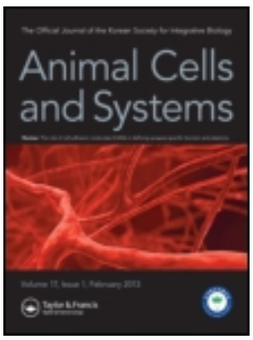

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Effects of intraperitoneal administration of Papaver rhoeas L. extract on mouse ovaries

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This research studies the effect of water-alcohol Papaver rhoeas L. (P. rhoeas) extract on mouse ovaries and in vitro development (IVD) of oocytes. Different dosages of P. rhoeas extract (25, 50, 100, 200 mg/kg body weight) were injected intraperitoneally (i.p.) during a period of 12 days. Following superovulation, the numbers of ovulated oocytes, the rates of in vitro fertilization, IVD and the cellularity of blastocysts were recorded. Additionally, effect of the best dosage on ovarian follicle population and the ability of immature oocytes to mature in vitro were evaluated. Administration of 200 mg/kg significantly increased the percentage of 4–8 cells, morula and blastocyst embryos compared to the control group ($p < 0.05$). Furthermore, total cellularity of blastocysts was significantly higher with the administration of 200 mg/kg of extract in comparison to control group ($p < 0.05$). Therefore, the most effective dosage was considered to be 200 mg/kg. With the administration of 200 mg/kg no marked changes were observed in the IVM rate of retrieved oocytes from treated group in comparison to control group. Furthermore, the percentage of ovarian follicles was not significantly different when compared to control group. Also, during visual evaluations no abnormal apoptosis was detected in follicles of ovaries treated with 200 mg/kg when compared to control group. Higher IVD and blastocyst cellularity in the group treated with defined dosage of P. rhoeas indicates that the extract affects ovaries in a dose dependent manner. The extract possibly increases the quality of ovulated oocytes and IVD competence of oocytes.

Keywords: embryo; oocyte; maturation; Papaver rhoeas L; ovary

Introduction

The consumption of fruits and vegetables have been shown to have protective roles on health (Millner 1999), which include reductions in cancer, heart disease, hypertension and stroke (Wolfe et al. 2003). During the past few decades, the medical value of plants has been established based on data indicating that plant extracts not only contain minerals and primary metabolites, but also have secondary metabolites with antioxidative potentials (Akinmoladun et al. 2007). Natural antioxidants are plants such as herbs (Xuejiang et al. 2001), legumes (Lin et al. 2001) and teas (Roedig-Penman & Gordon 1997). Numerous reports indicate that the antioxidative ability of medical plants are related to their phenolic compounds, which include phenolic acids, flavonoids, anthocyanins and tannins (Djeridane et al. 2006; Wong et al. 2006). Anthocyanins are secondary components of Papaver rhoeas (P. rhoeas L.) extract (Matysik & Benesz 1991) with the potential to scavenge free radicals (Philpott et al. 2006). There are numerous reports about the beneficial effects of anthocyanins on health and prevention of diseases such as heart disorders by the consumption of fruits rich in anthocyanins (Tsuda et al. 1996; Wang & Jiao 2000; Tsuda et al. 2002, 2003). There are investigations about the effects of plant extracts and their compounds on the female reproductive system (Celik et al. 2004; Gwehenberger et al. 2004; Owolabi et al. 2008; Mokhtari et al. 2009; Chen et al. 2010; Zafari Zangeneh et al. 2010; Nogueira et al. 2010). The effect of some medicinal plants on mice (Gupta et al. 2004; Gan et al. 2006; Mokhtari et al. 2009; Khazaei et al. 2011) and rat (Manneras et al. 2010) ovaries have been previously evaluated. It has been shown that defined dosages of plant extracts with antioxidative properties such as saffron (Mokhtari et al. 2010), Foeniculum vulgare (Khazaei et al. 2011) and Tribulus terrestris (Addy & Mosa 2012) improves folliculogenesis in mouse. Studies on the effects of plant extracts indicate that the positive effects of plants on the reproductive system are due to their antioxidative components (Mokhtari et al. 2009; Nogueira et al. 2010). P. roheas has been known as 'corn poppy,' and it belongs to the family of Papaveraceae. It is a wild plant found in different parts of the world, such as Asia (Gubruz et al. 2003). This plant has been used as a remedy for diseases such as sleep disorder and cough (Zargari 1994). Aside from antibacterial and antiseptic properties, it is effective against

