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Lycopene Improves Maturation Rate and Antioxidant Status of in vitro Matured Mouse Oocytes

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ABSTRACT

The present study aimed to investigate the effect of lycopene supplementation to the in vitro maturation (IVM) medium of mouse oocytes on their maturation rate and oxidative biomarkers levels. Lycopene concentrations of 50, 100, 200, and 400nM were tested. The effects of lycopene on maturation rate of mouse oocytes under in vitro oxidative and heat stress conditions were also explored. Cumulus-oocyte complexes (COCs) were collected from ovaries of super-ovulated mice via puncturing technique and incubated in the IVM medium for 17h. To simulate oxidative stress, COCs were incubated in an IVM medium containing 100µM hydrogen peroxide (H_2O_2) with or without lycopene. Additionally, to mimic heat stress, COCs were in vitro matured at 40℃ with or without lycopene. Among the four tested lycopene concentrations, the 200nM lycopene achieved the highest improvement in oocyte maturation. The oocyte concentrations of H_2O_2, malondialdehyde (MDA), total antioxidant capacity (TAC), reduced glutathione (GSH), catalase (CAT), and superoxide dismutase (SOD) were altered by lycopene treatment: H_2O_2 and MDA were significantly decreased, meanwhile TAC, GSH, CAT and SOD were increased. IVM rates of mouse oocytes revealed a significant decline following their exposure to heat and oxidative stresses. Interestingly, lycopene supplementation rescued the IVM rate of the oxidative stressed or the thermally challenged mouse oocytes as they appeared comparable to those of the control oocytes. Taken together, our data report beneficial effects of lycopene on mouse oocytes and suggest lycopene as a candidate factor for improving the quality of mammalian oocytes and embryos.

Key words: Heat stress; In vitro maturation; Lycopene; Mouse oocytes; Oxidative stress.

INTRODUCTION

In vitro maturation (IVM) is the initial and most critical step of in vitro embryo production (IVEP) during which oocytes obtain their ability to support further embryo development. It involves a series of complex and diverse events of nuclear and cytoplasmic changes that provide oocytes the inherent potential to promote embryo development (Ferreira et al. 2009). The IVM efficiency is influenced by various factors including the quality of oocytes and the cullure conditions (Koo et al. 2008).

Oxidative stress is triggered during IVM of mammalian oocytes by excessive production of reactive oxygen species (ROS), a natural by-product of cellular respiration and metabolism (Jiang et al. 2018). They primarily consist of superoxide anions (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radicals (OH) (Halliwell and Gutteridge 2015).

Lipid peroxidation involves deterioration of unsaturated fatty acids by action of ROS (Oborna et al. 2010). Malondialdehyde (MDA) is a stable end product of lipid peroxidation and its levels are usually utilized as an indicator of oxidative stress in oocytes (Yalçınkaya et al. 2013).

The total antioxidant capacity (TAC) is the sum of endogenous and food-derived antioxidants within the oocyte (Young 2001). Enzymatic and non-enzymatic components are involved in intracellular antioxidant processes. The superoxide dismutases (SODs) catalyze the conversion of O_2^- to H_2O_2 (Wang et al. 2018), and the catalase (CAT) decomposes H_2O_2 into water and oxygen, are examples of antioxidant enzymes (Nandi et al. 2019). Reduced glutathione (GSH) is the most abundant thiol in mammalian cells, and it plays an important antioxidant role by neutralizing reactive oxygen species (Wu et al. 2004; Mandouh et al. 2020).

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The rising global temperature exposes animals to stressful environmental circumstances, particularly in the summer. This results in a reduction in domestic animal fertility due to an increase in body temperature exceeding physiological limitations, a condition known as heat stress (Boni 2019). Heat stress represents a potential risk for female infertility since it causes a series of physiological, metabolic, cellular, and molecular alterations in the reproductive tract (Roth 2017).

