tract leading to hyperactivation, motility and acrosome reaction [31]. Acrosome reaction occurs when membrane surrounding the acrosome fuses with the plasma membrane of the oocyte, releasing the acrosome enzymes enabling sperm to penetrate the egg and initiate fertilization.

No significant differences were calculated for mean percentages of acrosome reacted sperm in this study but the value for CPA treated group of mice was seen to show an increased number of acrosome-reacted sperm especially at 3 hours as compared to the control group (Fig. 4). This study did not incorporate the use of oocytes to trigger the process of acrosome reaction by contact between sperm and the zona pellucida. However, *in vitro*, acrosome reaction can be initiated by the encounter of sperm with naturally occurring substances such as progesterone or others in the media. Acrosome reaction, therefore, need not occur in the presence of an oocyte and this has been supported by studies such as that by Jin, M. *et al*. [32]. Gianaroli *et al*., (2008) had previously showed that in intracytoplasmic sperm injection (ICSI) procedure for IVF, the oocytes injected with acrosome-reacted sperm (39.0%) has a higher implantation rate than that injected with non-reacted spermatozoa (8.6%) [33].

From the observation after 3 hours, nearly 90% of sperm in CPA treated group have undergone acrosomal reaction. ROS is required in a very small amount in the testis to initiate sperm capacitation, acrosome reaction and fertilization and as a large dose of CPA has been introduced to the mouse 33 days earlier, the process of acrosome reaction in the resultant sperm was somehow to be expected. This therefore gives an indication that a higher percentage of acrosome-reacted sperm observed in the CPA alone treated group is not a foolproof sign of increased rates of fertilization. Elangovan *et al*., (2006) earlier stated that exposure to CPA may cause the sperm incapable of penetrating and fertilizing the oocyte due to decreased motility. The highly proliferative and differentiating spermatogonia cells have been shown to

be the most affected by cyclophosphamide in a study by Kamarzaman, *et al*. [34] and at the end of 32 days, they would have become sperm with an array of abnormalities and altered functions. CPA also produces ROS which causes lipid peroxidation in sperm cell. Aitken *et al*. [35] has long since proven that ROS is significantly increased in damaged sperm and this condition is known to reduce ability of sperm to bind to the oocyte thus preventing fertilization.

On the other hand, the incubation of oocytes with more acrosome-reacted sperm may well increase chances of fertilization [33] and the results of the NSE group in this study concur as an increased acrosomal reaction compared to controls was observed at 6 hours. The effects of CPA on the resulting acrosomal reaction at 6 hours also seemed to be ameliorated with the supplementation with NSE as an even higher percentage of acrosome reaction was observed. This is believed to be due to the antioxidant properties of NSE protecting the normal sperm cell that is exposed to ROS in agreement to the findings of Chi, H.J. *et al.*, (2008) that showed the effect of the antioxidant enzyme catalase in significantly increasing the acrosome reaction rate of human spermatozoa [36]. In addition, studies done by Bashandy (2007) showed that sperm motility was increased in hyperlipidemic rats when treated with *Nigella sativa* [37]. Thus the results obtained in this study seemed appropriate to support the antioxidative property of *Nigella sativa* in scavenging free radicals produced by CPA including weakening the negative effects of lipid peroxidation hence protecting spermatogenic cell development. DNA damage inhibits maturation of sperm cell and the acrosomal cap which is formed from Golgi derivatives seems to be affected by the over production of ROS. *Nigella sativa* may prove to be very useful due to its antioxidant activity which has the capacity to overcome the effects of oxidizing agents.

Nonetheless, the increase in percentage of acrosome-reacted sperm which is shown in the CPA group at 3 hours may be due to spontaneous acrosome reaction and impaired sperm motility [25, 35]. Another interesting test on human and mouse sperm was earlier done by Mizuno *et al.*, (2002), whereby both type of sperm were immobilized for 1to 24 hours before incubation with the mouse oocyte to emulate the protocols in ICSI. The human sperm at 24 hours was still able to fertilize the egg but mouse sperm was found to have lost its activating ability after only 6 hours indicating the higher susceptibility of the mouse reproductive cells to alterations in its environment [38]. The acrosome-reacted status of above 90% in all treatment groups as seen at 24 hours may well indicate that acrosome has been lost and all sperm cells have been affected by the accumulation of oxidative agents.

CONCLUSION

The experiments in this study showed that the manifestation of histology of mouse testis in the control and CPA+NSE groups seemed similar indicating that NSE may have restorative and protective properties primarily against oxidative damage induced by CPA in the male reproductive organs. The supplementation of NSE may also promote acrosome reaction facilitating the process of fertilization but effects are very much time-based and further investigations in the incubation of the male and female reproductive cells with NSE might shed better light on the possible positive application of the herbal product.

