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# QUERCETIN IMPROVES DEVELOPMENTAL COMPETENCE OF MOUSE OOCYTES BY REDUCING OXIDATIVE STRESS DURING *IN VITRO* MATURATION

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**Abstract**

**Quercetin is a natural flavonoid with strong antioxidant activity. In the present study, we evaluate the influence of different concentrations of quercetin (QT) on intracytoplasmic oxidative stress and glutathione (GSH) concentration, during *in vitro* maturation (IVM) and fertilization in mouse oocytes. IVM was carried out in the presence of control (QT0), 5 (QT5), 10 (QT10), and 20 (QT20) µg/mL of QT. Nuclear maturation, intracellular GSH and ROS content were evaluated following the IVM. In these oocytes, we subsequently evaluated the effect of QT supplementation on embryo development, including 2-cell, 8-cell, and blastocyst rate. The results of the present study showed that the supplementation of 10 µg/mL QT in maturation medium increased the number of MII oocytes. In addition, fertilization and blastocyst rate in QT10 treatment group were significantly higher in comparison to the other groups, and elevated the amount of intracellular GSH content compared to other QT concentrations and control groups. The intracellular ROS level was the lowest among oocytes matured in Q5 and Q10 treatment groups. This result suggested that quercetin dose-dependently improves nuclear maturation and embryo development, via reducing intracytoplasmic oxidative stress in mature oocyte.**

**Key words: *in vitro* maturation, quercetin, oxidative stress, glutathione**

Modern assisted reproductive technologies (ART) use ovarian stimulation to expand the quantity of accessible oocytes and the result. However, the utilization of ovarian stimulation builds the patients' cost and suffering, and is related with side effects including ovarian hyperstimulation syndrome (OHSS) and potential cancer risk. The recovery of immature oocytes followed by *in vitro* maturation (IVM) and

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fertilization is an alternative to stimulated cycle *in vitro* fertilization (IVF). A few techniques are ordinarily used to survey the quality of IVM oocytes, for example, assessment of developmental competence of oocytes or parthenogenetic activation (PA), detection of intracellular glutathione (GSH) and reactive oxygen species (ROS) content (Abazari-Kia et al., 2014), and expression evaluation of genes related with oocyte maturation (Silva et al., 2014).

The intracellular glutathione (GSH) and reactive oxygen species (ROS) levels are perceived to assume vital part in oocyte IVM and embryo generation. Glutathione (GSH) is the major nonprotein sulfhydryl compound in mammalian cells and is known to play a critical role in protecting the cell from oxidative damage (de Matos et al., 2002). Increased oxidative stress is one of the reasons of impaired *in vitro* embryo development. Reduction of intracellular GSH content can lead to lower oocyte developmental competence (Kwak and Hyun, 2012). The production of ROS is a fundamental cellular process. However, inordinate quantity of ROS can change cellular molecules like proteins, lipids, and nucleic acids (Rajabi-Toustani et al., 2013). The production of excessive amount of ROS during *in vitro* cultures has an effect on fertilization rate, embryo development and the change of clinical pregnancy rates (Tavana et al., 2012).

In many reviews, lower intracellular ROS and greater GSH content suggests better developmental competence of IVM oocytes (Abazari-Kia et al., 2014; Mukherjee et al., 2014). Meanwhile, the change of GSH/ROS content and developmental potential may be related with differential expression of genes required in metabolism, proliferation, apoptosis, and oxidative stress (Silva et al., 2014).

