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Original Research

Effects of grape seed extract, quercetin and vitamin C on ovine oocyte maturation and subsequent embryonic development

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Abstract: The present study aimed to evaluate the effects of grape seed extract (GSE) versus quercetin and vitamin C on *in vitro* oocyte maturation and embryo development in sheep. The free radical scavenging activity of different concentrations of each product was measured by 1, 1- diphenyl-2-picryl hydrazyl (DPPH). Oocytes were collected from ovaries of slaughtered ewes and matured in TCM-199 medium containing fetal calf serum, follicle stimulating hormone (FSH), estradiol-17 β, sodium pyruvate, and gentamicin sulfate. The *in vitro* fertilization and culture were performed using Bracket and Oliphant's (BO) medium and modified Charles Rosenkrans medium with amino acids (mCR2aa), respectively. The results showed that the hydroalcoholic extract of grape seed had free radical scavenging activity. IC_{50} value for GSE, vitamin C, and quercetin was found to be 585 µg/mL, 53 µg/mL, and 43 µg/mL, respectively. The concentrations, which showed beneficial effects on oocyte maturation and early development based on the mean number of cleavage, morula and blastocyst rates, were 25-200 µg/mL, 5 or 15 µg/mL, and 800 µg/mL, respectively, for vitamin C, quercetin and GSE. However, there were no significant differences between different concentrations of GSE and control. Findings also highlight the great effect on blastocyst rate while adding GSE at 800 µg/mL. However, the best rate of blastocyst production was obtained in presence of quercetin. Findings suggested the need for further studies on special molecules derived from GSE.

Key words: Antioxidant; Blastocyst; Grape seed extract; Quercetin; Vitamin C; Sheep.

Introduction

In vitro embryo production from unstimulated ovaries in domestic animals appears to be a routine practice although it is considered as an important platform applied in the technology of artificial breeding, cloning, and transgenic animal production (1). The necessary components are prepared by the oocyte in order to support development until the embryonic genome is activated and can take over growth (2). Thus, IVM can be categorized as an important aspect of *in vitro* embryo production since it has such a potential to help utilize the vast supply of oocytes within an ovary (1). In recent years, there have been many research scholars who have made their attempts to improve IVM systems. However, *in vitro*-matured oocytes still possess poorer developmental competence in comparison with *in vivo*-matured ones (2).

In vitro culture conditions have been found to contain higher concentrations of O_2 than *in vivo* conditions. Oxygen tension in the oviduct is observed to be approximately from one-quarter to one-third that of atmospheric tension (3). Oxidative stress resulting from high external oxygen concentration have the potential to produce reactive oxygen species (ROS), which may have the responsibility regarding damaging embryos and inducing early embryonic developmental blocks (4). The formation of ROS such as superoxide anions (O_2•-), hydroxyl radicals (OH•) and hydrogen peroxide (H_2O_2) is carried out within a normal process taking place in the cell when deviation of electrons to oxygen (O_2) exists during electron transfer reactions (5). However, some exogenous oxidants, such as H_2O_2, may provide the optimal condition for over production of ROS, which probably induces multiple cellular damages, including lipid peroxidation, nuclear DNA strand breaks, and mitochondrial alteration, resulting in disturbing the development of preimplantation embryos *in vitro* (6).

As non-enzymatic antioxidants, vitamins (e.g. A, C, and E) and glutathione (GSH) can react with ROS and maintain appropriate levels. In addition, enzymatic defense systems, namely as superoxide dismutase, glutathione peroxidase and catalase attempt to suppress oxidative injury efficiently and weaken the damage caused by ROS as well (6). However, when IVM and embryo culture takes place, the levels of antioxidants are lower than *in vivo*, which is due to the fact that the oocytes or embryos are divorced from the donor body and are not provided with enough opportunity to benefit from the maternal antioxidant protection (5).

