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Effects of Thymoquinone Supplementation on Cyclophosphamide Toxicity of Mouse Embryo *In vitro*

[1]*Saheera Kamarzaman,* [2]*Azantee Yazmie Abdul Wahab and* [1]*Suzanah Abdul Rahman*

[1]Department of Biomedical Science, Faculty of Allied Health Sciences,
International Islamic University Malaysia (IIUM), Malaysia
[1]Fertility Centre, Faculty of Medicine,
International Islamic University Malaysia (IIUM), Malaysia

Abstract: Thymoquinone is the major active component derived from the traditional medicinal plant *Nigella sativa,* which has been shown to exhibit antioxidant property through different mechanisms in animal models. This study evaluates the prophylactic effect of thymoquinone supplementation on culture medium to ameliorate cyclophosphamide-induced alterations in cellular differentiation and proliferation during embryo development *in vitro*. Male and female mice were exposed to cyclophosphamide via a single intraperitoneal (i.p.) injection at 200 mg/kg. Sperms and oocytes were collected at day 33 and day 10 respectively, for insemination and fertilization in medium supplemented with thymoquinone (1µM, 10µM and 100µM). The stages of fertilization, embryo division, morphological effects and fragmentation were examined and compared between groups, 24 hours post-fertilization. Thymoquinone supplementation improved fertilization rates, significantly reduced the percentage of defects blastomeres of Type C (p<0.001) and significantly decreased the percentage of embryo fragmentation Grade IV (>50%, p<0.05) following paternal and maternal exposures to cyclophosphamide. The good quality embryos of Type A and Grade I fragmentation were not observed in the group without thymoquinone supplementation. The findings of this study showed that thymoquinone is a suitable exogenous antioxidant for preserving fair-quality embryos which can result into full term pregnancy.

Keywords: Cyclophosphamide · Thymoquinone · *In-vitro* Fertilization · Blastomeres · Embryo · Fragmentation

INTRODUCTION

Reactive oxygen species (ROS) are oxygen-derived free radicals that are formed during the intermediate steps of oxygen reduction [1]. They can cause direct oxidation by combining with other molecules which can lead to structural and functional changes and result in cellular damage [2,3]. In the event of excessive ROS production exceeding the antioxidant defense mechanism of the cells, it results in oxidative stress accompanied by other adverse effects [4].

Oxidative stress has been reported as one of the leading factors negatively affecting assisted reproductive techniques (ART) outcome due to the lack of embryo protection by oxygen radical scavengers [5,6]. Various factors can lead to the generation of oxidative stress, from either internal sources (e.g. Endogenous production of the spermatozoa, oocytes and embryo) or external sources (e.g. Culture media, oxygen concentration, visible light, freeze-thawing and ART procedures) in the *in-vitro* fertilization (IVF) setup [7]. Pathological effects of increased level of oxidative stress in the IVF setting are associated with the low cleavage rate, low blastocyst rate, high embryonic fragmentation, low fertilization rates, impaired embryo development and higher rates of pregnancy loss [8].

Cyclophosphamide (CPA), a commonly used anticancer drug, is one of the most damaging alkylating agents that affects the DNA of replicating cells and rapidly multiplying cells especially in the gonads and

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, Fax: +609-5716776.

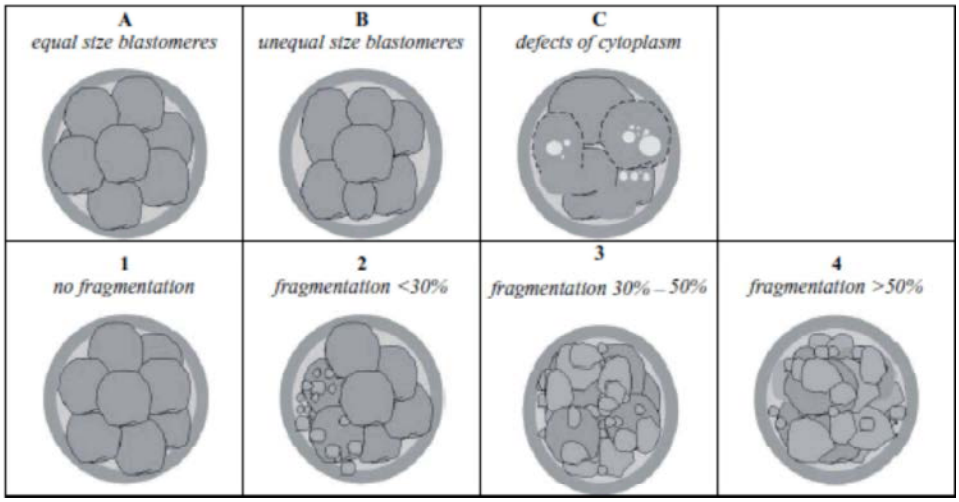

Fig. 1: Cleaving embryo scoring according to Baczkowski *et al*., [24].