Regular antioxidants are elements that assume an essential role in prevention of oxidative stress and pertinent diseases. Plants are regarded as main origin of natural antioxidants (Vijaya et al., 2010). Quercetin is a plant originated flavonoid from vegetables and fruits that has antioxidant activity as a free radical scavenger. Quercetin is one of the most representative elements in the flavonoid family and has been referred to a number of potential health benefits, including antioxidant, cancer prevention, DNA protection, anti-inflammatory action, and cardioprotective activity (Sestili et al., 1998; Rao et al., 1999; Yamamoto et al., 1999; Boots et al., 2008; Moskaug et al., 2004; Rogerio et al., 2010; Suematsu et al., 2011; Kleemann et al., 2011). The oral organization of quercetin expanded plasma antioxidant capacity and acted against hepatic ischemia–reperfusion injury significantly in rats (Su et al., 2002; 2003; Camargo et al., 2011). Also, quercetin has protective influences on cell function *in vitro* and *in vivo* (Cho et al., 2006; Zhu et al., 2007).

The use of quercetin to avoid oxidative stress *in vivo* and *in vitro* has been reported in several studies. Kang et al. (2016) investigated the effect of QT on nuclear maturation of porcine oocytes and demonstrated that a significantly higher proportion of QT-treated (1 μg/mL) oocytes developed into blastocysts compared to controls and that treatment groups had significantly lower levels of ROS than controls, and concluded that high concentration (50 μg/mL) of QT appeared to be toxic to oocytes. Sovernigo et al. (2017) reported ROS reduction and increased levels of GSH in groups with quercetin in comparison with control group and demonstrated that nuclear maturation cleavage and hatched blastocysts rates did not differ between

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groups, however, blastocyst rates after IVF in quercetin group were higher than in the control. Also, Wang et al. (2017) demonstrate that quercetin relieved deterioration in oocyte quality and improved subsequent embryo development and showed that quercetin prevented the decline in oocyte quality during postovulatory aging of oocytes and also reported that quercetin treatment reduced aging-induced morphological changes and reactive oxygen species accumulation. Moreover, Lee et al. (2016) investigated the effects of quercetin glycosides on the acetylcholine induced peak inward current ($I_{ACh}$) in *Xenopus* oocytes expressing the α7 nAChR and showed that in oocytes injected with α7 nAChR copy RNA, quercetin enhanced $I_{ACh}$, whereas quercetin glycosides inhibited $I_{ACh}$ and also demonstrated that quercetin glycosides mediated an inhibition of $I_{ACh}$, which increased when they were pre-applied and the inhibitory effects were concentration dependent.

Dimethyl sulfoxide ($(CH_3)_2SO$) is an amphipathic molecule with a highly polar domain and two apolar groups, making it soluble in both organic and aqueous media. In this manner, DMSO is commonly utilized as a solvent for chemicals that have little or almost no solubility in water (Santos et al., 1997). Past reviews have demonstrated that DMSO has multiple effects on the cell cycle, differentiation, aging, and apoptosis (Choi, 2011; Santos et al., 2003).

The objective of this study was to evaluate the influence of different concentrations of quercetin (QT) on intracytoplasmic oxidative stress and glutathione (GSH) concentration during *in vitro* maturation (IVM) and fertilization in mouse oocytes.

## Material and methods

### Experimental design

In order to identify the effective quercetin concentration for improving oocyte maturation (experiment 1), IVM medium was supplemented with four concentrations (0, 5, 10 and 20 µg/mL) of quercetin during the entire 24-h maturation period. For experiment 2, we evaluated the effects of the same four concentrations of quercetin in IVM medium on IVF rate. In experiment 3, we assessed the effects of these concentrations of quercetin in IVM medium on the ROS levels in oocytes to assess the effect of antioxidant activity of quercetin. The groups investigated in this study include: four concentrations (0, 5, 10 and 20 µg/mL) of quercetin, *in vivo* matured oocyte (IVO) and DMSO as the solvent quercetin.

### Animals

Sixty female NMARI mice, 21 days old, were used in this study. Animals were housed in a room with ambient temperature of 20–24°C, a 12 h light/dark cycle and free access to water and food. All experiments were performed in accordance with the recommendations and policies of the International Association for the Study of Pain (Zimmermann, 1983) and the Institutional Animal Welfare Law. All study protocols were approved by the internal deputy for animal research and the respective local government committee which is advised by an independent ethics committee

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in Shahid Beheshti University of Medical Sciences. The animals were selected randomly, and assigned into three equal groups as treatment, vehicle and control groups.