ACKNOWLEDGEMENT

This paper was partially funded by the International Islamic University Malaysia Endowment fund and authors would like to acknowledge the statistical assistance by Madam Norhafizah Abd. Manan, Faculty of Medicine of Cyberjaya University College of Medical Sciences (CUCMS) and the technical help by Madam Sriviowarti Noerdin from the Faculty of Pharmacy and Sr. Azantee Yazmie Abdul Wahab from the Fertility Centre of International Islamic University Malaysia (IIUM).

REFERENCES

1. Tabish, S.A, 2008. Complementary and Alternative Healthcare: Is it Evidence-based? Int J. Health Sci., 2: V-IX.
2. Dubick, M.A, 1986. Historical perspectives on the use of herbal preparations to promote health. J. Nutr., 116: 1348-54.
3. Ghazanfer, S.A, 1994. Handbook of Arabian medicinal plants. Boca Raton, FL: CRC Press, pp: 180.
4. Katoh, C., S. Kitajima, Y. Saga, J. Kanno, I. Horii and T. Inoue, 2002. Assessment of quantitative dual-parameter flow cytometric analysis for the evaluation of testicular toxicity using cyclophosphamide and ethinylestradiol treated rats. J. Toxicol. Sci., 27: 87-96.
5. De Smet, P.A.G.M., 1997. The role of plant-derived drugs and herbal medicines in health care. Drugs, 54: 801-40.

6. Patti, F. and S.L. Fermo, 2011. Lights and Shadows of Cyclophosphamide in the Treatment of Multiple Sclerosis. Autoimmune Diseases, pp: 1-14.
7. Aguilar-Mahecha, A., B.F. Hales and B. Robaire, 2002. Chronic Cyclophosphamide Treatment Alters the Expression of Stress Response Genes in Rat Male Germ Cells. Biology of Reproduction, 66: 1024-1032.
8. Shanafelt, T.D., T. Lin and S.M. Geyer, 2007. Pentostatin, cyclophosphamide and rituximab regimen in older patients with chronic lymphocytic leukemia. Cancer, 109: 2291-8.
9. Meriggiola, M.C., A. Costantinoa and S. Cerpolinia, 2002. Recent advances in hormonal male contraception. Contraception, 65: 269-272.
10. Brinkworth, M.H., 2000. Paternal transmission of genetic damage: findings in animals and humans. International Journal of Andrology, 23: 123-135.
11. Das, T., C. Purkayastha-Mukherjee, J. D'Angelo and M.A. Weir, 2002. Conserved F-box gene with unusual transcript localization. Dev. Genes E, 212: 134-140.
12. Ghosh, D., U.B. Das, S. Ghosh, M. Mallick and Debnath, 2002.Testicular gametogenic and steroidogenic activities in cyclophosphamide treated rat: a correlative study with testicular oxidative stress. J. Drug Chem Toxicol., 25: 281-92.
13. Awoniyi, D.O., 2010. The Role of Rooibos (*Aspalathus linearis*), Green Tea (*Camellia sinensis*) and Commercially Available Rooibos and Green Tea Antioxidant Supplements on Rat Testicular and Epididymal Function," (Ph.D. thesis, Cape Peninsula University of Technology, pp: 1-126.
14. Tariq, M, 2008. *Nigella sativa* Seeds: Folklore Treatment in Modern Day Medicine. Saudi J. Gastroenterology, 14: 1-5.
15. Gali-Muhtasib, H., N. El-Najjar and R. Schneider-Stock, 2006. The medicinal potential of black seed (*Nigella sativa*) and its components. Lead Molecules from Natural Products, pp: 133-153.
16. Randhawa, M.A. and M.S. Alghamdi, 2011. Anticancer Activity of *Nigella sativa* (Black Seed). A Review the American Journal of Chinese Medicine, 39: 1075-1091.
17. Ashraf, S.S., V. Rao and F.S. Kaneez, 2011. *Nigella sativa* Extract as a Potent Antioxidant for Petrochemical-Induced Oxidative Stress. Journal of Chromatographic Science, 49: 321-326.
18. Ahmed, W.A.A., S.A. Hassan, F.M. Galeb, M.A. El-Taweel and F.A. Abu-Bedair, 2008. The *in vitro* promising therapeutic activity of thymoquinone on hepatocellular carcinoma (HepG2) cell line. Global Veterinaria, 2(5): 233-241.
19. Mohammad Yaheya Mohammad Ismail, 2009. Therapeutic role of prophetic medicine *Habbat El Baraka* (*Nigella sativa* L.) – A review. World Applied Sciences Journal, 7(9): 1203-1208.
20. Farah, I.O. and R.A. Begum, 2003. Effect of *Nigella sativa* (L.) and oxidative stress on the survival pattern of MCF-7 breast cancer cells. Biomed Sci. Instrum, 39: 64.
21. Musa, D., N. Dilsiz, H. Gumushan, G. Ulakoglu and M. Bitiren, 2004. Antitumor activity of an ethonal extracts on *Nigella sativa* seeds. Biologia Bratislava, 59: 735-740.
22. Zaher, K.S., W.M. Ahmed and S.N. Zerizer, 2008. Observations on the Biological Effects of black cumin seed (*Nigella sativa*) and green tea (*Camellia sinensis*). Global Veterinaria, 2(4): 198-204.
23. Khalil, W.K.B., F.K.H. Abdel-Gawad, N. Belattar, Senator Abderrahmane and M.A. Abdel-Wahhab, 2011. Protective Effects of *Nigella sativa* extract against Chromiumvi-Induced Genotoxicity in Nile Tilapia (*Oreochromis niloticus*) and Zebrafish (*Danio rerio*). Global Veterinaria, 7(3): 283-293.
24. Shohayeb, M. and E. Halawani, 2012. Comparative antimicrobial activity of some active constituents of *N. sativa* L. World Applied Sciences J., 20(2): 182-189.
25. Elangovan, Namasivayam, Chiou, Tzeon-Jye, Tzenge, Woan-Fang, Chua, Sin-Tak, 2006. Cyclophosphamide treatment causes impairment of sperm and its fertilizing ability in mice. Toxicology, 222: 60-70.
26. Obembe, O.O. and Y. Raji, 2012. Reproductive Toxicity of *Quassia amara* Extract: Action on Sperm Capacitation and Acrosome Reaction. Academic Journal of Plant Sciences, 5: 60-69.
27. Bradshaw, A.D., D.C. Graves, K. Motamed and E.H. Sage, 2003. SPARC-null mice exhibit increased adiposity without significant differences in overall body weight. Proceedings of the National Academy of Sciences of the United States of America, 100: 6045-6050.
28. Katoh, C., S. Kitajima, Y. Saga, J. Kanno, I. Horii and T. Inoue, 2002. Assessment of quantitative dual-parameter flow cytometric analysis for the evaluation of testicular toxicity using cyclophosphamide and ethinylestradiol treated rats. J. Toxicol. Sci., 27: 87-96.
29. Fadwa, K.H., M. Tawfeek Suha, J. Ahmed Soulaf and Kakel, 2006. Effect of *Nigella sativa* oil treatment on the sex organs and sperm characters in rats exposed to hydrogen. Mesopotamia J. Agric, pp: 34.