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various inflammations. The components of the extract have been detected to contain alkaloids (Kalva & Sariyar 1989) and anthocyanins (Matysik & Benesz 1991). The antioxidative effects of P. rhoeas L. extract, which is mainly due to its anthocyanins, have been demonstrated in previous research (Kostic et al. 2010). There is evidence that whole plant extracts often have greater in vitro or in vivo. In this research, we intended to evaluate the effect of P. rhoeas extract on mouse ovaries using assisted reproductive technology and histological assay.

Materials and methods

Unless indicated, all materials and chemicals were purchased from sigma.

2.1. Animals

Animal experiments were carried out according to the Declaration of Helsinki and the Guiding Principles in the Care and Use of Animals (DHEW publication, NIH, 80–23).

Adult male and female N-MRI mice (purchased from Pasture Institute) were maintained in controlled conditions including controlled light:dark (12:12 hours) schedule, temperature and humidity and free access to enough water and food.

2.2. Plant material, the extract preparation and dosage injection

P. rhoeas extract collected from the Kermanshah region (Iran) was prepared in Shahid Beheshti University of Medical Sciences, (Tehran, Iran), Department of Pharmacognosy, Faculty of Pharmacy. Total dried plant powder (100 g) was soaked in 500 ml of 50% ethanol for three days at room temperature. The extract was filtered and ethanol evaporated at low pressure and temperature of 33°C (Sahraei et al. 2006). The soft extract was dissolved in normal saline and i.p. injected into female mice. The extract was evaluated for the presence of anthocyanins and flavonoids using spectrophotometer. About 10 mg P. rhoeas L. extract was dissolved in 1 ml of 50% ethanol. The light absorbents were detected in 530 nm and 300 nm for anthocyanins and flavonoids, respectively (Tonon et al. 2008). The extract was injected i.p. as milligram per kilogram of body weight. Experimental dosages were selected according to previous researches on the dosage determination (Sahraei et al. 2006) and toxicity (Soulimani et al. 2001). Dosages including 25, 50, 100 and 200 mg/kg of the P. rhoeas extract were injected daily during 12 days folliculogenesis of mouse (Nagy et al. 2003).

2.3. Oocyte collection, in vitro fertilization and in vitro development

All media were prepared using deionised water filtered through 0.22 μm sterile filters and stored albumin free. Each medium was incubated overnight in 5% CO_2, 37°C temperature and saturated humidity. After 12 days of injection, animals were superovulated by the i.p. injection of 7.5 IU pregnant mare serum gonadotropin (Intervet Inc, Netherland) followed by 7.5 IU human chorionic gonadotropin (HCG) 48 hours later. About 14–16 hours after HCG injection, female mice were sacrificed by cervical dislocation. The T_6 medium supplemented with 15 mg/ml bovine serum albumin (BSA) was used during cumulus oocyte complex (COC) collection, sperm capacitation and IVF. For COC collection, oviducts were removed and transferred to T_6 medium supplemented with 15 mg/ml BSA, disrupted by 26 gauge needles. The number of ovulated COCs was recorded.

COCs were transferred to 100 μl IVF droplets (T_6 medium supplemented with 15 mg/ml BSA) covered with mineral oil and incubated overnight in 5% CO_2, 37°C temperature and saturated humidity. Adult male mice (6–8 weeks old) were sacrificed by cervical dislocation and epididymides were removed, disrupted and transferred to 2 ml sperm capacitation medium (T_6 medium and 15 mg/ml BSA; Golkar-Narenji et al. 2010). Sperms were capacitated for two hours in CO_2 incubator. Sperms with the concentration of 2×10^6 cells/ml were added to IVF droplets in which COCs where placed before. About 7 hours after IVF, eggs were monitored by invert microscope (Nikon) and the percentage of 2PNs (two pronuclear stage) was recorded for evaluation of fertilization rate. IVF rates in all groups were compared to each other.

