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Influence of Media Supplementation with Zinc and/or Thymoquinone on *in vitro* Fertilization and Mouse Oocytes Development

Ibrahim A.H. Barakat,[1,2]* Jamilah O. L. Al-Shammari,[1] Saleh A. Khandeal,[1,3] and Nadia A. Al-Eisa[1]

[1]*Department of Zoology, College of Science, King Saud University, P.O. Box 2455, Riyadh 11451, Kingdom of Saudi Arabia*

[2]*Department of Cell Biology, National Research Centre, 33 Bohouth st., Dokki, Giza, Egypt*

[3]*Department of Animal Production, Faculty of Agriculture, Cairo University, Egypt*

Abstract. In the present study, thymoquinone (TQ) and zinc were added to fertilization and embryos culture media to check if this supplementation could improve the fertilization rate and development of *in vivo* matured mice oocytes. The effects of ovarian side (R and L) and strain of mice (C57BL/6J and SWR) on the *in vitro* fertilization and developmental competence of mice oocytes after *in vitro* fertilization were also investigated. The concentration used was 5 µg/ml for TQ and zinc was and 10 µg/ml media for zinc, respectively. Eighty adult female mice (40 from each strain) were used and subdivided randomly into 4 groups: group 1 was untreated (control); group 2 was for treatment with zinc; group 3 was for TQ and group 4 was for combined action of zinc and TQ. The results showed that fertilization rate and embryo development up to two cell stage improved when zinc was added in the culture media compared to TQ alone or zinc. The mean number of fertilized oocyte was significantly ($P \leq 0.05$) increased when C57BL/6J and right ovary were used. However, the type of strain used had no effect on the embryo development, whereas the ovarian side had a significant effect on the oocyte development. To conclude fertilization rate and fertilized oocyte development were better when the medium supplemented with zinc, right ovary and C57BL/6J strain was used.

Key words: Thymoquinone, zinc, *in vitro* fertilization, mouse strain, oocyte development.

INTRODUCTION

The *in vitro* embryo production (IVEP) system includes three major steps, namely *in vitro* maturation (IVM) of the primary oocytes, *in vitro* fertilization (IVF) of the matured oocytes and *in vitro* culture (IVC) of presumptive embryos, until transferred to recipients or cryopreserved for future use (Gandolfi *et al.*, 2005; Zhu *et al.*, 2007). Therefore, IVEP needs to be optimized to produce healthy and viable embryos yield. A major factor affecting *in vitro* mammalian embryo production is increased oxidative stress (Gaspmini *et al.*, 2000), which is caused by an increase of reactive oxygen species (ROS), also called free radicals (Miesel *et al.*, 1993). ROS, such as hydrogen peroxide (H_2O_2), superoxide anions (O_2-), and hydroxyl radicals (OH), are produced by aerobic activity of organisms, oocytes and embryos, external environment and culture media surrounding embryos during IVF (Guerin *et al.*, 2001; Martin-Romero *et al.*, 2008). This can adversely influence the early development of mouse, hamster, and bovine embryos (Blondin *et al.*, 1997; Harvey *et al.*, 2002; Watson *et al.*, 1994), sperm motility and axonemal protein phosphorylation (Aitken *et al.*, 1993), and block *in vitro* two cell embryos (Nasr-Esfahani and Johnson, 1992). The role of these end products is not fully understood; however, some authors reported they have important roles in cell signaling and homeostasis (Devasagayam *et al.*, 2004). If ROS levels increase dramatically in the culture environment, this may result in significant increase of oxidative stress, which can harmfully affect the cell membrane (Aitken *et al.*, 1989), DNA integrity (Halliwell and Aruoma, 1991), apoptosis (Yang *et al.*, 1998), gene expression and transcriptional factors (Sikka, 2003). This is a process known as oxidative stress which occurs as a consequence of an alteration in the equilibrium of the production of ROS and antioxidative processes in favor of the overproduction of ROS. An excessive oxidative stress, and/or inadequate antioxidant defense, may thus occur as the result of a pro-oxidant-antioxidant imbalance, and leading to