30. Zhang, Y., Q.X. Xie, S.P. Pan, C.X. Zhang, L.J. Xiao and Y.L. Peng, 2005. Comparison of three methods for evaluating acrosome reaction in human spermatozoa. Zhonghua Nan Ke Xue, 11: 419-425.
31. Chang, M.C., 1984. The Meaning of Sperm Capacitation. Journal of Andrology, 5: 45-50.
32. Jin, M., E. Fujiwara, Y. Kakiuchi, M. Okabe, Y. Satouh, S.A. Baba, K. Chiba and N. Hirohashi, 2011. Most fertilizing mouse spermatozoa begin their acrosome reaction before contact with the zona pellucida during *in vitro* fertilization. Proc. Natl. Acad. Sci. USA, 108(12): 4892-6.
33. Gianaroli, L., M.C. Magli, A.P. Ferraretti, A. Crippa, M. Lappi, S. Capitani and B. Bacetti, 2010. Birefringence characteristics in sperm heads allow for the selection of reacted spermatozoa for intracytoplasmic sperm injection. Fertil. Steril., 93(3): 807-813.
34. Kamarzaman, S., M. Sha'ban and S. Abdul Rahman, 2013. Effects on Mouse Spermatogenesis and DNA Fragmentation Following Exposure to Cyclophosphamide and Thymoquinone. European International Journal of Science and Technology, 2(7): 119-136.
35. Aitken, R.J., J.S. Clarkson and S. Fishel, 1989. Generation of reactive oxygen species, lipid peroxidation and human sperm function. Biology of Reproduction, 40: 183-197.
36. Chi, H.J., J.H. Kim, C.S. Ryu, J.Y. Lee, J.S. Park, D.Y. Chung, S.Y. Choi, M.H. Kim, E.K. Chun and S.I. Roh, 2008. Protective effect of antioxidant supplementation in sperm-preparation medium against oxidative stress in human spermatozoa. Human Reprod., 23(5): 1023-1028.
37. Bashandy, A.E.S., 2007. Effect of Fixed Oil of *Nigella Sativa* on Male Fertility in Normal and Hyperlipidemic Rats. International Journal of Pharmacology, 3: 27-33.
38. Mizuno, K., K. Hoshi and T. Huang, 2002. Fertilization and embryo development in a mouse ICSI Model Using Human and Mouse Sperm after Immobilization in Polyvinylpyrrolidone, 17(9): 2350-2355.