pituitary which results in miscoding, cross-linking and DNA breakage by the transfer of alkyl groups to the guanine compound of the DNA [9]. Cyclophosphamide treatment causes significant systemic toxicity due to the overproduction of reactive oxygen species (ROS) that cause oxidative stress [10-12]. In male rodents, several studies have reported that administration of cyclophosphamide once a week for 5 weeks cause oligospermia, azoospermia, testicular damage and germ cell toxicity [13-15]. This drug has been shown to cause ovarian toxicity and induce depletion in the primordial follicular (PMF) reserve of female mice [16]. Cyclophosphamide has also been reported to be teratogenic in rodents. Single intraperitoneal injection of 20mg/kg at 10^{th} day of gestation was found to induce cleft palate, spina bifida, exencephaly, skeletal anomalies and sternum defects in mice foetuses [17].

Antioxidants provide a defense mechanism through three levels of protection; (i) prevention, (ii) interception and (iii) repair [18]. *In vitro* antioxidants and scavengers supplementation are able to minimize the development of a state of imbalance in oxidants and antioxidants. Various animal studies indicate that supplementation of culture medium with antioxidants, vitamin C and E, amino acids, ROS scavengers, disulphide reducing agents and divalent chelators of cations can reduce oxidative stress and be beneficial to embryo survival and blastulation rates [19].

Nigella sativa, from the Ranunculacea family, has been one of the most widely used herbal medicines for the treatment of various diseases. The pharmacological properties of the oil have been reported to include anti-cancer [20], anti-diabetic [21], anti-microbial [22], anti-viral [23], anti-bacterial [24], anti-clastogenic [25] and hypotensive effects [26,27]. Thymoquinone is the active compound of the essential oil with anti-oxidative effect that works as a scavenger of various radical oxygen species including superoxide radical anion and hydroxyl radicals through different mechanisms [28-30].

The present study, therefore, was undertaken to evaluate the prophylactic effect of thymoquinone supplementation on culture medium to ameliorate cyclophosphamide-induced alterations in fertilization rate, blastomere structure and grade of fragmentation during preimplantation embryo development.

MATERIALS AND METHODS

Animals: A total of 40 Balb/c mice (20 males and 20 females), aged 8 to 15 weeks, weighing about 20-30g, were obtained from the local laboratory (Kuala Lumpur, Malaysia) and provided with food pellets and water *ad libitum*.

Treatment Protocols: Cyclophosphamide, CPA (Sigma-Aldrich) was dissolved in normal saline and administered intraperitoneally by a single injection of 200 mg/kg. Mice (n=5/group) were assigned randomly into 4 experimental groups: (i) Group 1: Vehicle-treated control (normal saline) (ii) Group 2: Vehicle-treated positive control (culture media was supplemented with thymoquinone) (iii) Group 3: Male mice pre-treated with CPA and (iv) Group 4: Female mice pre-treated with CPA. The culture media for *in-vitro* fertilization in groups 2, 3 and 4 was supplemented with 1µM, 10µM and 100µM of thymoquinone. Male and female mice were sacrificed on day 33 and day 10 post-CPA exposure,respectively.

Mouse Superovulation: Each female mouse received 15 IU Gonal F (follicle stimulating hormone, Merck Serono) between 4 to 6 p.m. on day 7 post-CPA exposure, to stimulate follicle growth in the female mouse ovaries. The same female mice were given 10 IU hCG (human chorionic gonadotrophin, Merck Serono) 48 to 60 hours post-Gonal F (on day 9 post-CPA exposure) for ovulation to take place.

Sperm and Oocyte Collection: Sperms from the cauda epididymis were used as these sperms have already undergone some maturational changes in their biochemistry and morphology, thus have the potential to be progressively motile and therefore be able to fertilise an oocyte. Epididymis was placed in the SAGE® Sperm Preparation Media (LabIVF) and left in the CO_2 incubator for 30 to 40 minutes to allow the sperm to swim up.

