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Effect of *Phoenix dactylifera* pollen grain on maturation of preantral follicles in NMRI mice

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ABSTRACT

Introduction: Optimizing an ideal culture system that is comparable to follicle environment to obtain mature oocytes is one of the important criteria in in vitro maturation (IVM). *Phoenix dactylifera* L. commonly known as date palm is an important herb in Asia folk medicine that is used to improve fertility in women from ancient time. The aim of this study was to investigate the effect of date palm pollen grain extract on IVM of mouse follicles.
Methods: In this study follicles with 1 or 2 layers of granulosa cells and round oocytes were isolated from 2-3 weeks old female NMRI mice ovaries. Follicles were cultured in IVM media with different concentrations 0, 10, 20, 30, 40 µg/ml of palm pollen grain extract for 12 days. Then, the effect of date palm pollen grain on follicular growth and maturation were analyzed.
Results: There was a significant increase in follicle growth and maturation rate in all treated groups as compared to the control group, but maturation rate was significantly higher in the presence of 20 µg/ml palm pollen grain.
Conclusion: Supplementation of IVM media with date palm pollen grain extract improves the IVM of follicles.

Implication for health policy/practice/research/medical education:
Date palm pollen has antioxidant property and may have a beneficial effect on in vitro maturation (IVM) of follicle and improve survival and growth rates of preantral follicles. Supplementation of IVM media with date palm pollen grain extract may improve IVM of follicles

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Introduction

Ovarian follicles are the main structural and functional units of female reproductive system which supply the essential microenvironment for the advantages of oocyte growth and maturation. Preantral follicles are considered as a potential source of oocytes to play an important role in in vitro studies of folliculogenesis and embryo production (1). In vitro maturation (IVM) of immature oocyte in laboratory conditions have been considered as an additional approach in assisted reproductive technology (ART) (2). The in vitro embryo production (IVEP) system involves IVM of the primary oocytes, in vitro fertilization (IVF) of the matured oocytes and in vitro embryo culture (IVC). IVM has been considered as one of the most critical part of this process towards successful IVEP (3). Currently, IVM system has been improved and applied as an effective infertility treatment for women with polycystic ovary syndrome (PCOS) to reduce the rate of ovarian hyper stimulation and to preserve fertility in patients at high risk of fertility-threatening disease or treatment regimens like chemotherapy (4). The combination of these reproductive technologies with cryopreservation is a useful method of fertility preservation for cancer patients before undergoing chemotherapy or radiation therapy (5). Maturation of oocyte depends on 2 essential aspects of cytoplasmic and nuclear maturation. So, the loss of complete maturation

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can reduce the success of oocyte developmental competence. However nuclear and/or cytoplasmic maturation are not supported properly by current culture systems (6). Providing effective culture medium comparable to follicular environment is a critical step toward obtaining mature oocytes (3). The intracellular glutathione *(*GSH) and reactive oxygen species (ROS) levels are recognized to play important role in oocyte IVM and embryo production. Reduction of intracellular GSH content can cause lower oocytes developmental competence (7). The generation of ROS is an essential cellular process. However, excessive amounts of ROS can change cellular molecules like lipids, proteins and nucleic acids (8). The production of high levels of during in vitro cultures leads to impress fertilization rate, embryo development and the change of clinical pregnancy rates (6). Natural antioxidants are compounds that play an important role in prevention of oxidative stress and associated diseases. Plants are considered as excellent sources of natural antioxidants (9).

Phoenix dactylifera L. generally known as the date palm is one of the important main plants of the Middle East (10). Date (*Phoenix dactylifera*) is a member of the palm family arecaceae or palmae (11). Palm pollen grains contain a wide range of nutritional contents such as: vitamins, minerals, trace elements, carbohydrates, lipids, organic acids, sterols, nucleic acids, free amino acids, enzymes and cofactors (10). The antioxidant activities of date palm pollen (DPP) is mainly due to phenolic components, such as flavonoids, phenolic acids, and phenolic diterpenes (12,13). Usually, the different parts of Phoenix dactylifera are used in traditional medicine for the cure of wide range of disorders like memory disturbances, fever, inflammation, paralysis, loss of consciousness and nervous disorders (14). The ancient Chinese and Egyptians used pollen as a medicinal agent (15). It has been demonstrated that DPP has gonadal stimulating potency and in ancient times was used by Egyptian to improve fertility in women (16).

Materials and Methods

Chemicals

All the reagents were obtained from Sigma-Aldrich (Germany) unless otherwise specified.