Oxidative damage of oocyte components is among the alterations induced by heat stress which could result in structural and functional changes and induce apoptosis (Ahmed et al. 2017). As well, heat stress has been reported to alter oocyte nuclear and cytoskeletal architecture and delay early embryonic development (Andreu-Vázquez et al. 2010). Heat stress stimulates excessive production of free radicals within maturing oocytes (Combelles et al. 2009). In cattle, the increased generation of free radicals within the oocyte following the heat stress is associated with meiotic arrest, poor oocyte quality, and a lower rate of embryo development (Sakatani 2017; Diaz et al. 2021).

The impact of antioxidants on oocytes is dependent on the nature and mode of action of the utilized substance. Among the substances that have been used to improve to the quality of in vitro matured oocytes are thiols, quercetin, carotenoids, vitamins C and E, resveratrol, and melatonin (Sovernigo et al. 2017; Budani and Tiboni 2020).

Lycopene is a red-colored carotenoid pigment that presents in tomatoes and a number of fruits and vegetables (Lugasi et al. 2003). Lycopene has been reported to have a strong free radical scavenging ability (Miller et al. 1996; Wenli et al. 2001). Supplementation of IVM media of bovine oocytes with 0.2µM lycopene resulted in a significant improvement in the oocyte quality and increase in oocyte maturation (Chowdhury et al. 2018; Sidi et al. 2022). Lycopene treatment also reduced intracellular ROS levels and increased mitochondrial activities in oocytes and preimplantation embryos (Chowdhury et al. 2018).

The present study aimed to investigate the effect of lycopene supplementation to the IVM medium on the maturation rate of mouse oocytes. The effects of lycopene on oocytes matured under in vitro oxidative and heat stress were also evaluated.

MATERIALS AND METHODS

Ethical Approval

The protocol of this study was permitted by the Committee for Research Ethics at the Faculty of Veterinary Medicine, Mansoura University, Egypt.

Animals

The present study was performed at the Reproductive Biology Research Laboratory (RBRL), Department of Theriogenology, Faculty of Veterinary Medicine, Mansoura University (Egypt). Thirty-five mature female mice (8-12 weeks old; ~ 25g bwt) were used for the IVM, oxidative and heat stress studies. The mice were maintained in a temperature-controlled environment (21-23ºC) under a 12h light/dark cycle and allowed to freely access feed and water ad libitum in the Medical Experimental Research Center (MERK), Faculty of Medicine, Mansoura University (Egypt). All mice were administrated intraperitoneal injections of 10IU pregnant mare's serum gonadotropin (PMSG, Gonaser®, HIPRA, Spain) 48h before their scarification (Takahashi et al. 2003; Jia et al. 2019; Zhang et al. 2019).

Collection of Ovaries and Recovery of Oocytes

After ensuring death of mice, the abdomen was opened, and the ovaries were located and extracted out from the body. Then, the ovaries were cleaned out from remnants of the surrounding fat and washed in pre-warmed (37ºC) sterile physiological saline (0.9% NaCl) to remove blood stains (Jia et al. 2019). The ovaries were then placed in a sterile 60mm Petri dish containing G-MOPS™ plus medium (Vitrolife, Sweden) for oocytes recovery.

Cumulus–oocyte complexes (COCs) were released from the ovaries via gentle puncturing of the antral follicles using a sterile insulin syringe needle under an SZ61 zoom stereomicroscope (Olympus, Japan) according to Monti and Redi (2016). Released COCs were picked out and sorted on the basis of their morphological appearance. Only good quality COCs in which oocytes appeared with evenly granular ooplasm and surrounded by compact multilayers of follicular cells were selected and used for subsequent studies (Zhang et al. 2019).

Lycopene Preparation

Lycopene extract (≥98%) with molecular weight 536.87g/mol was purchased from Nawah Scientific Inc. (Cairo, Egypt, HIKA2010). For preparing lycopene stock solution, 10mg of lycopene were dissolved in 18.6mL of dimethyl sulfoxide (DMSO; Sigma-Aldrich, D8418) to achieve a concertation of 1mM. Lycopene stock solution was immediately aliquoted, protected from light and stored at -20°C till the day of use. Lycopene working solutions were freshly prepared every time via diluting the stock solution with appropriate amounts of the IVM medium.