Oocytes were collected 13 to 15 hours post-hCG injection at the time of ovulation. The gametes will gradually lose viability after 15 hours. The oocytes were collected from the oviduct as a cumulus-oocyte-complex (COCs) consisting of the oocytes surrounded by the granulosa cells. Using a pair of sterile needles, a small hole was pierced in the ampulla and the COCs were floated out in the SAGE® Quinn's Advantage Medium with HEPES (LabIVF) and Human Serum Albumin (LabIVF). The COCs was picked-up with the micro-pipette.

***In-vitro* Fertilization in Medium Containing Thymoquinone (Antioxidant):** The calculated sperm volume for insemination was aliquoted from the sperm preparation media together with the sperm mixture and placed into each 50 µl drop of SAGE® Quinns Advantage Protein Plus Fertilization (HTF) media (LabIVF). The media was supplemented with 5% of thymoquinone, approximately 200µl from each concentration: 1µM, 10µM and 100µM.

Embryo Culture: The droplets were checked 2 hours post insemination for signs of fertilization. The fertilized oocytes were transferred into a pre-prepared SAGE® Quinn's Advantage Protein Plus Cleavage Media (LabIVF). Embryo development was observed at 24 hours post-fertilization.

Embryo Evaluation and Grading: Embryo evaluation was made 24 hours after insemination. The presence of first cleavage, blastomere symmetry and the extent of fragmentation are examined. A total of 94 embryos was evaluated morphologically using the Inverted Microscope (Olympus). The embryos were divided into classes depending on three morphological parameters (i) the number of cells, (ii) the appearance of blastomeres and (iii) the presence of fragmentation or cytoplasm defects. The morphology of 2 to 8 blastomeres were divided into 4 grades as described by Baczkowski *et al.* [31]; Grade A: equal size blastomeres; Grade B: distinctly asymmetric blastomeres (unequal size) and Grade C: defects of cytoplasm. Additionally, the embryos were graded as the following; Grade I: no fragmentation; Grade II: fragmentation less than 30%; Grade III: fragmentation between 30 to 50% and Grade IV: fragmentation above 50% (Figure 1).

Statistical Analysis: Descriptive data are presented as percent. For statistical evaluation, Chi-Square test and Fisher's exact test were applied. p-value<0.05 was considered significant in this study.

RESULTS

Embryo Cleavage: Table 1 presents the morphological features of mice embryos following 24 hours post-insemination. There were no significant differences (p=0.096) in the number of fertilization between the groups, 24 hours following *in-vitro* culture as indicated by non-parametric Chi-Square test. Our IVF model indicated that the number of arrested oocytes was significantly higher (p<0.001) in the culture media of control group 1 without thymoquinone supplementation. Supplementation of media with 100µM of thymoquinone resulted in higher number of oocytes compared to the media supplemented with 1µM and 10µM of thymoquinone in all groups. Groups supplemented with thymoquinone were associated with an embryo contained 2 to 4 cells.

The Structure of Blastomeres: The percentage of the appearance of the blastomeres is presented in Table 2. Fisher's Exact test (Monte Carlo 2-sided) indicated that there was a significant difference (p<0.001) in the equality of the blastomere structure between control and treatment groups. Thymoquinone supplementation of 1µM, 10µM and 100µM was effective in producing a higher percentage of equal sized Type A embryo (28.7%). Type A blastomeres was not developed in group 1 without thymoquinone supplementation. The proportions of Type C blastomeres were not developed in culture media supplemented with 100µM thymoquinone in group 3 following paternal exposure to CPA and were significantly reduced to 16.7% in group 4 following maternal exposure to CPA. Fifty percent (50%) of the embryos in group 1 showed defects in cytoplasm.

Table 1: The morphological features of mice embryos following 24 hours *in-vitro* culture

Group	No. of oocytes	No. of arrested oocytes	No. of fertilized oocytes	Cell cleavage
Group 1: Control	27	17	10	>9 cells
Group 2 : Positive Control:				
1µM TQ	13	2	11	2 to 4 cells
10µM TQ*	0	0	0	0
100 µM TQ	17	4	13	2 to 4 cells
Group 3: CPA-treated male:				
1µM TQ	8	0	8	2 to 6 cells
10µM TQ	11	1	10	2 to 4 cells
100µM TQ	14	1	13	2 to 5 cells
Group 4: CPA-treated female:				
1µM TQ	10	2	8	2 to 3 cells
10µM TQ	5	2	3	2 to 3 cells
100µM TQ	20	2	18	2 to 4 cells

*Group 2 supplemented with 10uM TQ was excluded from the statistical analysis due to no oocytes.

