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RESEARCH NOTE

Effect of *Papaver rhoeas* extract on in vitro maturation and developmental competence of immature mouse oocytes

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Abstract
Purpose This experiment examined the effect of *Papaver rhoeas* L. extract on in vitro maturation, in vitro fertilization (IVF) and subsequent developmental competence of mouse oocytes.
Materials and methods Cumulus oocyte complexes (COCs) at germinal vesicle stage were collected from female Naval Medical Research Institute (NMRI) mouse ovaries. The COCs were transferred to maturation medium supplemented with different concentrations of *P. rhoeas* extract. Two trials were carried out to examine the effect of low concentrations (0, 10, 15, 20, 25 μg/ml) and high concentrations (0, 50, 100, 200 μg/ml) of the extract. The maturation rate was recorded. After IVF, embryos were cultured and their developmental process was monitored for 96 h.
Results Maturation rate and blastocyst formation improved by using low concentrations of the extract; however, no significant increase was observed when compared to the control group. In addition no significant differences were observed in the fertilization rates of oocytes treated with both low and high concentrations compared to the control group. However, among higher concentrations, 100 μg/ml, *P. rhoeas* extract significantly increased both the in vitro maturation rate and in vitro developmental (IVD) competence when compared to the control group ($P < 0.05$).
Conclusions It was concluded that natural extracts increase the IVD competence of oocytes. The improved effect on oocyte maturation was dependent on the addition of optimum concentrations of *P. rhoeas* extract to the maturation medium.

Keywords Antioxidant · Embryo · In vitro · Oocyte · *Papaver rhoeas* L.

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Introduction

Fully-grown oocytes free from the inhibitory effects of follicles resume meiosis spontaneously [1]. The culture conditions of oocytes during in vitro maturation play a critical role in the rate of embryo production and quality [2]. Reactive oxygen species (ROS) such as superoxide anions ($O_2^{\cdot -}$), hydroxyl radicals (OH·) and hydrogen peroxide (H_2O_2) are produced through normal chemical pathways in cells. Furthermore production of endogenous ROS through different enzymatic reactions in oocytes and embryos is inevitable [3]. ROS change cellular molecules such as lipids, proteins and nucleic acids [4]; they are also agents of oxidative stress that has a detrimental effect on

early in vitro embryo development [5, 6]. In vitro culture conditions for oocytes and embryos contain higher concentrations of oxygen than in vivo conditions, leading to an increased level of ROS [7]. Anti-oxidative substances prevent detrimental actions of free radicals [8]. Previous investigations have indicated that the addition of anti-oxidants such as ethylene diamine tetra-acetic acid (EDTA) [9], diethylene triamine penta-acetic acid (DTPA) [10], vitamin C and vitamin E [11] during in vitro culture increased embryonic developmental competency. Numerous studies have shown that plant extracts have several in vitro anti-oxidative properties [12–15]. The anti-oxidative properties of many plant species such as *Papaver rhoeas* L. (*papaveraceae*), an annual plant found in limestone soil [13] have been demonstrated in previous studies [14, 15]. In this survey the effect of *P. rhoeas* extract on in vitro maturation, in vitro fertilization (IVF) and subsequent embryo development was evaluated.

Materials and methods

Unless indicated, all chemicals were purchased from Sigma.

Animals

Male and female Naval Medical Research Institute (NMRI) mice (6–8 weeks old) were used (purchased from Pasteur Institute, Tehran, Iran) for the experiment. Animals were kept on 12 h light: 12 h dark photoperiod and controlled temperature with ad libitum access to water and food.

Plant material and extract preparation method

P. rhoeas was obtained from the Kermanshah region (western Iran). Plant materials were transferred to Shahid Beheshti University, characterized by M. Kamalinejad and a voucher number p-147 was deposited at the Herbarium Department [16]. Dried petals (100 g) were added to 500 ml ethanol (50%) and macerated at room temperature for 5 days. After filtration, ethanol was evaporated at low pressure at 33°C. About 15 g extract was obtained from 100 g of dried petals of *P. rhoeas* [16]. The soft extract was dissolved in maturation medium.

In vitro maturation

Female mice were sacrificed by cervical dislocation. The ovaries were excised and placed in a medium that contained minimum essential medium alpha (MEMα) [17] supplemented with 5% fetal bovine serum (FBS), 100 IU penicillin and 100 IU streptomycin. Antral ovarian follicles were punctured using 26-gauge needles. Cumulus oocyte complexes (COCs) at the germinal vesicle (GV) stage were collected and washed three times in maturation medium droplets including MEMα supplemented with 100 IU penicillin, 100 IU streptomycin, 5% FBS, 7.5 IU/ml recombinant human follicular stimulating hormone (rhFSH) (Organon, Holland), and 100 IU/ml human chorionic gonadotrophin (HCG) (Organon, Holland). Low concentrations of *P. rhoeas* extract (0, 10, 15, 20 μg/ml) in the first trial group and higher concentrations of the extract (0, 50, 100, 200 μg/ml) in the second trial group were added to the maturation medium. The media were equilibrated at 37.5°C in 5% CO_2. About 10–15 COCs were placed in 25-μl maturation medium droplets overlaid with mineral oil. After the incubation of oocytes for 12–18 h, the granulosa cells were removed by gentle pipetting and the percentage of oocytes at the GV stage, the germinal vesicle breakdown (GVBD) stage and the metaphase II (MII) stage were recorded using an inverted microscope (Nikon).