**Cumulus-oocyte complexes collection**

Cumulus-oocyte complexes were obtained from female mice at age of 7–8 weeks old in control groups that were injected with 10 IU PMSG (Pregnant Mare Serum Gonadotropin, Gestyl, Organon, Oss, The Netherlands) and killed 46–48 h later. Both ovaries were immediately excised, and antral follicles present on the ovary surface were punctured using 27 G needles in HTCM, HEPES-buffered TCM199 (Sigma Aldrich, St Louis, MO, USA), supplemented with 5% (v/v) heat-inactivated FBS (Fetal Bovine Serum, Gibco, Invitrogen, Barcelona, Spain). Germinal vesicle stage oocytes containing an intact vestment of cumulus cells (COCs) were collected and transferred into 20 µl drops of TCM199 culture medium and immediately were used for the study.

To obtain metaphase II (MII) stage oocytes, female mice were intraperitoneally injected with 5 IU HGC (Human Chorionic Gonadotrophin, Pregnyl, Organon, Oss, The Netherlands) 48 h after PMSG injection. After 14–16 h, mice were killed and the cumulus–oocyte complexes were isolated from the oviducts into HTCM medium, containing 100 IU/ml hyaluronidase, to separate cumulus cells from the oocytes for the study. Oocytes were immediately transferred into 20 µl drops of TCM199 culture medium. Denuded oocytes were observed under stereomicroscope and camera (Luxeo 6Z Stereozoom Microscope, Labo America Inc., Roseville, CA, USA). Oocytes with a round clear zona pellucida, a small perivitelline space and a pale, moderately granular cytoplasm that does not contain inclusions were considered to be "normal" (Ubaldi and Rienzi, 2008).

***In vitro* maturation**

For *in vitro* maturation (IVM) of oocytes, COCs were transferred into 20 µl drops of TCM199 culture medium with different concentrations of quercetin (Sigma Aldrich, St Louis, MO, USA) (0, 5, 10 and 20 µg/mL) and were placed in an incubator at 37°C, 5% $CO_2$ pressure. After 24 h of maturation, oocytes of each group were denuded of cumulus cells to determine maturation efficiency by pipetting using a narrow-bore pipette. The stage of meiotic maturation was determined by examination of the presence or absence of the first polar body (metaphase II). Also denuded oocytes were treated according to the experimental design.

***In vitro* fertilization**

For IVF, epididymal sperm suspensions were prepared from adult male NMRI mice and incubated for 1 hour in human tubal fluid (HTF) to ensure sperm capacitation. Then maturated oocytes were incubated for 6 h at 37°C with capacitated spermatozoa in IVF media. Fertilized oocytes were washed and cultured to the blastocyst stage in potassium simplex optimization medium (KSOM) containing BSA 5 mg/ml under mineral oil for 96 h, at 37°C and at 5% $CO_2$ and saturated humidity. The numbers of two-cell, eight-cell and blastocyst embryos were recorded with inverted microscope (Nikon, Tokyo, Japan) daily.

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To approve the anti-oxidative effect of quercetin isolating the functional interference of DMSO from Sigma-Aldrich (St. Louis, MO, USA) used to dissolve quercetin, the development of mouse oocytes exposed to DMSO was investigated.