There is a great tendency in natural antioxidants presented in medicinal and dietary plants replacement of synthetic types, paving the way for preventing oxidative damages (5). Phenolic compounds are very important because of their positive health effects since they are able to protect cell against oxidative damage during the occurrence of antioxidant and pro-oxidant imbalances. Indeed, reactive species play both a beneficial and toxic role while cautious should be made regarding the balance maintenance between them (7). Grape seed proan-

thocyanidin is a new generation antioxidant, which not only causes the removal of excess amount ROS produced in the disease process, but it is also applied as a signaling molecule (8). Grape (*Vitis vinifera*) is considered as one of the most commonly used fruit growing worldwide (9). Resveratrol, quercetin, catechin, flavone, flavonols, procyanidin, anthocyanin, gallic acid, epicatechin include the phenolic compounds isolated from the black grapes (10). Research has shown that the total phenolic content of grape seed was higher than that of the peel and pomace making grape seeds as a valuable source of phenolics and antioxidants(9).

Quercetin (3,3',4',5,7 – pentahydroxyflavone) is a plant derived flavonoid from fruits and vegetables with the feature of antioxidant action, which function as a free radical scavenger. Abundance of studies has also been carried out regarding the investigation of hamster embryo cells, Chinese hamster V79 cells, human sperm, human HepG2 hepatocytes and porcine oocytes (11). It has been found that quercetin may have anti-inflammatory and antioxidant properties caused by its free radical scavenging and metal chelating activities (12).

The current study was an attempt to investigate the effects of grape seed extract (GSE), vitamin C or quercetin when supplemented in maturation medium at various concentrations on cleavage, morula, and blastocyst rates of sheep oocytes.

Materials and Methods

All the culture media, growth factors and other chemicals were obtained from Sigma-Aldrich (St. Louis, MO, USA) and the plasticware were purchased from Falcon (Paignton, UK) unless stated otherwise.

The GSE was obtained from the Ebnemasouyeh pharmaceutical company (Tehran, Iran). It was prepared by steam distillation (1:5 herb/water, in w/v ratio) for 4 h using a Clevenger apparatus. The dry matter of GSE was set at 16 mg/mL.

Secondary metabolites identification was carried out on GSE according to the method of Crozier *et al*. (1997)(13). High performance liquid chromatography (HPLC) analysis was performed using a Waters Series HPLC (Waters, USA) system equipped with 1580 pump and a UV-1570 detector. Peaks were separated by reversed-phase HPLC on a 150×3.9 mm I.D., 5 µm particle diameter C18 Symmetry column. The column eluted at flow rate 1 mL/min with 27 min gradient of 10-90% acetonitrile in water, adjusted to pH 2.5 with trifluoroacetic acid (TFA). To identify peaks, the spectral patterns and retention time of the samples were compared with standard peaks.

The free radical scavenging activity of different concentrations of GSE, quercetin, and vitamin C was measured by 1, 1- diphenyl-2-picryl hydrazyl (DPPH). The DPPH method was determined as described by Shekhar and Anju (2014) (14) and Andzi-Barhé *et al*. (2015) (15). Briefly, 0.1 mM solution of DPPH in ethanol was prepared. This solution (2.85 mL) was added to 0.15 mL of different concentrations (1, 5, 15, 25, 50, 100, 200, 400, 800, 1000 µg/mL) of quercetin and vitamin C and different concentrations of GSE (1, 5, 15, 25, 50, 100, 200, 400, 800, 1000, 2000, 4000 µg/mL). The mixture was shaken vigorously and allowed to stand at room temperature for 30 min. Then, absorbance was measured at 517 nm by using a spectrophotometer.

The activity is defined by the index of reduction of the radical scavenger activity (RSA), expressed as a percentage, where the absorbance of the reaction mixture which contains the free radical and the sample of antioxidant is compared with the absorbance of the mixture without the antioxidant (control solution).

$RSA\% = ((AT - AS)/AT) \times 100$

AT: absorbance of the control (DPPH° only)

AS: absorbance of the sample (antioxidant + DPPH°)

The IC_{50} value of the sample, which is the concentration of sample required to inhibit 50% of the DPPH free radical, was calculated using regression equations of $y=0.0705x+8.7491$ ($R^2=0.9835$), $y=0.4318x+9.3746$ ($R^2=0.9849$) and $y=0.6921x+13.20$ ($R^2=0.88$) for GSE, vitamin C and quercetin, respectively.