In vitro fertilization and embryo development

Adult male mice (6–8 weeks old) were sacrificed by cervical dislocation and the epididymides were dissected out, disrupted and transferred to the IVF medium. T_6 medium was applied through IVF and in vitro development (IVD) [17]. IVF and sperm capacitation medium consisted of T_6 medium supplemented with 15 mg/ml BSA (equilibrated at 37.5°C in 5% CO_2). An incubation period of approximately 2 h was considered sufficient for sperm capacitation. Mature oocytes from each treatment were transferred to 100 μl IVF medium droplets and 2×10^6 sperm/ml was added to the IVF droplets. After 4–6 h incubation of oocytes with sperm, the oocytes were removed, washed and transferred to IVD medium which consisted of T_6 medium supplemented with 4 mg/ml BSA (equilibrated at 37.5°C in 5% CO_2). About 7 h after IVF, the oocytes were monitored by inverted microscope and the percentage of both male and female pronuclear (2 PN) formation was recorded to evaluate the fertilization rate. During IVD (96 h) the numbers of 2-cell, 4- to 8-cell, morula and blastocyst embryos were recorded.

Statistical analysis

The ANOVA and Duncan protected least-significant tests, using Statistical Analysis System (SAS) v1.9 program, were applied for all statistical analysis. All percentages of values were subjected to arc sine transformation prior to analysis. All data were expressed as mean ± SEM. A probability of $P < 0.05$ was considered to be statistically significant.

Table 1 Effect of the *P. rhoeas* extract on in vitro oocyte maturation

Extract concentration (μg/ml)	Maturation stage of oocytes (%)			
	Total COCs	GV (mean ± SEM)	GVBD (mean ± SEM)	MII (mean ± SEM)
Trial 1: low concentrations				
0	137	13.1 ± 2.4	7.3 ± 0.2	62.6 ± 2.6
10	163	11.4 ± 1.6	7.4 ± 0.2	64.5 ± 4.6
15	137	11.3 ± 1.8	7.4 ± 0.3	70.1 ± 6.1
20	162	10.4 ± 1.6	7.1 ± 0.2	70.9 ± 3.1
25	168	9.2 ± 1.5	7.1 ± 0.2	71.5 ± 2.7
Trial 2: high concentrations				
0	91	17.5 ± 2.6	29.9 ± 3.4[a]	56.3 ± 3.4[b]
50	91	14.1 ± 2.5	16.6 ± 3.7[b]	64.8 ± 3.6[ab]
100	102	13.4 ± 2.7	15.2 ± 3.1[b]	70.7 ± 4.8[a]
200	97	12.2 ± 2.3	12.5 ± 2.7[b]	72.9 ± 5.4[a]

Percentage of metaphase II oocytes (MII), percentage of oocytes arrested at vesicle germinal (GV) and percentage of oocytes at germinal vesicle break down (GVBD) stage. All experiments were repeated seven times. Data are expressed as mean ± SEM. Different superscripts show significant differences in a column ($P < 0.05$)

Results

In the first trial group (low concentrations of the extract were added to maturation medium), maturation and blastocyst formation improved; however, the improvement was not significant when compared to the control group. In addition the percentage of arrested oocytes at GV and GVBD stages did not show significant differences compared to the control group (Table 1). In the second trial group (higher concentrations of the extract were added to the maturation medium), the addition of 100 and 200 μg/ml extract significantly ($P < 0.05$) increased the maturation rate compared to the control group. In all the experimental groups the numbers of oocytes that arrested at the GVBD stage significantly ($P < 0.05$) decreased. IVF improved in all concentrations in both trials; however, no significant difference was observed between the experimental and control groups (Table 2). In the first trial group, there were no significant differences in the percentage of 2-cell, 4- to 8-cell, morula and blastocyst formation throughout the 96 h period of embryonic development (Table 3). In the second trial group, the oocytes treated with 50 and 100 μg/ml extract achieved the highest rate of embryo development and significantly increased the percentages of 4- to 8-cell morula and blastocyst formation compared to the control group ($P < 0.05$). As shown in Table 3, the percentage of 2- to 8-cell morula and blastocyst formation in oocytes treated with 200 μg/ml extract showed no significant difference when compared to the control group (Table 3).