* Corresponding author: ibrahimahb@yahoo.com, ibarakat@ksu.edu.sa

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consequential damage to lipids, carbohydrates, proteins, and DNA. Therefore, maintenance of the balance between the oxidant and antioxidant systems represents a vital aspect both of normal and abnormal cell function and survival (Faris *et al.*, 2013). The success rates of IVF depend to a large extent on the antioxidant supplementation to neutralize the effect of ROS on oocytes and embryos quality (Agarwal *et al.*, 2008). Barakat *et al.* (2014) showed that using of green tea axtract as a source of antioxidants in the maturation medium improvement the *in vitro* maturation and embryo development of sheep cumulus oocytes complexs (COCs) to blastocyst stage. Vahedi *et al.* (2009) showed that addition of some useful materials such as growth factors, estradiol, gonadotropins, and antioxidant (Balasubramanian and Rhob, 2007) are necessary for improvement of embryo production. The antioxidants supplementation to the culture medium can be divided into two large groups: one that is non-enzymatic or metabolic with low molecular weight, such as L-ascorbic acid (vitamin C), cysteine, α-tocopherol, and β-mercaptoethanol and ones with an enzymatic action, such as superoxide dismutase (SOD), glutathione peroxidase, and catalase (Nordberg and Arnér, 2001). Within the body, oocytes and embryos can be protected from oxidative stress by free radical and enzymatic scavenging by the aid of antioxidants that exist within the follicular and oviductal fluid (Gupta *et al.*, 2010; Wang *et al.*, 2002).

Natural herbal constituents are extensively studied for their ability to protect cells from miscellaneous damages. Currently, the use of phytochemicals as a therapy in diseases related to oxidative stress has gained immense interest for their ability to quench free radicals by electron or proton donation and their capability to protect body tissues against oxidative stress (Nabavi *et al.*, 2012). *Nigella sativa* (black seeds) has a long history in medicinal use for centuries. Thymoquinone (TQ), the major bioactive constituent of *Nigella sativa* seed has been reported to exhibit many pharmacological effects (Norwood *et al.*, 2006; Wilson-Simpson *et al.*, 2007; Ragheb *et al.*, 2009; Bouchra *et al.*, 2009; Woo *et al.*, 2011).

Zinc is an essential trace element found in small amounts in a variety of cells and tissues. It is a cofactor of more than 300 enzymes (Tapeiro and Tew, 2003), and is involved in several cell functions including signal transduction, transcription and replication (Cousins *et al.*, 2006). It has been known that a non adequate level of zinc can alter not only gene expression but also a variety of cellular functions with severe consequences on animal health (Selker *et al.*, 2007). Several studies showed that men with high dietary intake of antioxidants have a lower frequency of sperm aneuploidy and improved semen quality compared with men with low intake (Silver *et al.*, 2005; Young *et al.*, 2008). As a result, over the last decade, several antioxidant nutraceutical formulations have been developed and proposed as a therapy for male infertility. Recently, a new nutraceutical formulation containing zinc, D-aspartate (D-Asp) and Coenzyme Q10 (CoQ10), a combination of antioxidants and micronutrients, has been developed by Merck Serono. Zinc is a cofactor for several metalloenzymes involved in DNA transcription and protein synthesis and also has anti-apoptotic and antioxidant properties (Ebisch *et al.*, 2007). Zinc therapy in men with asthenozoospermia resulted in a significant increase in sperm concentration, progressive motility, sperm integrity and improved conception and pregnancy rates (Omu *et al.*, 1999). Recently, major efforts have been made to determine the effect of trace elements on performance of embryo production *in vitro*. Best to our knowledge there is no report regarding use of TQ with or without zinc as a source of exogenous antioxidant supplementation in culture media during *in vitro* production of mice embryos system. Thus, the objectives of the present study were to evaluate the impact of TQ with or without addition of zinc to fertilization and culture media, on developmental competence of mice oocytes *in vitro*.

MATERIALS AND METHODS

Chemicals and materials

All chemicals were purchased from Sigma Chemical Company (St. Louis, MO, USA) unless otherwise indicated. Polystyrene plastic culture dishes (35x10 mm, 60x10 mm) and 0.22 µm millipore membrane filters syringe were purchased from Nunclon, Nalge Nunc International, Roskide, Denmark.

Experimental animals

A total of healthy adult 20 male and 80 females mice of C57BL/6J and SWR strain (*Mus musculus*) approximately 6 to 10 weeks old ranging in weight from 25 to 30 g obtained from animal house, Faculty of Pharmacy, King Saud University were used in the present study. Animals were housed in 35x25x25 cm polypropylene rodent cages at 25°C on a 12: 12 h light cycle and were kept in the laboratory under constant conditions for at least one week before use. Each cage contained five females, while males were housed separately. Animals were provided with feed and water *ad libitum*. The experiments were approved by the state authorities and followed king Saud University rules on animal protection.