**Measurement of intracellular ROS and GSH levels**

MII stage oocytes were sampled from control and treated groups for determination of their intracellular reactive oxygen species (ROS) and glutathione (GSH) levels by methods described previously (Wang et al., 2014). Briefly, 2-7-dichlorodihydrofluorescein diacetate (H2DCFDA; Invitrogen, Barcelona, Spain) and 4-chloromethyl-6, 8-difluoro-7-hydroxycoumarin (Cell Tracker Blue CMF2HC Molecular Probes; Invitrogen, Barcelona, Spain) were used to detect intracellular ROS as green fluorescence and the GSH level as blue fluorescence, respectively. For each group, a total of 35–40 oocytes was incubated for 30 minutes in dark and in PBS, which contained 1 mg/ml polyvinyl alcohol (PBS/PVA) containing 10 μM H2DCFDA and 10 μM Cell Tracker Blue. After incubation, the oocytes were washed with PBS/PVA, placed in 10 μL droplets, and then the fluorescence was observed using fluorescence microscope (Labomed Lx 400 fluorescence microscope; Labo America Inc., Roseville, CA, USA) with UV filters (460 nm for ROS and 370 nm for GSH) (Wang et al., 2014). The fluorescent images were saved as graphic files in TIFF format. The fluorescence intensities of the oocytes were analyzed using Image-J software (Version 1.40; National Institutes of Health, Bethesda, MD) and normalized to that of the control oocytes. The experiment was replicated three times.

**Statistical analysis**

The analyses were carried out using the statistical software GraphPad PRISM 6.0 (GraphPad Software, Inc., La Jolla, CA, USA) and the results are presented as the mean ± SEM. One-way analysis of variance was used to determine significant differences in the data followed by a Tukey test to determine statistical differences among groups. A probability of $P<0.05$ was considered statistically significant.

## Results

The results of quercetin supplementation to the different maturation media on oocyte nuclear maturation is shown in Table 1. The addition of 10 μg/ml quercetin to maturation media increased the number of oocytes at MII stage (93.21±1.21%), when compared to QT20, QT0, QT5 groups and DMSO-treated oocytes (85.08±2.32%, 86.37±1.51%, 86.55±1.41%, and 85.53±1.81, respectively). Highest (20 μg/ml) and lowest concentration (5 μg/ml) of quercetin significantly decreased the rate of fully expanded cumulus cells 24 h after *in vitro* maturation (IVM) compared with the *in vivo* matured IVO group ($P<0.05$). At the same time, the rate of cumulus cell expansion significantly increased (from 97.43± 0.6% to 87.87±2.1%) by supplementing 10 μg/ml of quercetin to control group.

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Table 1. Maturation of mouse oocytes cultured in IVM media supplemented with various concentrations of quercetin. Values of three replicates are shown as the mean ± SEM

| Groups | No. oocytes | Ab. (%) | GV (%) | MI (%) | MII (%) | Exp. (%) |
|---|---|---|---|---|---|---|
| Control (IVO) | 205 | 2.68 ±0.41 a | 1.3±0.12 a | 2.5±0.37 a | 93.62±0.9 a | 98.81±0.28 a |
| Control (IVM) | 215 | 4.18±0.42 b | 3.27±0.17 b | 6.75±0.53 b | 86.37±1.51 b | 87.87±2.1 b |
| QT 5 μg/ml | 201 | 4.01±0.26 b | 3.33±0.16 b | 6.7±0.73 b | 86.55±1.41 b | 92.8±1.44 b |
| QT 10 μg/ml | 210 | 2.56±0.15 a | 1.37±0.11 a | 2.6±0.43 a | 93.21±1.21 a | 97.43±0.6 a |
| QT 20 μg/ml | 206 | 4.8 ±0.24 b | 3.4±0.23 b | 7.1±0.43 b | 85.08±2.32 b | 89.77±1.55 b |
| DMSO | 205 | 4.53±0.37 b | 3.01±0.14 b | 6.88±0.69 b | 85.53±1.81 b | 88.96±1.47 b |

IVO: *in vivo* maturation; IVM: *in vitro* maturation; QT: quercetin; GV: germinal vesicle; MI: metaphase; MII: metaphase II; Exp: expansion, Ab: abnormal. Within columns, different superscripts denote significant difference (P<0.05).