In vitro Maturation of Mouse Cumulus–oocyte Complexes (COCs)

Immature mouse oocytes were in vitro matured in pre-equilibrated Global total medium (LifeGlobal, USA) as described by Harada et al. (2021). Briefly, COCs were washed three times in handling medium followed by another three times wash in IVM medium. The COCs were randomly cultured in groups of 20-25 oocytes in a 50µL prewarmed droplet of IVM medium covered with sterilized mineral oil for 17h at 37ºC in 5%CO_2 in air with maximum humidity (95%) according to Eppig et al. (2009) for experiments 1-3 and at 40ºC for experiment 4 to mimic in vivo heat stress.

Assessment of Maturation Rate

Oocyte maturation was judged by the degree of cumulus cell expansion and the nuclear maturation changes. After 17h of maturation, oocytes were denuded from the cumulus cells by pipetting several times. Oocytes that revealed breakdown of the germinal vesicle with extrusion first polar body were considered mature (Nikseresht et al. 2015).

Biochemical Analysis

Three replicates were analyzed per each group. Each replicate consisted of 20 oocytes in 100µL of IVM

medium. The oocytes were pelleted by their centrifugation at 3000rpm for 10min at 4ºC. The cell pellets were gently rinsed twice with 500µL of cold phosphate-buffered saline (PBS). The pellets were disrupted in 800µL of lysis buffer (50mM sodium phosphate, 300mM NaCl, pH=8.0) via passing them in three cycles of repeated freezing and thawing followed by vortexing. Lysed oocytes were centrifuged at 12,000rpm for 15min at 4ºC and the supernatant was decanted and used for biochemical analysis (Rakha et al. 2022).

The levels of H_2O_2 (Fossati et al. 1980) and MDA (Ohkawa et al. 1979) within the matured oocytes were assessed calorimetrically using commercially available kits (Biodiagnostics, Egypt) per manufacturer's instructions.

The concentration of TAC (Koracevic et al. 2001), GSH (Beutler et al. 1963), SOD (Nishikimi et al. 1972), and CAT (Aebi 1984) within the matured oocytes were estimated using colorimetric assay kits (Biodiagnostics, Cairo, Egypt) per manufacturer's instructions.

Experimental Design

Experiment 1: Effect of lycopene addition to IVM medium on maturation rate of mouse oocytes: mouse COCs were divided into five groups based on the concentration of lycopene added to the IVM medium. The concentrations of lycopene were 0 (control), 50, 100, 200 and 400nM. After 17h of IVM, oocyte maturation rates were calculated and compared between different groups to select the best concentration of lycopene to be used.

Experiment 2: Effect of lycopene addition to IVM medium on oxidative stress and antioxidant biomarkers levels of in vitro matured mouse oocytes: mouse COCs were divided into two groups: control (matured without lycopene) and lycopene-treated (matured with 200nM lycopene) groups. At the end of the incubation period (17h), COCs were collected, denuded, lysed, and the oocyte concentrations of H_2O_2, MDA, TAC, GSH, CAT and SOD were measured and compared between different groups.

Experiment 3: Effect of lycopene on maturation rate of mouse oocytes matured under oxidative stress conditions: the immature oocytes were divided into three groups: control group (oocytes matured without lycopene), oxidative stress group (oocytes cocultured with 100µM H_2O_2) and oxidative stress + lycopene group (oocytes cocultured with 100µM H_2O_2 and 200nM lycopene).

Experiment 4: Effect of lycopene on maturation rate of mouse oocytes matured under heat stress conditions: the immature oocytes were divided into three groups: control group (oocytes matured at 37℃ without lycopene), heat stress group (oocytes matured at 40℃ without lycopene) and heat stress + lycopene group (oocytes matured at 40℃ with 200nM lycopene).

Statistical Analysis

Each experiment was performed three times. Data were analyzed in GraphPad Prism 7 (GraphPad Software, CA, USA). Group pairs and multiple groups were compared by the Student's t-test and one-way analysis of variance (ANOVA), respectively. The data were expressed as the mean±SEM. Differences of $P<0.05$ were utilized to indicate statistical significance.