Non-parametric Chi-square test was not significant, p=0.096

Table 2: The percentage of the structure of mice blastomeres following 24 hours *in-vitro* culture

Group	Type A (equal size)	Type B (Unequal size)	Type C (defects of cytoplasm)	Total
Group 1: Control	0	50% (5)	50% (5)	100% (10)
Group 2 : Positive Control:				
1µM TQ	81.8% (9)	9.1% (1)	9.1% (1)	100% (11)
10µM TQ*	0	0	0	0
100 µM TQ	30.8% (4)	61.5% (8)	7.7% (1)	100% (13)
Group 3: CPA-treated male:				
1µM TQ	50% (4)	50% (4)	0	100% (8)
10µM TQ	20% (2)	70% (7)	10% (1)	100% (10)
100µM TQ	7.7% (1)	92.3% (12)	0	100% (13)
Group 4: CPA-treated female:				
1µM TQ	0	100% (8)	0	100% (8)
10µM TQ	100% (3)	0	0	100% (3)
100µM TQ	22.2% (4)	61.1% (11)	16.7% (3)	100% (18)
Total Count	27	56	11	
% within treatment groups	28.70%	59.60%	11.70%	100% (94)

*Group 2 supplemented with 10uM TQ was excluded from the statistical analysis due to no oocytes.

Data is presented in percentage.

The number of embryo is presented in parentheses.

Fisher's Exact Test (Monte Carlo Sig 2-sided) was significant at p<0.001.

Table 3: The percentage of mice embryo fragmentation following 24 hours in-vitro culture

Group	Grade I (No fragmentation)	Grade II (Fragmentation <30%)	Grade III (Fragmentation 30% -50%)	Grade IV (Fragmentation >50%)	Total
Group 1: Control	0	0	50% (5)	50% (5)	100% (10)
Group 2 : Positive Control:					
1µM TQ	45.5% (5)	36.4% (4)	9.1% (1)	9.1% (1)	100% (11)
10µM TQ*	0	0	0	0	0
100 µM TQ	30.8% (4)	38.5% (5)	23.1% (3)	7.7% (1)	100% (13)
Group 3: CPA-treated male:					
1µM TQ	37.5% (3)	37.5% (3)	25% (2)	0	100% (8)
10µM TQ	20% (2)	60% (6)	20% (2)	0	100% (10)
100µM TQ	30.8% (4)	38.5% (5)	15.4% (2)	15.4% (2)	100% (13)
Group 4: CPA-treated female:					
1µM TQ	0	62.5% (5)	37.5% (3)	0	100% (8)
10µM TQ	0	100% (3)	0	0	100% (3)
100µM TQ	27.8% (5)	50% (9)	5.6% (1)	16.7% (3)	100% (18)
Total Count	23	40	19	12	94
% within treatment groups	24.50%	42.60%	20.20%	12.80%	100%

*Group 2 supplemented with 10uM TQ was excluded from the statistical analysis due to no oocytes.

Data is presented in percentage.

The number of embryo is presented in parentheses.

Fisher's Exact Test (Monte Carlo Sig 2-sided) was significant at p<0.05, p=0.017.

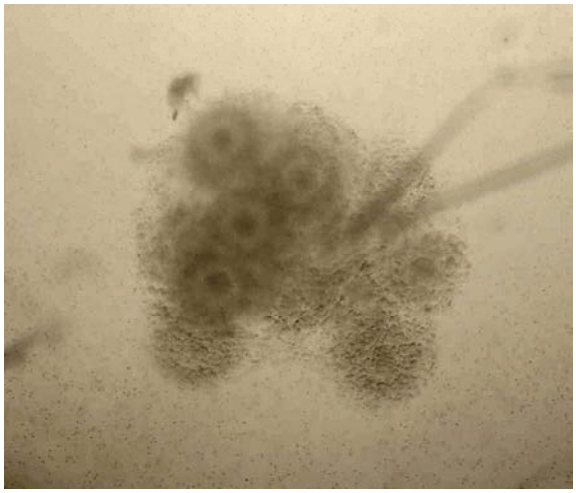

Fig. 2: Cumulus-oocyte-complex (COCs)