Super-ovulation and mature oocytes collection

Oocytes were collected from super-ovulated adult female mice inbred strain (C57BL/6J and SWR) as previously described by Rinaudo and Schultz (2004) with little modifications. Briefly, female mice were injected intraperitoneally (i.p) with 10 IU follicle stimulating hormone (FSH) and 48 h later with 10 IU human chorionic gonadotropin (hCG). The following morning, after 16 h from the last injection, females were sacrificed by cervical dislocation and mature COCs were obtained from the ampullae of the oviduct in a collection medium. The cumulus cells surrounding the oocytes were removed by pipetting in collection medium containing 80 IU ml^{-1} hyaluronidase and randomly divided into two groups.

Sperm preparation

Spermatozoa were obtained from the cauda epididymis of the mature C57BL/6J and SWR male mice into 0.4 ml of M2 media. The sperm were left for 5 – 10 min. in CO_2 incubator at 37°C for dispersion. Sperm motility and concentration measurement were performed at 37°C in M2 medium using a hot plate. The sperm motility was assessed and classified as progressive or not progressive. Progressive motility was defined as the sperm head that moved in a forward direction. Numbers of sperm were counted in four chamber of hemocytometer slide and was expressed per milliliter of suspension. The sperm suspension was covered by mineral oil and incubated under 5% CO_2 in humidified air at 37°C to induce sperm capacitation for acquisition of fertilizing ability.

Experimental design

To evaluate the role of zinc and TQ on *in vitro* mice embryo production, 1179 *in vivo* matured oocytes from C57BL/6J and SWR strains were used; 335 oocytes were used for control group, 315 oocytes were used for studying the effects of zinc in fertilization and culture media, 268 mature oocytes were used for evaluating the role of TQ added to the fertilization and culture medium and 261 mature oocytes were used to study the effect of interaction between zinc and TQ on the embryo production when supplemented to fertilization and culture media. Each treatment was repeated five times as replications. According to the capacitation and culture medium used oocytes were divided into 4 groups: group I, spermatozoa were capacitated in basic capacitation medium (M2) and zygotes were cultured in basic culture medium (M16); group II, spermatozoa were capacitated in M2 plus $ZnCl_2$ at 10 µg ml^{-1} fertilization medium and zygotes were cultured in the M16 culture medium plus 10 µg ml-1 $ZnCl_2$; group III, TQ was added to the fertilization and culture media with 5 µg ml^{-1} concentration, and group IV, ZnCl2 and TQ were added in combination to the fertilization and culture media with the same concentrations used in the second and third groups, respectively. Group I served as the control group and groups II, III and IV were considered as treatment groups.

In vitro fertilization and embryo culture

For *in vitro* fertilization (IVF) 10 µl of sperm suspension either treated with zinc or TQ or zinc plus TQ as treated groups and without additives as control group were added to 400 µl drop of M2 medium containing oocytes and left for 6–8 h. in CO_2 incubator. After completion of fertilization period, fertilized oocytes were classified into four categories; a) fertilized oocytes that characterized by presence of two pronuclei and second polar body, b) unfertilized oocytes that did not have any polar bodies and degenerated first polar body in some oocytes, c) shirked oocytes that appeared with shrinkage cytoplasm and d) fragmented oocytes,

cytoplasm was converted into small pieces with different sizes and each part was surrounded by a plasma membrane. Concerning embryo development, fertilized oocytes were cultured in 500 µl M16 medium covered with mineral oil as control group and supplemented with zinc or TQ or zinc plus TQ as treated groups under 5% CO_2 in humidified air at 37°C for 5 consecutive days.

Statistical analysis

Replicates of experiments were performed on different days with different batches of oocytes and semen. Statistical analyses for all data were carried out using analysis of variance (ANOVA). Statistical differences were considered significant at $P \leq 0.05$ levels by using Duncan's Multiple Range Test procedure (Duncan, 1955). Results were expressed as mean ± SEM (standard error of mean). All the calculations were performed using the SPSS statistical program.

RESULTS

Effect of zinc and /or TQ supplementation

Table I showed that there was a significant ($P \leq 0.05$) difference in the number of fertilized oocytes, where zinc treatment gave the highest mean (1.3) compared to other treatment groups. There was no significant difference between the group treated with TQ and the group treated with zinc and TQ together. The same trend of results found for rate of fertilization was also true for development of mice oocytes *in vitro*.