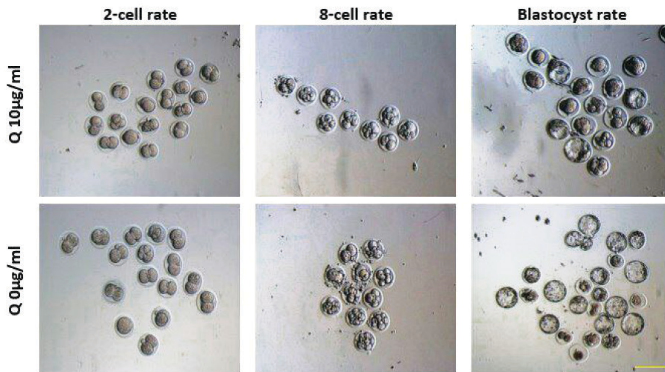


Figure 1. Oocytes Q10 treatment group showed the highest blastocyst rate (P<0.05) compared to control group

Table 2. Effect of different concentrations of quercetin in maturation media on mice oocyte fertilization rates in comparison to *in vivo* maturation and DMSO media

| Groups | 2-cell rate | | 8-cell rate | | Blastocyst rate | |
|---|---|---|---|---|---|---|
| | average | SEM | average | SEM | average | SEM |
| Control (IVO) | 95.33±3.44 a | 1.40 | 84.67±6.53 a | 2.66 | 63.67±7.87 a | 3.20 |
| Control (IVM) | 83.33±4.89 b | 1.99 | 69.17±6.62 b | 2.69 | 50.33±3.50 b | 1.42 |
| QT 5 | 88.42±3.20 ab | 1.30 | 81.50±2.59 ac | 1.05 | 57.00±7.27 b | 2.96 |
| QT 10 | 91.33±4.18 a | 1.70 | 82.83±2.40 a | 0.97 | 61.50±3.83 a | 1.56 |
| QT 20 | 82.17±3.92 b | 1.60 | 75.50±5.05 bc | 2.05 | 48.00±4.98 b | 2.02 |
| DMSO | 82.17±3.82 b | 1.55 | 68.83±3.25 b | 1.32 | 48.33±2.58 b | 1.05 |

Values with different superscripts differed significantly (P<0.05).

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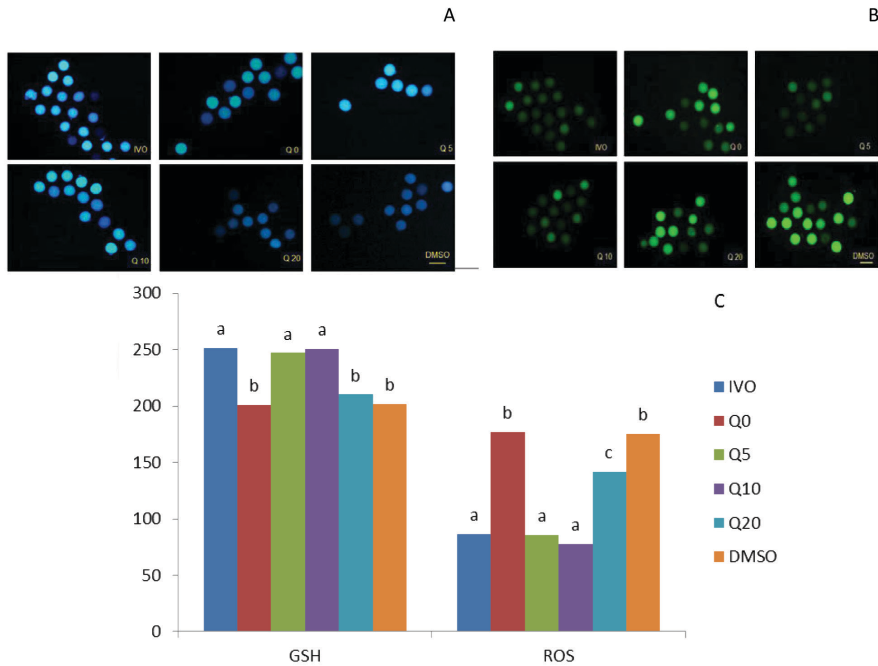
The results of quercetin supplementation to the different maturation media on IVF are shown in Figure 1 and Table 2. As shown, the blastocyst rate was higher (P<0.05) for Q10 (61.5±3.83%) than for Q0 (50.33±3.50%), Q20 (48±4.98%), DMSO (48±2.58%) and Q5 (57±7.27%) treatment groups. The blastocyst rate of oocytes in Q10 group was statistically similar to those of *in vivo* groups.