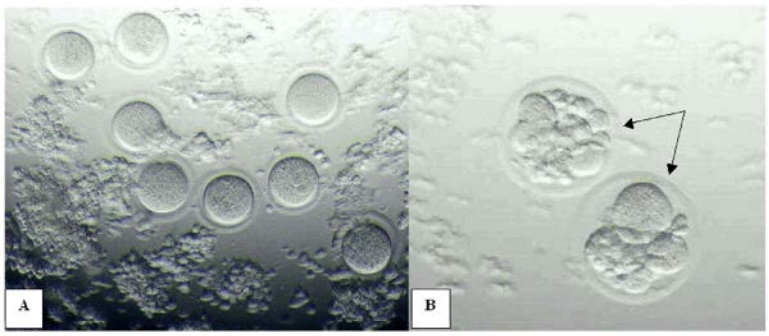

Fig. 3: Embryos of Group 1 (Vehicle-treated control without thymoquinone supplementation) following 24 hours *in-vitro* culture showing (A) arrested oocytes and (B) Grade IV fragmented embryos (>50%, arrows)

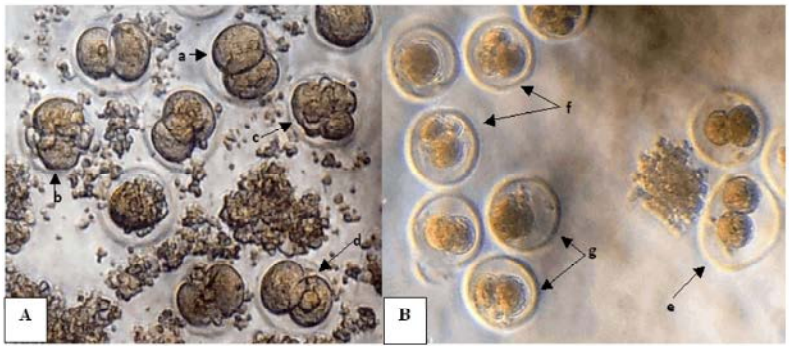

Fig. 4: Embryos of Group 2 (Positive control) supplemented with (A) 1uM of thymoquinone and (B) 100uM of thymoquinone, at different cleavage stages following 24 hours *in-vitro* culture.

a-Equal size blastomeres (Type A) with no fragmentation (Grade I).
b-Equal size blastomeres (Type A) with <30% fragmentation (Grade II).
c-Unequal size blastomeres (Type B) with no fragmentation (Grade I)
d-Defects of cytoplasm (Type C) with no fragmentation (Grade I).
e-Equal size blastomeres (Type A) with no fragmentation (Grade I).
f-Unequal size blastomeres (Type B) with no fragmentation (Grade I).
g-Unequal size blastomeres (Type B) with <30% fragmentation (Grade II).

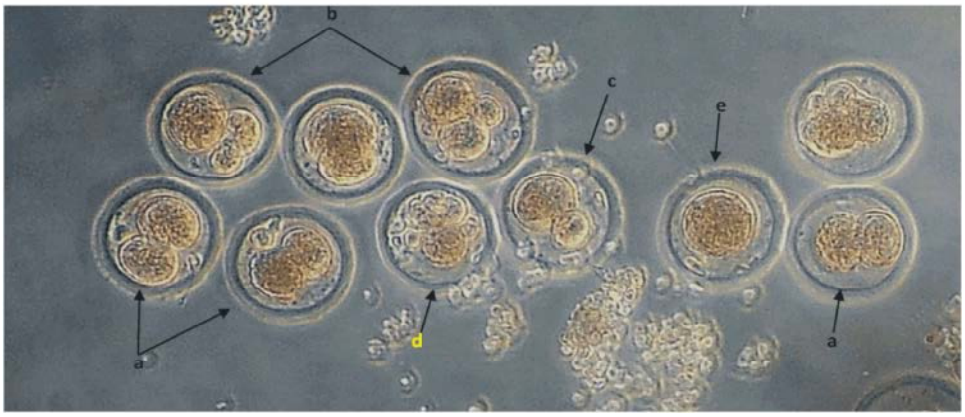

Fig. 5: Embryos of Group 3 (CPA-treated male mice) supplemented with 100µM of thymoquinone at different cleavage stages following 24 hours *in-vitro* culture.
a - Equal size blastomeres (Type A) with no fragmentation (Grade I).
b - Unequal size of 3-cells blastomeres (Type B) with no fragmentation (Grade I).
c - Unequal 2-cell blastomeres (Type B) with no fragmentation (Grade I).
d - Unequal blastomeres of more than 3 cells (Type B) with <3-% fragmentation (Grade II).
e - Arrested oocyte.