2.4. Evaluation of embryo development and blastocyst quality

For in vitro development, fertilized eggs generated with the effect of each dosage were transferred to IVD medium containing T_6 medium supplemented with 4 mg/ml BSA. Embryo development was monitored 24, 48, 72 and 96 hours after IVF and the number of 2 cells, 4–8 cells, morula and blastocyst embryos were recorded using an inverted microscope (Nikon). In order to evaluate cellularity of blastocysts, all blastocysts were exposed to solution containing 1% Triton X-100 and 100 μg/ml propidium iodide for 15 seconds and finally embryos were fixed overnight in 100% ethanol containing 25 μg/ml bisbenzimide (Calbiochem, Germany). The embryos were mounted on cleaned glass slides using glycerol and kept in a dark chamber until being observed by fluorescent light using a Leica

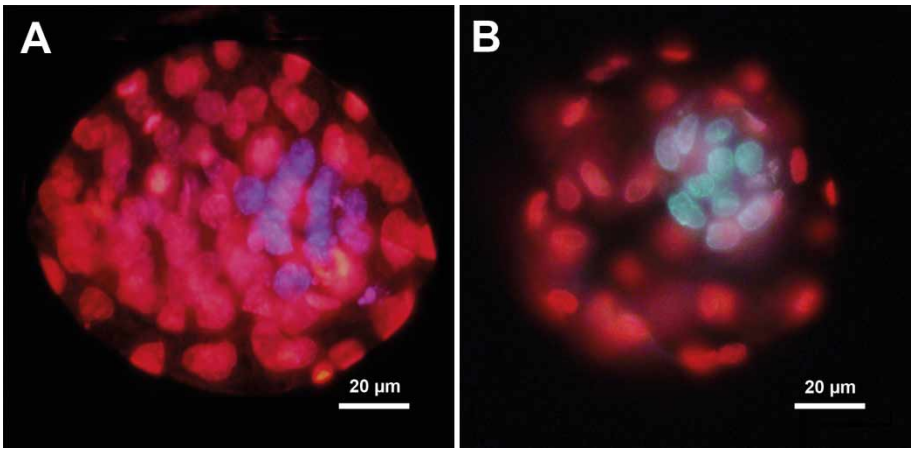

Figure 1. Differential staining showing countable inner cell mass and trophectoderm around inner cell mass of a blastocyst embryo. (A) A blastocyst generated from oocytes with the effect of 200 mg/kg P. rhoeas L. extract. (B) A blastocyst generated from control group.

microscope (Olympus). Inner cell mass (ICM) was blue and trophectoderm (TE) was red around ICM (Figure 1). Cells of ICM and TE were counted at $\times 100$ magnification.

2.5. Selection of the most effective dosage

IVF, IVD and blastocyst cellularity were considered for selection of the most effective dosage of the extract. The dosage with the highest rate of IVD and cellularity was selected as the most effective one. This dosage was considered for more evaluations by in vitro maturation and histological assays.

2.6. In vitro maturation and subsequent embryo development

Results indicated that 200 mg/kg was the most effective dosage. Therefore, 200 mg/kg P. rhoeas extract was injected (i.p.) into 10 female mice daily for 12 days. After 12 days of treatment, ovaries were immediately transferred to MEMα medium supplemented with 5% fetal bovine serum (Golkar-Narenji et al. 2010), and oocytes were released using 26 gauge needles. Immature oocytes were transferred to maturation medium and about 16 hours later the maturation rate was recorded using an inverted microscope (Nikon). COCs were denuded by gentle pipetting to remove granulosa cells to see the first polar body. In vitro matured oocytes were exposed to IVF and IVD procedures according to the above mentioned protocol for IVF.

2.7. Histological assay

Ovaries were fixed in 10% buffered formalin, embedded in paraffin wax, serially sectioned at 6 µm, stained with haematoxylin and eosin and analysed under a light microscope. Follicles were counted and classified into four groups: (1) primordial: follicles with one oocyte surrounded by one layer of flattened cells; (2) primary: follicles with one oocyte surrounded by one layer of cuboidal cells; (3) preantral: follicles with more than one layer of cuboidal cells without an antrum and (4) antral: follicles with more than one layer of cuboidal cells with an antral cavity in granulose cells. Sections were selected from different parts of ovaries randomly for TUNEL test, which was applied according to our previous published research (Eimani et al. 2009). An in situ cell death detection kit [horseradish peroxidase (POD); Roche, Mannheim, Germany] was used according to the manufacturer's instructions. Briefly, after deparaffinisation, hydrated sections were incubated in a humidified chamber with 0.15% Triton X-100 (Sigma-Aldrich, St Louis, MO, USA) at room temperature for 15–30 minutes, and the slides were then rinsed twice with PBS. The TUNEL reaction mixture was added, and the slides were incubated in a humidified atmosphere in the dark; slides were again rinsed three times with PBS, then converter–POD was added and the slides were incubated in a humidified chamber for 30 minutes at 37°C. The slides were again rinsed three times in PBS. Diaminobenzidine substrate or alternative POD substrates were added. Slides were incubated three times with PBS and were then counterstained with haematoxylin.