Values with different superscripts differed significantly (P<0.05). GSH: glutathione; ROS: reactive oxygen species.

Figure 2. The intracellular GSH (A) and ROS (B) evaluation in different groups. C: The intracellular GSH and ROS levels in different concentrations of quercetin in maturation media compared to *in vivo* maturation and DMSO media

The presence of 10 μg/ml quercetin in the maturation medium increased the 2-cell rate fertilized oocytes compared with the control group (91.33% vs 83.33±4.89).

Supplementation of media with 5 μg/ml – 10 μg/ml quercetin increased the 8-cell rate fertilized oocytes compared with the control group (81.5±2.58% and 82.83±2.40% vs. 6917±6.16, respectively).

The results for the intracellular GSH and ROS levels were shown in Figure 2. After IVM, significantly higher (P<0.05) intracellular GSH level was detected for oocytes matured *in vivo* (251.55±21.93) and oocytes matured in Q5 (247.09±17.38) and Q10 (250.64±7.15) treatment groups than Q0, Q20 and DMSO medium. The lowest intracellular ROS level was observed in oocytes matured *in vivo* (86.82±5.06) and

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those oocytes matured in Q5 (85.64±11.47) and Q10 (77.91±17.18) treatment groups compared to Q0 (177.19±13.14), Q20 (141.82±28.02) and DMSO (175.55±20.03) medium (P<0.05).

## Discussion

The accessibility of suitable metabolic substrates such as glucose, proteins, oxygen, pyruvate, amino acids, vitamins and hormones in the culture media influences the *in vitro* oocyte maturation, fertilization, and embryo development and plays an important role in the continuation of meiosis in oocyte during IVM and entire embryonic development. To meet this goal, the COC should get the nutrients from its environment at the appropriate levels.

In the present study, we examined the effect of quercetin as an antioxidant on *in vitro* maturation of oocytes. The present study showed that quercetin supplementation of the maturation medium *in vitro* enriched the maturation rates of the oocytes.

To investigate the effect of quercetin on oocyte nuclear maturation, supplementary quercetin was included in the maturation medium in different doses. In experiment 1, the treatment of mouse oocytes with quercetin during IVM linearly increased the nuclear maturation rate from control to 10 μg/mL of quercetin. Indeed, the addition of 10 μg/mL quercetin to maturation media significantly increased the number of oocytes at MII stage, as compared with other groups. The result of our study showed that fertilization rate was higher for Q10-treated oocytes in comparison to Q0, Q20, and Q5 groups. Percentage of 8-cell formation was higher in Q5 and Q10 as compared with other groups. Also, the percentage of blastocyst formation was higher in Q10 as compared with other groups. Kang et al. (2013) reported that quercetin treatment did not improve oocyte nuclear maturation in the porcine oocytes, but significantly higher blastocyst rates of parthenogenetically activated oocytes were achieved when the IVM medium was supplemented with an adequate concentration of quercetin (1 μg/mL). However, cleavage rates and blastocyst cell numbers were not affected. Also, Kim et al. (2014) reported that the developmental rates to morula and blastocyst increased when porcine IVF embryos were cultured in medium supplemented with low concentrations of quercetin (1–10 μM), but significantly decreased at high concentrations of quercetin (25–50 μM). In the case of our experiment, the maximum developmental rate to blastocysts among all concentrations of quercetin was reported at quercetin 10 μM (P<0.05).