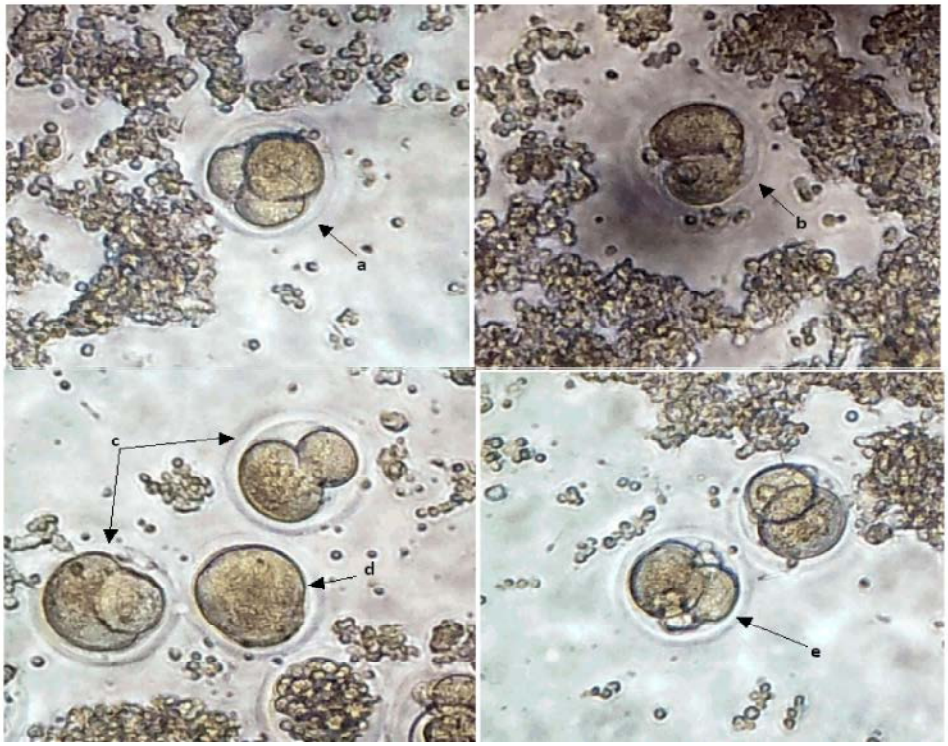

Fig. 6: Embryos of Group 4 (CPA-treated female mice) supplemented with 100uM of thymoquinone, at different cleavage stages following 24 hours *in-vitro* culture.
a-Unequal size blastomeres (Type B) with no fragmentation (Grade I).
b-Equal size blastomeres (Type A) with <30% fragmentation (Grade II).
c-Unequal size blastomeres (Type A) with <30% fragmentation (Grade II).
d-Arrested oocyte.
e-Unequal size blastomeres (Type B) with 30%-50% fragmentation (Grade III).

Embryo Fragmentation: Table 3 presents the percentage of embryo fragmentation following 24 hours *in-vitro* culture. The percentage of embryo fragmentation between groups are significantly different (p=0.017, p<0.05) as determined by Fisher's Exact test (Monte Carlo 2-sided). The overall proportion of Grade I non-fragmented embryos were 24.5% with thymoquinone supplementation and was not developed in group 1 media without thymoquinone. Most of fragmented embryos in media supplemented with thymoquinone were found to be of intermediate grade, i.e. Grade II (42.6%). Supplementation of 1µM and 10µM thymoquinone showed effectiveness in protecting the embryo from the development of grade IV fragmentation in groups pre-treated with cyclophosphamide, 0% in groups 3 and 4, compared to 50% in group 1. Culture media supplemented with 100µM thymoquinone showed low percentage of grade IV fragmentation with 15.4% and 16.7% following paternal and maternal exposures to CPA, respectively.