2.8. Statistical analysis

ANOVA and Duncan's multiple range test were applied for all statistical analysis using SAS program (SAS v9.1 2002). All percentage values were subjected to arc sine transformation before the analysis. All data were

expressed as mean ± SEM. A probability of $p < 0.05$ was considered statistically significant.

3. Results

3.1. Superovulation, IVF, IVD and determination of the best dosage

There were no significant differences in the number of oocytes obtained from treated mice after superovulation compared to the control group. Furthermore, no significant differences were observed in fertilization rate of oocytes obtained from each treated groups compared to control group (Table 1). However, between experimental doses there was a significant increase ($p > 0.05$) in the percentage of 4 cells, morula and blastocysts in the treated group with 200 mg/kg extract compared to control group (Table 2). The highest ICM cell number and TE cell number were observed in blastocysts with 200 mg/kg extract treatment. Furthermore, total cell number of blastocysts was the highest in the group treated with 200 mg/kg and there were significant difference ($p > 0.05$) when compared to the control group (Table 3).

Table 1. Ovulation and fertilization rates per female.

Extract dose (mg/kg of body weight)	Replication (Number)	Ovulated oocytes (Number)	Fertilized oocytes (%)
0	6	17.3 ± 4.7	77.5 ± 7.3
25	6	15.2 ± 1.9	81.0 ± 4.5
50	7	22.5 ± 5.7	79.4 ± 2.4
100	6	16.4 ± 2.8	83.2 ± 3.2
200	8	17.3 ± 3.3	86.0 ± 4.1

Note: Numbers and percentages are expressed as mean ± SEM.

3.2. Oocyte maturation and subsequent developmental competence

As shown in Table 4, maturation rates of oocytes recovered from ovaries treated with 200 mg/kg extract was not significantly different compared to the control group. Also, the percentages of arrested oocytes at vesicle germinal and vesicle germinal break down stages were not significantly different compared to the control group. Table 5 shows that IVF and IVD rates including 2 cell, 4–8 cell, morula and blastocyst formation in oocytes recovered from mouse ovaries treated with 200 mg/kg extract were not significantly different compared to the control group.

Table 2. In vitro development of embryos 24, 48, 72 and 96 hours after IVF.

		In vitro development			
Extract dose (mg/kg)	Replication (No.)	2 cell (%) 24 h	4–8 cell (%) 48 h	Morula (%) 72 h	Blastocyst (%) 96 h
0	6	60.2 ± 7.7	36.1 ± 5.2^{b}	25.1 ± 5.0^{b}	10.3 ± 3.8^{b}
25	6	69.9 ± 7.2	39.1 ± 7.3^{b}	25.2 ± 9.0^{b}	12.2 ± 8.0^{b}
50	7	71.4 ± 5.1	41.0 ± 5.3^{b}	25.2 ± 6.1^{b}	9.0 ± 3.1^{b}
100	6	62.6 ± 3.4	45 ± 8.0^{ab}	34.6 ± 7.0^{b}	14.2 ± 5.0^{b}
200	7	69.2 ± 6.4	63.6 ± 8.0^{a}	58.1 ± 5.8^{a}	33.1 ± 3.2^{a}

Note: Data are expressed as mean ± SEM. Percentages with the same superscripts (a or b) in each column are not significantly different. Different superscripts (a versus b) in a column shows significant difference (a versus b; $p < 0.05$).

Table 3. Cellularity of blastocysts.