Effect of mouse strain

The results showed that there were significant ($P \leq 0.05$) differences for the average number of fertilized oocytes between C57BL and SWR strains, where the mean was 0.7 for the SWR strain, and 0.6 for C57BL/6J strain, while there were no any significant differences between strains for the development of the fertilized oocytes to two cells embryo stage (Table II).

Effect of ovarian side

Table III showed that the ovarian side (R & L) significantly ($P \leq 0.05$) affected the rate of oocytes fertilized *in vitro* and the number of embryos formed, where the average number of fertilized oocytes for left ovary was 0.5±01, while the right ovary was 0.81. Also, the mean number of embryos formed was significantly ($P \leq 0.05$) different where it was 11.83±1.6 for left ovary and 15.73 for right ovary.

Effect of the interaction between different treatments and strain of mice

Statistical analysis revealed that the results for the group treated with zinc gave the highest mean for the number of fertilized oocytes in both strains (1.3±1.0) compared to the control group (1.0±0.2) and (0.8±0.2) for SWR and C57 strain, respectively. In contrast, there was no significant difference between the treatment group with zinc and the group treated with zinc and TQ together in both strains (Fig. 1).

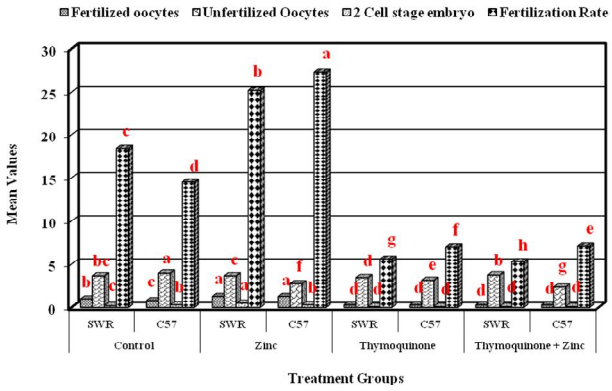

Fig. 1. Effect of different treatments and type breeds on *in vitro* fertilization and development of fertilized oocytes.

DISCUSSION

ROS are involved in several cellular signaling mechanisms and they can interact with lipids, proteins and DNA, leading to severe pathologic conditions. High ROS levels are detrimental to gametes, and compromise their function through lipid peroxidation, protein damage and DNA strand breakage (Aitken *et al.*, 2010). Although, spermatozoa physiologically produce ROS promoting sperm capacitation, in some pathological conditions the semen ROS levels exceeds the sperm antioxidant defenses and lead to a state of oxidative stress that could impair not only fertilization but

Table I. Effect of zinc and/or thymoquinone on *in vitro* fertilization and development of mouse oocytes.

Treatments	Fertilized oocytes	Unfertilized oocytes	Fertilization rate	Tow cell stage embryos
Control	0.9 ± 0.1 b	3.9 ± 0.1 a	16.45 ± 2.05 b	0.2 ± 0.1 b
Zinc	1.3 ± 0.1 a	3.2 ± 0.1 c	26.2 ± 2.56 a	0.4 ± 0.1 a
Thymoquinone	0.3 ± 0.1 c	3.3 ± 0.1 b	6.29 ± 2.2 c	0.1± 0.0 c
Zinc + Thymoquinone	0.3 ± 0.1 c	3.1 ± 0.2 d	6.17 ± 2.1 d	0.1± 0.0 c

* Mean values within the same column with different superscripts (a, b, c, d) differ significantly ($p \leq 0.05$).

Table II. Effect of mouse strain (SWR and C57BL/6J) on *in vitro* fertilization and development of matured oocytes

Treatments	Normal oocytes	Abnormal oocytes	Fertilized oocytes	Unfertilized oocytes	Fertilization rate	Tow cell stage embryos
SWR	4.4 ± 0.1 a	3.3 ± 0.1 a	0.7 ± 0.1 a	3.6 ± 0.1 a	13.6 ± 1.7 b	0.2 ± 0.0 a
C57BL/6J	3.7 ± 0.1 b	2.7 ± 0.1 b	0.6 ± 0.1 b	3.1 ± 0.1 b	14.0 ± 2.0 a	0.2 ± 0.0 a

* Mean values within the same column with different superscripts (a, b) differ significantly ($p \leq 0.05$).

Table III. Effect of ovarian side on *in vitro* fertilization and development of *in vivo* matured oocytes.