Animals

The NMRI mice were purchased from Razi Institute (Mashhad, Iran), and housed under standard conditions of temperature 25°C and 12 hours light: 12 hours dark. They were fed water and pellets ad libitum.

Preparation of extract

Samples of the plant were collected from botanical garden at Bushehr city (South of Iran). Pollen grains were washed with distilled water and then dried. The extract was prepared by mixing 0.1 g of palm pollen within 1 ml of PBS with vigorous shaking and vortexing.

DPPH free radical scavenging activity

The percentage of antioxidant activity of DPP extract was estimated by the scavenging free-radical DPPH as compared with butylated hydroxy anisole (BHA) as a standard compound (3). The different concentration of DPP extract was added to methanol solution of DPPH (250-4000 µg/ml). Then, absorbance was measured at 517 nm after 30 minutes incubation in dark with a micro plate reader. The scavenging activity percentage (%) was determined by using the following formula:

DPPH radical scavenging (%) = (control OD - sample OD/control OD) ×100

Isolation of preantral follicles

Female mice, 2-3 weeks old, were killed by cervical dislocation and ovaries were instantly transferred to culture medium that consisted of α-minimal essential medium (α-MEM, Gibco, UK) supplemented with 10% fetal bovine serum (FBS, Gibco, UK), 100 µg/ml penicillin and 50 µg/ml streptomycin under mineral oil. Preantral follicles from ovaries were mechanically isolated by using 30-gauge needles under a stereomicroscope. Isolated follicles that were preantral follicles with 120-150 µm diameter were chosen and cultured in culture medium of MEM-α supplemented with different concentrations of DPP (0, 10, 20, 30, 40 µg/ml extract) for 12 days. Then, the effect of DPP grain on follicular growth and maturation was analyzed (17).

Culture of preantral follicles

The preantral follicles isolated were cultured in 96-well plates which contained 19×35 µl droplets of α-MEM supplemented with 5% FBS, 100 mIU/ml recombinant follicle stimulating hormone, 1% insulin transferrin selenium mix (ITS; Gibco, UK), 100 µg/ml penicillin and 50 µg/ml streptomycin under mineral oil and was incubated at 37°C, humidified atmosphere of 5% CO2 in air for 12 days (18).

Evaluation of follicle parameters

Measurement of follicle diameter was performed with ocular micrometer at 100 X magnification and Image J imaging system software version 1.43 (National Institutes of Health, Bethesda, MD) every 48 hours during the culture period.

From the day four, we could not measure the exact diameter of the growing follicles because of overgrow of granulosa cells. The survival rate of the follicles was confirmed by evaluation of follicle morphology under an inverted microscope. Follicle survival in culture was considered positive during the time that maintained oocytes were surrounded by granulosa cells attached to the culture dish (1).

Ovulation induction

Ovulation was induced on day 12 of culture, by addition of fresh medium supplemented with 1.5 IU/ml human chorionic gonadotropin (HCG; Organon) to the droplets. The cumulus oocyte complexes (COC) were observed 14-16 hours later under the inverted microscope (5).

Statistical analysis

The data are presented as mean ± standard deviation (SD). Statistical analysis was performed using SPSS version 16. Criteria including survival rate, degeneration, germinal vesicle stage, antrum formation rate and oocyte maturation were analyzed by one-way analysis of variance (ANOVA). $P < .05$ was considered to be statistically significant.

Results

DPPH free radical scavenging activity

The anti-oxidative potential of the DPP grain extract was estimated by using the DPPH free radical scavenging activity assay. As shown in Figure 1, DPP grain extract showed dose dependent activity and also the DPPH scavenging effect was 65.6% at a concentration of 1 mg/ml. As a result, depending on concentration, the DPP grain extract demonstrated a moderate DPPH-scavenging activity as compared to the BHA as a standard compound (Figure 1).

Evaluation of follicle parameters

In the present study, we showed the promoting effect of different concentrations of DPP grain extract on IVM of mice follicles (Figure 2).

The results showed that there was a significant increase in follicle diameter in all treated groups as compared to the control but maturation rate was significantly higher in the presence of 20 µg/ml palm pollen grain ($P < .01$). Survival rate of follicles on day 12 of culture was significantly higher in the presence of 20 µg/ml palm pollen grain compared with the control group ($P < .05$).

As shown in Figure 3, the supplementation of DPP to maturation medium increased the rate of oocyte maturation. The survival rate, maturation rate, antrum formation rate were significantly improved in this group compared to the control group ($P < .05$). All three dosages of DPP improved IVM rate. However, a significant increase in the IVM rate was seen only in the group treated with 20 µg/ml ($P < .05$).