RESULTS

Effect of Lycopene Addition to IVM Medium on Maturation Rate of Mouse Oocytes

Among the four tested concentrations of lycopene, lycopene addition to the IVM medium of mouse oocytes at concentrations of 100nM and 200nM revealed significant increase ($P=0.005$ and $P=0.0001$ respectively) in their maturation rates (81.14±1.50% and 89.89±0.73% respectively) compared to the control group (66.69±2.40%) (Fig. 1A). Conversely, maturation rates of mouse oocytes did not change significantly ($P>0.05$) by addition of lycopene to the IVM medium of mouse oocytes at concentrations of 50nM and 400nM (69.71±3.62 and 62.59±1.24% respectively) (Fig. 1A). Based on these results, the 200nM lycopene concentration was used for subsequent experiments.

Effect of Lycopene Addition to IVM Medium on Oxidative Stress and Antioxidant Biomarkers Levels of in vitro Matured Mouse Oocytes

Addition of lycopene to the IVM medium of mouse oocytes at the concentration of 200nM resulted in a significant decrease in the intracellular levels of H_2O_2 of mouse oocytes compared to the control group by the end of the in vitro maturation period (0.153±0.003mmol/L vs. 0.190±0.006mmol/L, $P=0.005$) (Table 1). Similarly, the intracellular levels of MDA of mouse oocytes were found to be significantly decreased by lycopene addition to their IVM medium at a concentration of 200nM in comparison to the control group (3.467±0.219nmol/mL vs. 5.300±0.058nmol/mL, $P=0.001$) (Table 1).

Addition of lycopene to the IVM medium of mouse oocytes at the concentration of 200nM revealed a significant increase in the oocyte intracellular levels of TAC compared to the control group (0.663±0.024mmol/L vs. 0.547±0.009mmol/L, $P=0.010$) (Table 1). Moreover, lycopene addition to the IVM medium of mouse oocytes at the concentration of 200nM led to significant increases in the oocyte intracellular levels of other antioxidants namely GSH (4.283±0.261mmol/L vs. 3.097±0.078mmol/L, $P=0.012$), CAT (0.313±0.009U/L vs. 0.233±0.007U/L, $P=0.002$), and SOD (364.667±3.844U/mL vs. 285.000±7.371U/mL, $P=0.0007$) (Table 1).

Effect of Lycopene on Maturation Rate of Mouse Oocytes Matured under Oxidative Stress Condition

Oocytes matured in the presence of 100µmol/L of H_2O_2 showed a significant decrease in their nuclear maturation rates compared to the control oocytes (43.16±1.24% vs. 69.21±1.27%, $P<0.0001$). On the other hand, oocytes matured in the presence of both 100µmol/L of H_2O_2, and 200nM of lycopene revealed a significant increase in their nuclear maturation rate compared to H_2O_2 treated oocytes (66.70±0.99% vs. 43.16±1.24%, $P<0.0001$). No significant difference ($P=0.35$) in the oocyte nuclear maturation rate was seen between oocytes treated simultaneously with H_2O_2 and lycopene and the H_2O_2 untreated oocytes (69.21±1.27%) (Fig. 1B).