Treatments	Normal oocytes	Abnormal oocytes	Fertilized oocytes	Unfertilized oocytes	Fertilization rate	Tow cell stage embryos
Right	4.3 ± 0.1 a	3.3 ± 0.1 a	0.8 ± 0.1 a	3.5 ± 0.1 a	15.7 ± 2.0 a	0.2 ± 0.0 a
Left	3.8 ± 0.1 b	2.7 ± 0.1 b	0.5 ± 0.1 b	3.2 ± 0.1 b	11.8 ± 1.6 b	0.1 ± 0.0 b

* Mean values within the same column with different superscripts (a, b) differ significantly ($p \leq 0.05$).

also embryo development (Aitken *et al.*, 2010). *In vivo* studies suggested that oral administration of antioxidants improves semen quality and pregnancy rates in sub-fertile men (Gharagozloo and Aitken, 2011; Showell *et al.*, 2011). To this end, in the present study we evaluated the effects of zinc, TQ and their interaction on the *in vitro* fertilization rate and embryo developmental competence in mice. Main results demonstrated that the mean of fertilization rate and embryo development improved when zinc was added to the culture media than compared to TQ alone or zinc plus TQ. The mouse strain and ovarian sides also affected *in vitro* fertilization rate and embryo development. To our knowledge, there are no studies on the effect of TQ with or without zinc as antioxidant on fertilization rate and developmental competence. Zinc concentration in seminal plasma is generally higher than in serum and has been positively correlated with sperm count and motility (Ebisch *et al.*, 2007; Yuyan *et al.*, 2008; Akinloye *et al.*, 2011). On the other hand, a negative effect of high zinc levels in seminal plasma or in sperm tails on sperm motility has been reported by others (Henkel *et al.*, 1999; Wong *et al.*, 2001). Despite these contradictory results, the role of zinc as an antioxidant is well established. Zinc *in vitro* is able to inhibit both superoxide anion generation and SOD-like activity in spermatozoa of infertile men (Gavella *et al.*, 1999). Also, the sperm count, motility and fertilization capacity may be preserved by zinc supplementation (Saki *et al.*, 2010). Our results are in agreement with Alhimaidi (2005), who found that treatment with TQ immobilized spermatozoa and inhibit the embryo development *in vitro*. These results showed the opposite of what expected from the treatment of TQ to take the access of the free radicals in the medium. TQ is known to work as an antioxidant and effective inhibitor of lipid peroxidatin (Houghton *et al.*, 1995). High levels of ROS can induce DNA damage in spermatozoa. This damage can be produced during comigration of mature and immature spermatozoa from the seminiferous tubules to the caudal epididymis where

sperm are highly packed and this would facilitate ROS-induced DNA damage (Ollero *et al.*, 2001). Moreover, the process of DNA fragmentation in spermatozoa progresses even after ejaculation. *In vitro* incubation of swim-up selected human spermatozoa results in a progressive increase in the percentage of DNA fragmented sperm (Muratori *et al.*, 2003). In agreement with those studies, we found that sperm culture for 6 h induces an increase of sperm DNA fragmentation and, more interestingly, it is prevented by antioxidant treatment. According to Aitken *et al.* (2010) sperm oxidative stress not only could impair the sperm fertilizing ability but also its competence to sustain a correct embryo development. Moreover, DNA damage in human spermatozoa has been correlated with increased miscarriage rates and morbidity of the offspring (Muratori *et al.*, 2003; Robinson *et al.*, 2012). The treatment with TQ might act on the respiratory enzymes of spermatozoa or on the antigen of the cell membrane of spermatozoa, but this need more molecular studies by histochemistry and electron microscopy to be confirmed and clarified (Alhimaidi, 2005). Thymoquinone is the major active constituent of *Nigella sativa* (black seeds). Seeds have been used in traditional medicines to treat a variety of ailments and most of its biological effects are mainly attributed to TQ (Ilaiyaraja *et al.*, 2013). We think that the role of thymoqunione in improving *in vitro* embryo production needs further studies to investigate their effects on ultra-structural and physiological attributes of mammalian sperm, oocytes and embryos.

CONCLUSIONS

Our results demonstrate that the addition of zinc to the medium environment leading to improves mice oocytes fertilization percentage compared to thymoquinone and that the addition of thymoquinone in the fertilization medium needs to be more studied. The study also demonstrated that the direction of ovary and strain type affects the *in vitro* fertilization percentage, where we found that the right ovary and C57BL/6J strain increased the fertilization percentage.

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