The percentage of arrested oocytes at GV stage showed significant ($P < .05$) difference compared to the control group (Table 1). The percentage of GV stage oocytes in the control group was significantly higher than other treatment groups ($P < .05$). The oocytes treated with 20 µg/ml extract showed the lowest percentage of GV stage compared with control group, 10 and 40 µg/ml extract groups ($P < .05$); however, no significant difference was observed

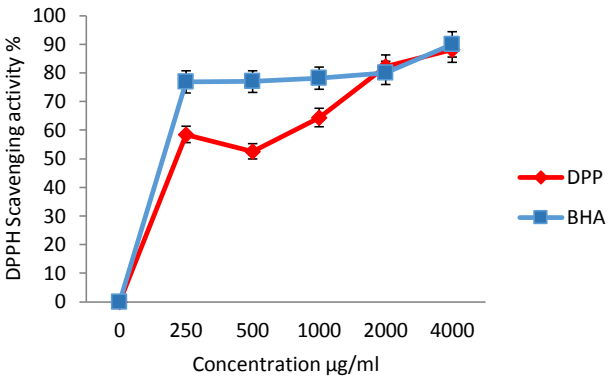

Figure 1. DPPH free radical scavenging activity of date palm pollen extract (sample) and BHA (Mean ± SD).

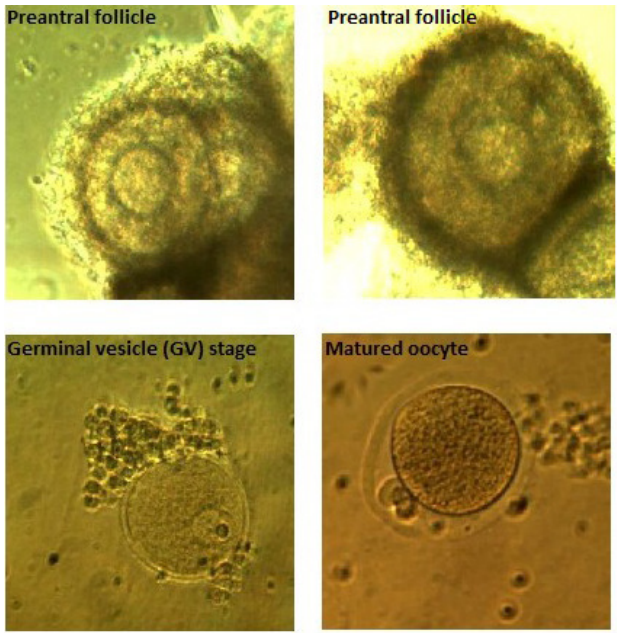

Figure 2. Development of follicles during in vitro culture. Preantral follicles on day 0 and 2, Germinal vesicle (GV), Matured oocyte under exposer with 20 µg/ml DPP.

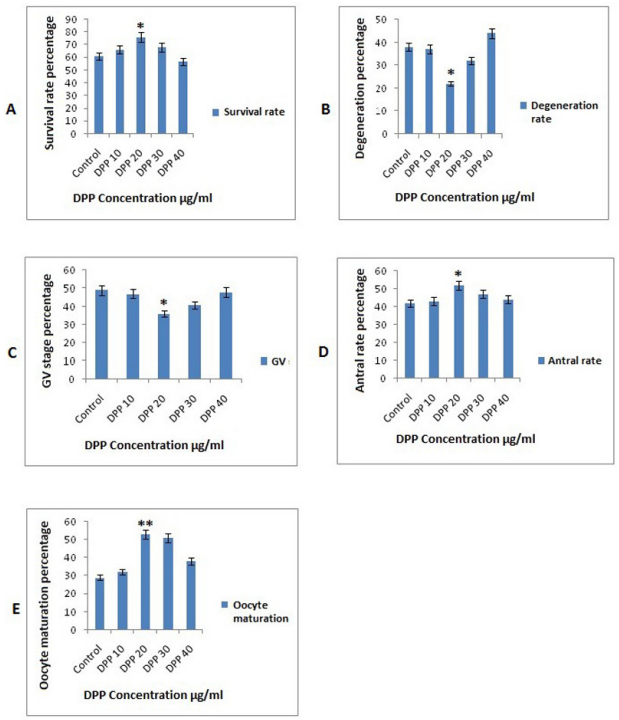

Figure 3. Effect of date palm pollen on follicles parameter. (A) Survival rate, (B) Degeneration, (C) Germinal vesicle stage, (D) Antral rate, (E) Oocyte maturation. (Mean ± SD, $P < .05$ was considered significant).