Four ram testes were gathered from a nearby abattoir immediately after slaughter and were transferred to the laboratory. In the laboratory, rinse of the testes twice with normal saline and trimmed in order to remove the extra testicular tissue. Connective tissue covering the cauda epididymis was cautiously removed as well to prevent from rupturing blood vessels or the epididymal duct. Epididymal sperm was collected as stated in Garde *et al*. (2003) (16). Therefore, sperm was recovered from the side of epididymis through injection of PBS retrogradely by the ductus deferens. Collected sperm was washed once by centrifugation by exposing to PBS medium at 600 g for 7 min. The pellet was suspended in Bracket and Oliphant's (BO) medium and subjected to evaluation for motility.

Oocyte collection as well as *in vitro* maturation was carried out according to the protocol proposed by hasanzadeh *et al*. (2016)(17). To this end, sheep ovaries were collected from a nearby abattoir and then carried to the laboratory in phosphate-buffered saline (PBS) consisting of 100 IU/mL penicillin and 50 mg/mL streptomycin at 30-34ºC within 3 h of slaughter. Through slicing the surface of an ovary with a surgical blade, cumulus-oocyte complexes (COCs) were released from all visible follicles (>2 mm diameter). Those possessing more than 3 layers of compact unexpanded cumulus cells and having homogenous evenly-granular ooplasm were transported to 100-µl droplets (15–20 COCs per droplet) of IVM medium, which included TCM-199 with 10% FBS, 10% sheep follicular fluid, 5 mg/mL follicle stimulating hormone (FSH), 1 mg/mL estradiol- 17β, 0.81mM sodium pyruvate and 50 mg/mL gentamicin sulfate, and subsequently were cultured under mineral oil in a CO_2 incubator (5% CO_2 in air) at 38.5ºC for 24 h.

To initiate *in vitro* fertilization, the oocytes were rinsed twice with washing BO medium (containing 10µg/mL heparin, 137.0µg/mL sodium pyruvate, and 1.942 mg/mL caffeine sodium benzoate) and were then forwarded to 50-µl droplets (15–20 oocytes per droplet) of capacitation and fertilization BO medium (washing BO medium containing 10 mg/mL fatty acid-free bovine serum albumin (BSA)). The provision of the spermatozoa was done for fertilization as previously explained by Chauhan *et al*. (1997) (18). Insemination of Oocytes was then carried out by using a final concentration of 1×10^6/mL motile spermatozoa and incubation was also taken place under mineral oil in a CO_2 incuba-

tor at 38.5ºC for 18 h. The presumed zygotes began to culture in modified Charles Rosenkrans medium with amino acids (mCR2aa) containing 0.6% BSA for 48 h in groups of 15–20 per droplet.

After that, the cleaved embryos were subjected to the *in vitro* culture (IVC) medium (mCR2aa+0.6% BSA+10% FBS) for up to 8 d until morulae and blastocysts were achieved, with every 48-h change of medium. As the literature has shown the use of different concentrations for quercetin (12) and vitamin C (19, 20) and the limitation of using high volumes of GSE due to change in viscosity and composition of maturation medium has also been the focus of previous research, thus, the present study aims to take into account different concentrations of vitamin C (25, 50, 100 and 200 µg/mL), quercetin (5, 15, 25, 50 µg/mL) and GSE (50, 200, 400, 800 µg/mL).

The HPLC experiments were independently repeated three times under the same conditions and concentrations and all analyses were performed in triplicate. Results are expressed as mg/g DW. As to the purpose of the study, each treatment regarding maturation of ovine oocytes was replicated at least four times. Quantitative data analysis was applied through statistical software program (SPSS 16, IBM, USA). Comparisons of multiple numeric datasets were done using one-way ANOVA followed by Duncan multiple-range test. Results are shown as mean±SEM, and statistical significance was indicated at $P<0.05$.