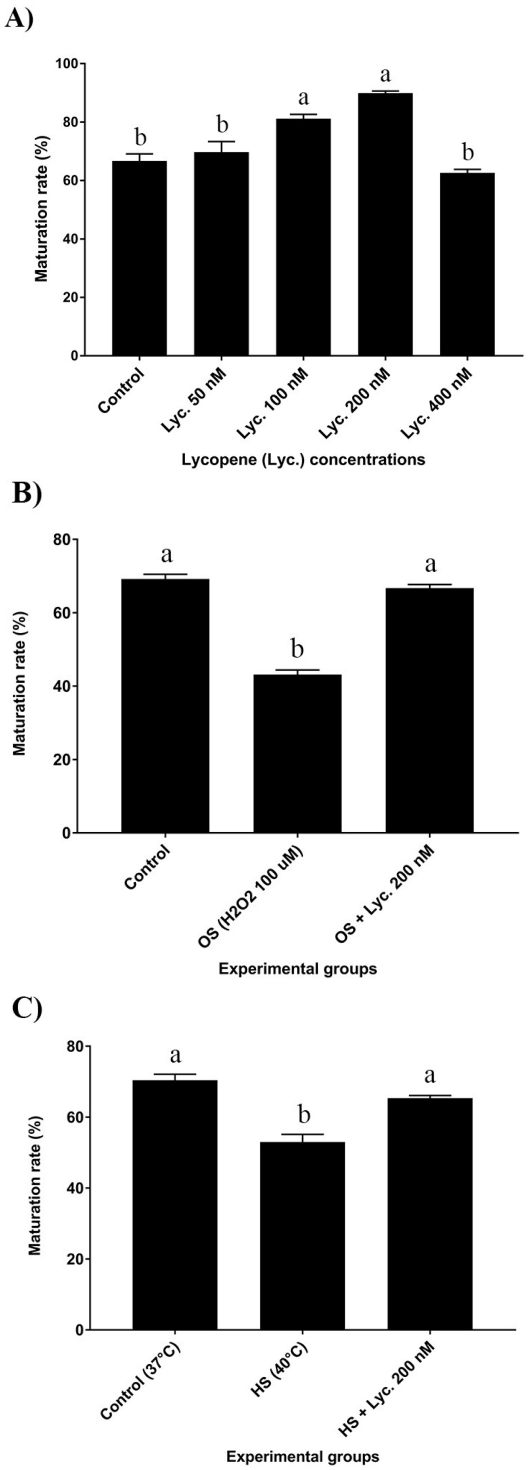

Fig. 1: Effect of lycopene addition to IVM medium on maturation rate of mouse oocytes. A) Effect of four different concentrations of lycopene on maturation rates of mouse oocytes cultured under standard laboratory conditions. Based on this experiment, the 200nM lycopene concentration was used for subsequent experiments. B) Maturation rates of mouse oocytes cultured under oxidative stress (OS) conditions in the presence or absence of 200nM lycopene. C) Maturation rates of mouse oocytes cultured under heat stress (HS) conditions in the presence or absence of 200nM lycopene. The error bars represent the standard errors of the means. Different superscript letters indicate significant differences (P<0.05).

Table 1: Effect of lycopene addition to IVM medium on oxidative stress and antioxidant biomarkers levels of in vitro matured mouse oocytes

Item	Control	Lyc. 200nM
H_2O_2 (mmol/L)	0.190±0.006	0.153±0.003**
MDA (nmol/mL)	5.300±0.058	3.467±0.219**
TAC (mmol/L)	0.547±0.009	0.663±0.024*
GSH (mmol/L)	3.097±0.078	4.283±0.261*
CAT (U/L)	0.233±0.007	0.313±0.009**
SOD (U/mL)	285±7.371	364.667±3.844***

Data are presented as mean ± SEM. *P<0.05, **P<0.01, ***P<0.001.

Effect of Lycopene on Maturation Rate of Mouse Oocytes Matured under Heat Stress Condition

Mouse oocytes matured at 40°C for 17h revealed a significant decrease in their nuclear maturation rates compared to those matured at 37°C for the same time period (53.00±2.14% vs. 70.42±1.70%, P=0.0007). On the other hand, oocytes matured at 40°C for 17h in the presence of 200nM lycopene revealed a significant increase in their nuclear maturation rates compared to those matured at 40°C (65.35±0.77% vs. 53.00±2.14%, P=0.004). However, they did not differ significantly (P=0.15) in their maturation rates from oocytes matured at 37°C for a similar time period (70.42±1.70%) (Fig. 1C).

DISCUSSION

The laboratory mouse, *Mus musculus*, is regarded as the reproductive biologists mammal of choice (Tevosian 2014). It is the most commonly used animal in laboratory research due to its small size and inexpensive cost (Hickman et al. 2017). The short reproductive life span and high number of offspring of mouse made it an excellent model for reproductive biology related studies (Bryda 2013).