Table 1. Effect of DPP on IVM of preantral follicle[a]

DPP concentrations µg/ml	Total (%)	Survival rate (%)	Degeneration rate (%)	GV (%)	Antral rate (%)	Oocyte Maturation (%)
Control (DPP 0)	92	61 ± 4.04	38 ± 5.56	49 ± 4.00	42 ± 4.35	29 ± 2.51
DPP 10	81	66 ± 4.50	37 ± 5.03	47 ± 6.65	43 ± 5.29	32 ± 6.02
DPP 20	79	76 ± 6.11	22 ± 5.13	36 ± 4.04	52 ± 3.00	53 ± 4.72
DPP 30	76	68 ± 6.42	32 ± 5.50	41 ± 4.00	47 ± 6.55	51 ± 3.00
DPP 40	87	57 ± 4.16	44 ± 4.00	48 ± 2.51	44 ± 6.42	38 ± 5.85

[a] The data were represented as mean ± SD.

when compared to 30 µg/ml extract ($P > .05$).

Discussion

Recently in vitro culture of preantral follicles plays important role in ART (18). IVM of oocytes has developed as one of the promising method of infertility treatment. Retrieval of immature oocytes without gonadotropin stimulation and culture in maturation media is used in women with ovarian hyper stimulation syndrome (OHSS) (19). IVM has been suggested as an effective strategy for fertility preservation, since it does not need gonadotropin stimulation and is consequently related with serum E2 levels that are in the normal range. As a result, IVM provides an opportunity for serious fertility preservation and also in women suffering from E-sensitive diseases (20,21). In vitro culture mediums of oocytes have higher concentrations of oxygen in comparison to in vivo conditions, which caused an increase of ROS levels. Nowadays natural antioxidants are gaining a great attention which may prevent oxidative damage and related diseases (8). Different culture media have been used to provide optimum conditions for appropriate in vitro oocyte maturation (4). In 2014 Barakat et al (3) evaluated the oocyte maturation and embryo development of sheep and demonstrated that the supplementation of maturation media with low concentration of GTE as an antioxidant can enhance the rate of oocytes maturation and formation of morula and blastula in sheep.

Heidari et al. indicated that fibroblast co-culture improves the growth and viability rate of preantral follicles through development of granulosa cell proliferation (1). The effects of fibroblast co-culture and activin A were evaluated on IVM and fertilization of mouse preantral follicles that elevated the growth and survival rate of preantral follicles (2). The effect of *Papaver rhoeas* L. extract on IVM of sheep oocytes indicated that the rate of oocyte maturation and MII stage increased compared to the control group (8).

Lately, a study revealed that the addition of quercetin in maturation media improved embryos development (22). It has been shown that the natural antioxidants saffron (*Crocus sativus* L.) aqueous extract in culture medium increases the oocyte maturation and embryo development (6). In another study it was demonstrated the antioxidant properties of saffron (*Crocus sativus* L.) aqueous extract and its component like crocin could improve the rate of IVM, IVF and embryo development of mouse oocytes. However, this study showed that saffron aqueous extract had a stronger effect than pure crocin (23). The previous studies documented the antioxidant activity of DPP grain. It was suggested that the supplementation of DPP as an antioxidant enhanced GSH and restored LPO in the testes of Cd-treated rats. In addition, DPP could improve Cd-induced oxidative stress in the testicular tissues (24). The antioxidant potential of DPP was demonstrated by its bioactive components that could protect the oral mucosa by blocking oxidative free radicals, preventing DNA damage, and neutralizing inflammatory reactions (25).

These studies exhibited that the administration of DPP as a source of antioxidant in the maturation medium increased maturation rate of oocytes. In other studies various antioxidants like β-mercaptoethanol (β-ME), cysteine, cysteamine and anthocyanin were applied to develop IVM of oocytes (7). DPP as an antioxidative substance prevented harmful actions of free radicals. Extract of DPP is an herbal mixture that is generally used as a folk remedy for curing male infertility in traditional medicine (16). It was concluded that the supplementation of IVM media with DPP grain extract may improve IVM of follicles.

Conclusions

Finally, the results of the present study demonstrated that DPP has antioxidant property and in proper dose to medium as an antioxidant may have a beneficial effect on IVM of follicle and improve survival and growth rates of preantral follicles. Supplementation of IVM media with DPP grain extract may improve IVM of follicles.

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Authors' contributions

All the authors wrote the manuscript equally.

Conflict of interests

The authors declared no competing interests.

Ethical considerations

Ethical issues (including plagiarism, misconduct, data fabrication, falsification, double publication or submission, redundancy) have been completely observed by the authors.

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