Results

RP-HPLC analyses were carried out to provide a quantitative measurement of the secondary metabolite profiles (Figure 1), expressed in mg/g DW on a dry weight of seed. Statistically significant differences ($p < 0.05$) were found between the each compound assayed. *V. vinifera* compounds were quantified in the group of phenolic acids. Generally, Epichatechin was the major phenolic acid in the seed, with an average level of 1.80 mg/ g DW and caffeic acid 1.25 mg/ g DW. Minor phenolic acids found in the grape were catechin, procyanidine $B_{1,}$ procyanidine B_2, proanthocyanidin timer, proanthocyanidin timer monogallete and procyanidin C_1 was present (0.22 mg/ g DW), (0.81 mg/ g DW), (0.64 mg/ g DW), (0.54 mg/ g DW), (0.44 mg/ g DW) and (0.35 mg/ g DW) respectively.

DPPH method was used to measure the antiradical capacity of hydroalcoholic extract of GSE. IC_{50} value of the GSE was found to be 585 µg/mL and 53 µg/mL and 43 µg/mL, respectively, for vitamin C and quercetin. (Figure 2).

The effective concentration of vitamin C, quercetin and GSE, based on their radical-scavenging activity, were used in this experiment. Results showed that the cleavage and morula rates were not significantly different among the control and groups treated with 25 or 50 µg/mL of vitamin C. However, these rates were significantly lower ($p<0.05$) for the oocytes given more than 100 µg/mL of vitamin C. Moreover, the blastocyst developmental rates were decreased by using more than 50 µg/mL of vitamin C (Figure 3A). As shown in figure 3B, the addition of 15 µg/mL quercetin to maturation medium significantly increased the rate of cleavage

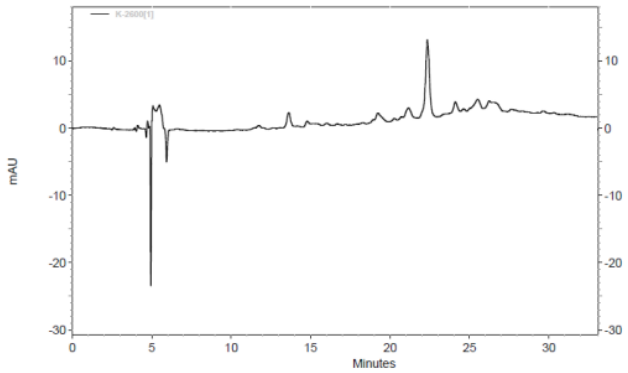

Figure 1. HPLC Chromatograph of the hydroalcoholic grape seed extract of Vitis vinifera. A: Galic asid, B: procyanidin B1, C: catechin, D: procyanidineB2, E: caffeic acid, F: epicatechin, G: proanthocyanidin timer, H: proanthocyanidin dimer mangollet, I: procyanidin C1.

compared to other groups, and also the rates of blastocyst formation significantly increased, when 5 or 15 µg/mL of quercetin were used ($p<0.05$). In contrast, the blastocyst rates were significantly lower ($p<0.05$) for those oocytes having more than 25 µg/mL quercetin compared to other groups. Higher concentration of GSE (800 µg/mL) was the most effective treatment (Figure 3C) based on the rates of cleavage, morula, and blastocyst. However, there were no significant differences between different concentrations of GSE and control.

Discussion

Previous research revealed that adding high concentrations of antioxidants to the IVM medium resulted in decreasing the rate of blastocyst formation in comparison with treatment with low concentrations, highlighting that benefiting from proper concentration of an antioxidant can provide optimal condition for the generation of high quality embryos (4). Naruse *et al.* (2007) found that a relatively low concentration of water-soluble minimum essential medium (MEM) vitamins during maturation of porcine oocytes could positively bring about remarkable subsequent development, whereas higher concentrations of MEM vitamins were found to be ineffective or detrimental to IVM and subsequent parthenogenetic development (21).