IVM of oocytes is a critical step during in vitro generation of embryos. Maintaining high-quality oocytes represents a great challenge due to different possible sources of stress that may affect oocytes during IVM (Hatırnaz et al. 2018). Oxidative stress stands at the top of these types of stress (Soto-Heras and Paramio 2020). Thus, continuous search for substances with the most potent antioxidant activity to minimize oocyte oxidative damage is an ongoing research subject. In line with this, the present study analyzed the effect of addition of four different concentrations of lycopene, namely 50, 100, 200, and 400nM, during IVM of mouse oocytes. Among these concentrations, addition of 200nM lycopene has been found to achieve the highest increase in oocyte IVM rate which was accompanied with a significant decline in H_2O_2 and MDA levels and an increase in TAC, GSH, CAT and SOD levels. Similar to our findings, bovine oocytes supplemented with 200nM lycopene have been found to improve their IVM rate which was associated with decreased production of ROS (Chowdhury et al. 2018). On the other hand, our finding doesn't coincide with those of Watanabe et al. (2010) in which supplementation of porcine oocytes with 7M lycopene didn't affect their meiotic competence. Though the concentration used by the latter study seems to be substantially high compared to the concentration used by the present study, the authors of the aforementioned study noted a rise in glutathione

concentration in oocytes matured with lycopene supplementation compared to control oocytes confirming the antioxidant effect of lycopene on oocytes.

In order to confirm the promoting effect of lycopene on maturation and antioxidant status of oocytes, lycopene was added to mouse oocytes that matured under oxidative stress. Towards this aim, mouse COCs were matured in an IVM with a final concentration of 100µM H_2O_2, a concentration known to form a proper threshold of oxidative stress on mouse oocytes (Goud et al. 2008; Zhou et al. 2016). As expected, the present study observed a significant decrease in the IVM rate of mouse oocytes challenged with H_2O_2 during their IVM compared to the control group. On the other hand, lycopene supplementation during IVM rescued mouse oocytes from the damaging effects of H_2O_2 which was reflected by their maturation rate which appeared close to that of the control oocytes.

Heat stress affects oocyte development and function. The best example for this effect is seen in buffalos which display a significant decrease in their fertility during hot weather (Das and Khan 2010). Oocytes have been proven to be much more sensitive than embryos. The thermoneutral zone of laboratory mice ranges from 20 to 26°C (Keijer et al. 2019). Incubating oocytes at high temperature during IVM disrupted their maturation rates (Payton et al. 2004; Roth and Hansen 2005; Wang et al. 2009). Oocyte response to heat shock usually involves generation of high amounts of ROS which were reduced by administration of antioxidants both in vitro and in vivo (Lawrence et al. 2004; Roth et al. 2008). Protective effect of lycopene on thermally challenged oocytes has been suggested (Kang et al. 2021; Sidi et al. 2021). Supplementation of bovine COCs with 0.2µM lycopene during their IVM at 40.5°C resulted in higher maturation rate than those matured at the same temperature with no lycopene (Sidi et al. 2021). Remarkably, maturation rate of heat-stressed lycopene treated oocytes was comparable to those of matured at standard laboratory temperature (38.5°C) (Sidi et al. 2021). Similar trends in oocyte maturation rates under elevated temperature have been observed by the present work, suggesting a conserved effect of lycopene on heat-challenged oocytes.

In conclusion, our data report a beneficial effect for lycopene during IVM of mouse oocytes via decreasing their oxidative damages. The results of the present work also suggest a protective effect of lycopene on mouse oocytes exposed to oxidative and heat stresses. Further research is needed to evaluate the effect of lycopene during in vitro fertilization and culture of preimplantation embryos of mouse, domestic mammals, and human.

Author's Contribution

Conceptualization, S.I.R. and S.M.Z.; methodology, S.I.R.; software, S.I.R. and M.A.E.; validation, S.I.R. and M.A.E.; formal analysis, S.I.R. and M.A.E.; investigation, S.I.R.; re-sources, S.I.R. and A.M.M.; data curation, S.I.R. and M.A.E.; writing original draft preparation, S.I.R. and A.Z.B.; writing review and editing, S.I.R. and M.A.E.; visualization, S.I.R.; supervision, A.M.M. and S.M.Z.; project administration, H.E.-S.A., A.M.M., and S.M.Z.; funding acquisition, S.I.R., H.E.-S.A., A.M.M., and S.M.Z. All authors have read and agreed to the published version of the manuscript.

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