				Number of cells		
The extract dosage (mg/kg)	Replication (Number)	Blastocysts (Number)	ICM index*	TE	ICM	Total
0	7	12	29.6 ± 1.6	52.7 ± 4.9^{b}	16.3 ± 2.1	65.9 ± 6.9^{b}
25	7	11	29.8 ± 0.9	52.0 ± 5.4^{b}	16.7 ± 1.4	68.7 ± 6.5^{b}
50	7	13	28.5 ± 0.6	58.7 ± 6.9^{ab}	19.7 ± 2.3	89.4 ± 8.8^{ab}
100	8	10	29.0 ± 1.3	69.6 ± 7.6^{ab}	16.7 ± 1.9	75.4 ± 8.8^{ab}
200	9	10	28.0 ± 1.3	75.4 ± 8.2^{a}	20.0 ± 2.4	95.4 ± 9.7^{a}

Note: Data are expressed as mean ± SEM. Percentages with the same superscripts (a or b) in each column are not significantly different. Different superscripts in a column shows significant difference (a versus b; $p < 0.05$).
TE, trophectoderm; ICM, inner cell mass.
*ICM index: ICM/total.

Table 4. In vitro oocyte maturation.

Extract dose (mg/kg body weight)	Replication (Number)	Total oocytes (Number)	GV (%)	GVBD (%)	MII (%)
0	9	111	22.0±3.0	16.3±4.0	57.6±4.2
200	9	115	18±5.0	22.0±3.0	67.2±4.0

GV, Germinal vesicle; GVBD, Germinal vesicle break down; MII, metaphase.

Table 5. IVF and IVD rates of in vitro matured oocytes.

			In vitro development			
The extract dosage (mg/kg)	Replication (Number)	IVF (%)	2 cell (%) 24 h	4–8 cell (%) 48 h	Morula (%) 72 h	Blastocyst (%) 96 h
0	9	60.0±4.1	48.2±6.4	28.0±6.0	17.1±4.0	14.0±2.0
200	9	70.0±4.6	53.1±6.2	34.0±5.0	17.3±4.0	13.0±3.0

3.3. Histological assay

The percentages of primordial, primary, preantral and antral follicles are shown in Figure 2. No significant difference was observed in the percentage of all types of follicles between ovaries treated with 200 mg/kg extract and control ovaries. Visual evaluation of sections by TUNEL staining showed that, there were no apoptotic follicles in selected sections of ovaries treated with 200 mg/kg and control ovaries. Approximately all antral follicles showed apoptosis in granulosa cells. Figure 3 shows a natural apoptosis in granulose cells during development to the late antral stage.

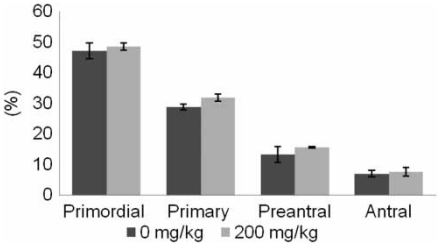

Figure 2. The percentage of follicles in ovaries treated with (200 mg/kg) or without (0 mg/kg) P. rhoeas L. extract.

4. Discussion

This study was undertaken to evaluate effect of peritoneal administration of P. rhoeas extract on mouse ovaries. IVF and subsequent IVD to blastocyst stage make it possible to appraise oocyte quality (Robker 2008). In this research, spectrophotometer showed ability of this extract to absorb 530 nm and 300 nm wavelengths, which are related to anthocyanins and flavonoids, respectively. It has been shown that anti-oxidants and reactive oxygen species (ROS) have physiological roles in reproductive process such as oocyte maturation, fertilization, luteal regression and endometrial shedding (Gupta et al. 2009). In this research, ovaries treated with 200 mg/kg P rhoeas L. extract ovulated oocytes with higher IVD rates. ROS are produced through normal chemical pathways in cells. It was found that, steroidogenic cells (Freeman & Crapo 1982), macrophages and neutrophils (Nakamura et al. 1999) are the main sources of ROS production in