Table 2 Effect of the *P. rhoeas* extract on in vitro fertilization rates

Extract concentrations (μg/ml)	No. of matured oocytes	2 PN (mean ± SEM)
Trial 1: low concentrations		
0	83	58.5 ± 2.4
10	84	62.8 ± 1.9
15	105	65.9 ± 3.1
20	103	62.3 ± 5.0
25	105	62.5 ± 3.0
Trial 2: high concentrations		
0	62	62.3 ± 4.8
50	72	72.5 ± 3.3
100	88	66.6 ± 2.7
200	88	64.5 ± 6.2

Percentage of fertilized ova with 2 PN (%) expressed as mean ± SEM. All experiments were repeated seven times. There were no significant differences ($P < 0.05$)

Discussion

This study evaluated the effect of *P. rhoeas* extract on oocyte maturation and subsequent embryo development. Investigations on the effect of botanical components such as green tea polyphenols by Wang and co-workers [18] demonstrated that supplementation of green tea polyphenols as anti-oxidants through maturation of bovine oocytes increased blastocyst formation. It was demonstrated that *P. rhoeas* extract exerted its anti-oxidative effects on mouse brain tissue [19]. The anti-oxidative potential of some plants such as *P. rhoeas* have been evaluated and it was shown that those plants are scavengers of hydrogen peroxide [15]. In this experiment the in vitro maturation rate and developmental competence of oocytes treated with lower concentrations of *P. rhoeas* extract improved; however, the addition of higher concentrations to the maturation medium was more effective. The addition of 50 μg/ml extract to the maturation medium improved the maturation rate and increased developmental competence. The highest maturation rate was observed with the addition of 200 μg/ml

Table 3 In vitro embryo development following insemination

Extract concentrations (μg/ml)	Fertilized ova with 2 PN (%)	Time after insemination and embryo stage			
		24 h, 2-cell	48 h, 4- to 8-cell	72 h, Morula	96 h, Blastocyst
Trial 1: low concentrations					
0	62	66.3 ± 2.9	47.7 ± 4.4	37.9 ± 6.9	26.9 ± 5.6
10	66	70.7 ± 3.4	49.8 ± 4.0	39.4 ± 4.6	33.0 ± 4.0
15	74	75.1 ± 2.6	51.0 ± 4.5	42.9 ± 5.5	35.7 ± 4.6
20	80	68.0 ± 5.1	54.4 ± 6.2	46.0 ± 5.6	35.0 ± 4.2
25	83	72.7 ± 3.8	49.2 ± 2.4	44.3 ± 4.7	39.4 ± 3.8
Trial 2: high concentrations					
0	48	62.3 ± 4.8	38.6 ± 1.9^{b}	32.5 ± 5.7^{b}	19.8 ± 3.8^{b}
50	65	72.6 ± 2.7	52.3 ± 5.5^{a}	46.5 ± 5.2^{a}	32.2 ± 3.8^{a}
100	74	66.6 ± 2.7	53.7 ± 3.8^{a}	48.5 ± 3.3^{a}	34.8 ± 3.1^{a}
200	65	64.4 ± 6.2	40.7 ± 3.2^{b}	32.2 ± 2.9^{b}	24.5 ± 2.1^{ab}

Percentage of embryos expressed as mean ± SEM. All experiments were repeated seven times. Different superscripts indicate significant differences ($P < 0.05$)

extract. The high Fe^{2+} chelating activity of *P. rhoeas* extract is the main cause of its anti-oxidative properties [20]. Heavy metals such as iron, zinc and copper produce harmful free radicals through chemical reactions [21] and it has been shown that iron is the most detrimental element. Production of hydroxyl radicals are catalyzed by iron [22] which directly effects lipids and promotes oxidative stress [23]. It has been concluded that traces of iron might be the agent of natural cleavage arrest in mouse embryos [10]. Olson and Seidel [24] have shown that the presence of appropriate concentrations of chelators such as EDTA and DTPA in developmental medium increased the number of bovine blastocyst formations. The alternative anti-oxidative effect of the extract may due to its flavonoids. Major chemical components of *P. rhoeas* extract include both alkaloids [25] and non-alkaloids [26]. Non-alkaloid components such as secondary metabolites have been characterized [26] and include anthocyanins with cyanidol as their major component [27]. Anthocyanins are water-soluble plant pigments [28] and belong to a larger group known as flavonoids [29]. They protect plants from oxidative stress induced by UV light and the metabolic process [30]. Recently it has been shown that anthocyanins are free radical scavengers [31] that protect cells against free radicals by gamma-glutamyl synthetase (gamma-GCS) activation. Activation of gamma-GCS elevates glutathione (GSH) levels in medium [32]. Increased GSH levels through oocyte maturation are associated with improvement in subsequent embryo development [33]. Because of electron deficiency in anthocyanins, they react with ROS and neutralize free radicals [34]. In this study the developmental competence of oocytes was reduced to the level of the control group when 200 μg/ml extract was added to the maturation medium. Reduction in embryo development might be due to deleterious effects of excessive concentrations of the extract. It has been shown that an excessive amount of chelators such as EDTA in developmental medium tends to be harmful [24]. High concentrations of anthocyanins reduce the proliferation ability and viability of human cells [35]. In addition, an excessive concentration of anthocyanins change the cell membrane structure and damage cell polarization [36]. Therefore, through morphological evaluations, the optimum concentration is 100 μg/ml in maturation medium. Our experiment indicated that the effect of a plant extract as an anti-oxidant on oocyte maturation and subsequent embryo development seemed to be beneficial. It was concluded that these increasing effects on oocyte maturation and subsequent developmental competence were dependent on the extract concentration in the maturation medium.

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