As shown in the study, 10 μM DMSO treatment, the concentration used to dissolve quercetin in our study, had no significant effect on the number of oocytes at MII stage and the rates of blastocyst formation, as compared with IVM control group.

In agreement to our results, Yu et al. (2014) reported that the low-dose quercetin exposure (1 mM) provided mild protection by an increase in the percentage of embryos that reached the morula stage. With a concentration of quercetin up to 5 mM, blastocyst formation was recovered to levels of the control group. When zygotes were exposed to quercetin of 10 mM, the rate of embryos reaching the blastocyst

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stage were extensively recovered as well. Our results are also in agreement with those reported by Kang et al. (2016) who investigated the effect of QT on nuclear maturation of porcine oocytes and reported that a significantly higher proportion of QT-treated oocytes developed into blastocysts compared to controls. Also, Wang et al. (2017) reported that quercetin prevented the decline in oocyte quality during postovulatory aging in mouse oocytes.

Interestingly, quercetin has contradictory roles – prooxidant or antioxidant depending on the experimental situation and dose (Yu et al., 2014). It is documented that high concentration of ROS may result in oxidative damage which subsequently causes apoptotic cell death in various cell types (Bayir et al., 2008). Consistently, our results showed that in quercetin-treated oocytes, ROS levels were significantly lower after maturation. Additionally, high doses of quercetin (20 μg/ml) did a poor job of reducing the levels of ROS. In Jurkat T-lymphocytes, adequate concentrations of quercetin kept cellular DNA from oxidant activity, while high concentrations of quercetin had the unfavorable effect (Johnson and Loo, 2000). In agreement with our finding, Kang et al. (2016) reported that quercetin treatment groups of porcine oocytes had significantly lower levels of ROS than controls. Also, Wang et al. (2017) showed that following quercetin treatment, oocytes showed a significant dose-dependent decrease in ROS accumulation in a way that 10 μM quercetin had the best preventive effects on aging induced morphological changes and ROS accumulation.

The antioxidative performance of quercetin has been reported in other cell types, including spermatogonial cells (Mi et al., 2007) and neuronal cells (Suematsu et al., 2011). Yu et al. (2014) investigated the effects of quercetin on the development of preimplantation embryos under oxidative stress and found that quercetin exposure highly intensifies the quality of $H_2O_2$-treated embryos, depicted by reduced apoptosis, increased blastocyst formation, and fewer incidences of fragmentation and developmental retardation. They concluded that this effect of quercetin on the development of preimplantation embryos may be connected with the antioxidant activity of quercetin in reduction of ROS levels in zygotes. Kang et al. (2013) reported that the relative ROS contents of oocytes matured in medium supplemented with 1 or 10 μg/mL quercetin were significantly lower than those in oocytes matured in the control medium. In agreement to our results, they found that the relative ROS levels were not significantly different between the control and oocytes treated with high concentration of quercetin.

In the present study, we found significant difference in GSH levels between the groups in the presence or absence of quercetin. The intracellular content of GSH in the QT5 and QT10 groups was higher as compared with those of QT20, DMSO and control groups. Previous studies have shown that quercetin has the ability to keep intracellular GSH at high levels (Gitika et al., 2006; Ishige et al., 2001). Maedomari et al. (2007) indicated that GSH synthesized by intact porcine cumulus cells during maturation culture, enhanced oocyte maturation, and played a key role in fertilization and embryonic development. Also, de Matos et al. (2002) reported that an increase in intracellular GSH is connected with a decrease in peroxide levels within oocytes and stimulates sheep embryo development.

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In conclusion the results of the present study indicated that higher levels of oocyte maturation rate, fertilization rate, and blastocyst formation registered at adequate concentration (10 µg/mL) of quercetin may be attributable to antioxidant enzymes activity in both oocyte and cumulus cells. Oocytes at the presence of 10 µg/mL concentration of quercetin represented a higher level of intracellular GSH and capability to produce the blastocyst stage.

**Conflicts of interest**

The authors declare no conflict of interests.

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