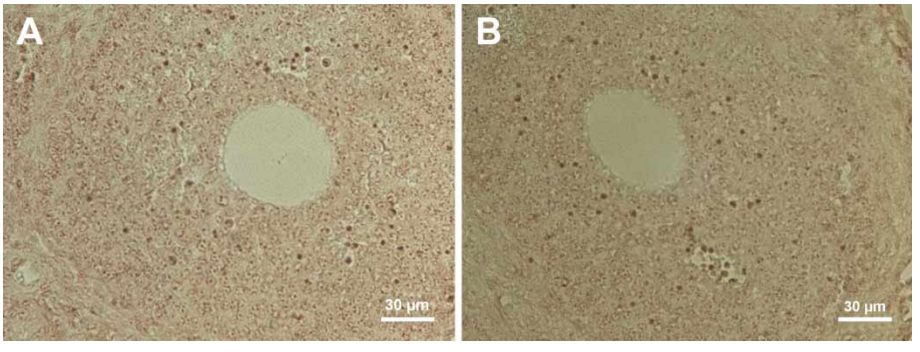

Figure 3. Ovarian follicles stained using TUNEL Kit. Dark spots are apoptotic granulose cells. (A) A follicle of mouse ovary treated with 200 mg/kg P. rhoeas L. extract. (B) A follicle of untreated mouse ovary (control group).

ovaries. Previous studies demonstrated that intake of antioxidants in animal models prevents ovarian senescence and reduction of oocyte quality. The microenvironment of oocytes plays a critical role in fertilization and embryo development (Tarin et al. 1998). Reduction of antioxidants in follicular and tubular fluids with presence of ROS inhibits oocyte development. Abundance of iron in biological systems makes it a major source of hydroxyl radicals. Iron produces free radicals through fenton reaction (Halliwell & Gutteridge 1990). It has been shown that the antioxidative effect of P. rhoeas extract is due to its high Fe^{+}-chelating activity (Schaffer et al. 2004). Observation of blastocysts with higher cellularity with administration of P. rhoeas extract indicated that oocyte released from treated ovaries have higher potential to develop in vitro to produce good-quality blastocysts. That was maybe due to antioxidative effects or effects of other unknown factors in this extract on ovaries. It has been mentioned that whole plant extracts may exhibit pharmaceutical properties which are due to interactions between their components (Wagner & Ulrich-Merzenich 2009). Effects of P. rhoeas L. extract are possibly related to positive interaction and synergies between its components. It has been reported that low levels of ROS in follicular fluid are needed for successful IVF (Attaran et al. 2000). Sato et al. reported that administration of antioxidant such as superoxide dismutase by intravenous injection inhibited ovulation of rabbits that were stimulated by gonadotropin (Sato et al. 1992). It was concluded that the presence of some superoxide radicals are needed for superovulation (Norihiro 2005). With peritoneal administration of P. rhoeas extract, superovulation was not suppressed and fertilization rate was with the same rate as control group. The highest IVD rates and blastocyst cellularity were observed when 200 mg/kg extract was administered. Therefore, effect of the extract is dose dependent. Our previous study showed that the administration of defined concentration of P. rhoeas extract to maturation medium increased IVM rates and subsequent developmental competence (Golkar-Narenji et al. 2010). In this research, P. rhoeas extract administered in vivo and immature oocytes were retrieved from treated ovaries and matured in vitro. In vivo administration of extract did not increase the IVM rate and subsequent IVD. Therefore, it seems that increasing effects of P. rhoeas extract on oocyte maturation is more effective when added to maturation medium in comparison with in vivo administration. Through evaluation of follicular population, the ratio of follicular types in the treated group was not different compared to the control group. This indicates that the extract did not affect the follicular population. Programmed cell death is a continuing process in ovaries through follicular development (Tilly 1996) Granulosa cells are essential for normal folliculogenesis (Tilly 1996), and their apoptosis seemed to have negative effects on the IVF rate (Oosterhuis et al. 1998). Evaluation of selected ovarian sections for apoptosis assessment by TUNEL test showed that there was no abnormal apoptosis in granolusa cells in the group treated with 200 mg/kg extract. Therefore, treatment with 200 mg/kg extract did not induce abnormal apoptosis in granulose cells. Lauren et al have shown that the iron chelators such as DTPA suppress ovarian cancer growth (Brard et al. 2006). Regarding the high chelating activity of P. rhoeas extract and effects of chelators on the treatment of ovarian cancer, it seems that future research on the effects of P. rhoeas on ovarian cancer could be worthwhile. This research indicates that a plant extract with proven antioxidative properties may positively affect oocyte quality and increase its ability for in vitro development.

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