Morphology of the Embryos: Figure 2 shows the cumulus-oocyte-complex (COCs) collected 13 hours post-hCG injection. In Figure 3, representative photomicrographs of arrested oocytes and highly fragmented embryos (Grade IV) of group 1 are shown. In Figures 5, 6 and 7, embryo blastomeres of group 2 (positive control), group 3 (CPA-treated male mice) and group 4 (CPA-treated female mice) respectively, supplemented with 100µM thymoquinone at different cleavage stages following 24 hours *in-vitro* culture are shown. The majority of the embryos of groups 3 and 4 showed an unequal size of blastomeres (Type B) with a low grade of fragmentation (Grade I and Grade II) as opposed to group 1.

DISCUSSION

Identification of embryos that are most likely to result in full term pregnancy are crucial to the success of *in vitro* fertilization (IVF). In most cases of IVF procedure, multiple embryos are generated and cultured. However, selecting good quality embryos for implantation is highly subjective and the procedure can end in IVF failure or multiple pregnancies. Non-invasive methods of embryo evaluation are useful in reproductive medicine. They help assess embryos without damage by observing the morphology and dynamics of embryo development [32].

This study was designed to determine the developmental competency of mouse embryo in the presence or absence of antioxidant supplementation in the IVF setup. We expected thymoquinone supplementation to (i) provide a bed of nutrients for embryos to fertilize in the laboratory, (ii) protect *in-vitro* produced mouse embryos from oxidative stress and (iii) improve conditions for embryonic development by elevating thymoquinone antioxidant action, against the alkylating effects of cyclophosphamide.

In this study, a decreased rate of fertilization, a higher number of arrested oocytes, a higher percentage of unequally cleaved blastomeres and defects in the cytoplasm as well as a higher percentage of Grades III and IV embryo fragmentations were observed in the culture media without the supplementation of thymoquinone as compared to effects seen when thymoquinone was added. Agarwal *et al.* [5] reported that pathological levels of ROS have a negative impact on embryo quality and may lead to early embryonic developmental block and retardation. Similar findings were reported by Bedaiwy *et al*. [33] which showed that elevated day 1 ROS levels in culture media were associated with slow development, high fragmentation and reduced formation of a morphologically normal blastocyst which ultimately leads to a lower clinical pregnancy rate. Furthermore, Goto *et al.* [34] found that ROS production was increased in embryos cultured under *in vitro* conditions compared with those cultured *in vivo*. These results could be due to the increased levels of oxidative stress produced by the embryo or the environment and techniques employed during IVF culture. The embryo can produce ROS via several pathways, i.e. oxidative phosphorylation, NAPDH and xantine oxidase systems. The embryo is a fast developing organism with high energy needs that are met by generation of adenosine triphosphate (ATP) through mitochondrial oxidative phosphorylation and glycolisis. As the embryo develops from the zygote stage, its needs and metabolism changes and these will lead to alteration to its redox state [35]. Therefore, excessive generation of ROS occurs at certain critical points, such as during embryonic genome activity, embryonic compaction and hatching, due to increased in energy demands [36]. The nature of fertilization procedure in ART techniques can also contribute to ROS generation [6]. In conventional IVF, the oocytes, cumulus cell mass and spermatozoa can all generate and contribute to the ROS levels in the media. Centrifugation of spermatozoa during preparation techniques has been shown to increase ROS production and oxidative stress in male gametes [37]. The media used in the IVF setting can directly influence the quality of oocyte and embryo [6]. Some commercially available culture media can contain metallic ions (i.e. Fe^{2+} and Cu^{2+})

that can incorporate into the cells and independently accelerate ROS generation within these cells during ART processing [35]. Supplements added to the media may also increase the oxidant load. Supplementation of serum containing amine oxidase can lead to the increased of ROS (H_2O_2) production. Concurrent with this, the *in vitro* formed embryos have a lower antioxidant mechanism when compared to *in vivo* generated embryos as shown by the work of Feugang *et al.* [38]. Fragmented embryos have a limited developmental potential and rarely result in implantation [39]. Thus, all the observations above have led us to investigate the extent of the antioxidative effect of thymoquinone in a media environment in the protection of the developing embryo against either intrinsic ROS levels or that generated in the external environment throughout the process of IVF.

In vitro studies on the effect of cyclophosphamide on fertilization and cleavage demonstrated a dose-related inhibition [40]. Previous studies have shown that germ cells are specifically sensitive to cyclophosphamide treatment. Our previous study reported that 200mg/kg cyclophosphamide treatment when observed at 32 days post-treatment, kills up to 52% of spermatogonia and 47.6% of elongated spermatid in male mice [15]. In female mice, cyclophosphamide administration of 75mg/kg destroyed more than 50% of primordial follicle (PMF) pool [16]. A recent study on the consequences of maternal cyclophosphamide treatment (75mg/kg) in pre-implantation mouse embryos reported a reduction in the oocyte fertilization rate, a reduction in 8-cell stage embryos formation from 48 to 72 hours post-fertilization and increased aneuploidy compared to controls [41]. Similar findings were reported by Pydyn and Ataya [42] that showed reduced oocyte fertilization and early cleavage rates when female mice were injected intraperitoneally with 100mg/kg cyclophosphamide. Treatment of oocytes with increasing concentrations (1 to 1000µg/ml) of cyclophosphamide for 48 hours resulted in dose-response inhibition of the rate of maturation of pig oocytes *in vitro* [43]. The particular sensitivity of the reproductive tissues to cyclophosphamide is due to the high proliferating activity [44]. It is also reported that exposure to single high dose of cyclophosphamide can produce DNA fragmentation in mouse testicular cells [15], probably resulted from cross-linking of DNA. It is therefore possible that cyclophosphamide can induce these defects during embryo culture *in vitro* and may play a role in the progeny outcome. These observations are indicative of the effects of 200 mg/kg cyclophosphamide used in this study on the reproductive functions and expected fertilization rates.

Previous studies of IVF media used for bovine and mouse embryo culture supplemented with antioxidants such as vitamins C and E, taurine, hypotaurine, thiols and B-mercaptoethanol showed great success of embryo development. Our results demonstrated that embryos cultured in medium containing thymoquinone during fertilization, following paternal and maternal exposure to cyclophosphamide resulted in the improvement of fertilization rates, low level of arrested oocytes, as well as a low percentage of cytoplasmic defects and fragmentation. A similar observation was reported when culture media was supplemented with 100µM vitamin E that resulted in significantly more expanded blastocysts than embryos cultured in control medium [45]. Vitamin E is a natural antioxidant that inhibits NADPH oxidase-mediated generation of superoxide anion and protects cell membranes against oxidative stress [46]. Similarly, vitamin C has been reported to protect DNA from exogenous oxidation [47], prevents apoptosis in rat and mouse follicles, improves blastocyst production in mice [48] as well as improves the developmental competence of porcine embryos *in vitro* [49]. It can reduce embryo oxidative stress by inducing hypotaurine and taurine in the oviduct which can neutralize hydroxyl radicals and prevent sperm lipid peroxidation even at low concentrations [35]. Exogenous antioxidant such as β-mercaptoethanol (βME) has been frequently used to increase antioxidant capacity of embryos via increasing intracellular levels of ROS scavengers such as glutathione (GSH) [50,51]. A recent study by Hosseini et al. [52] reported that βME was found to increase the cleavage rates and improve both hatching rate and quality of hatched embryos.

These results diverge from those obtained by Alhimaidi [53], who demonstrated that treatment of thymoquinone at 10 mg/ml into sperms and embryo culture media reduces the sperm motility, fertilization rate and embryo development *in vitro*. The defects could be due to the action of thymoquinone on the enzymes of the sperm or on the antigen of the cell membrane of the sperm.

In this study, media supplemented with thymoquinone were observed to allow the development of 4-cell embryos 24 hours following culture *in vitro*. A good quality embryo should have at least 4 cells on the second day and at least 8 cells on the third day of culture [31]. The defense mechanism of thymoquinone against ROS does not only act as a superoxide anion scavenger but also as a general free radical scavenger [54]. Thymoquinone is also known as an effective inhibitor of lipid peroxidation [55].

CONCLUSION

The results of the experiment indicated that the influence of cyclophosphamide exposure on pre-implantation embryo development can be ameliorated by supplementing the culture medium with thymoquinone. The addition of thymoquinone in the culture media plays a role in increasing the developmental competency as well as in increasing the resistance of mouse embryos cultured *in vitro* to ROS. From these results, further investigations are needed to determine the appropriate concentrations of the antioxidant, as an excess of antioxidant compounds in the medium may have deleterious effects on the embryo.

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