In their study, Kere *et al.* (2012) argued that Porcine embryos treated with vitamin C (50 µg/mL) led to improvement of cleavage and blastocyst rates and total cell

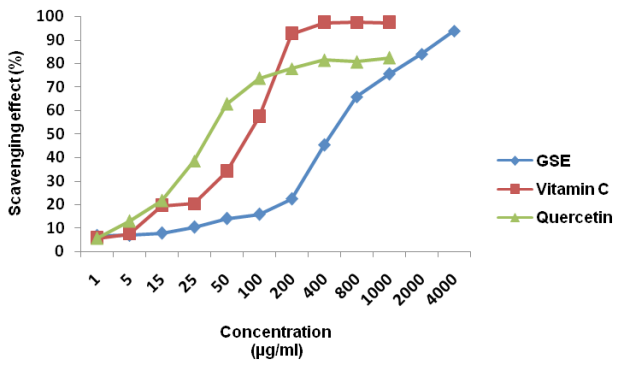

Figure 2. Antioxidant activities of different concentrations of GSE, vitamin C and quercetin.

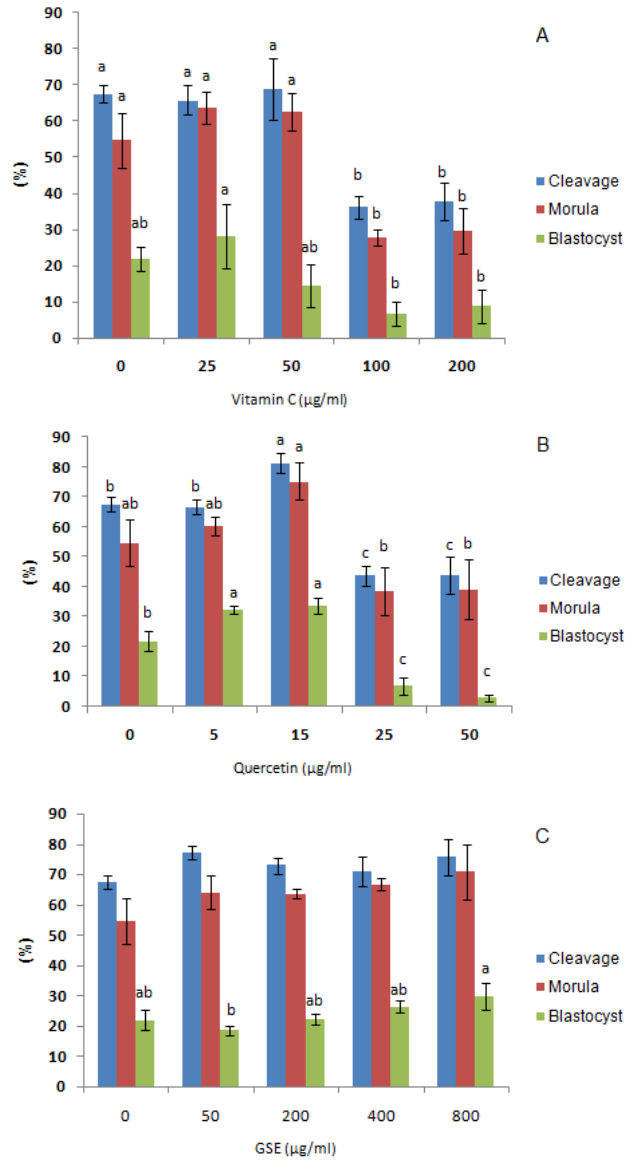

Figure 3. Cleavage, and embryo development of sheep oocytes treated with different concentrations of vitamin C (A), quercetin (B) and GSE (C) during *in vitro* maturation (P<0.05).

numbers per blastocyst and a reduction of apoptotic index as well (19). In fact, it was found that a suboptimal concentration of free-radical scavengers in the culture system would also help to the viability of poor embryo and, in turn, to progressive embryo losses during *in vitro* embryo production. Nonetheless, supplementation of 100 µg/mL of vitamin C to the culture medium caused to increase the apoptotic index, which supports the existence of a narrow window for the concentration dependency of vitamin C treatment. Hence, concerning the findings of the present study and investigating the synergistic effect of vitamin C and E in previous research (22), it can pave the way for future research to look into the lower concentration of vitamin C in combination with vitamin E in maturation medium of ovine oocytes.

Findings of the current research showed the significant improvement of the rate of blastocyst formation when 5 to 15 µg/mL of quercetin were used in maturation medium of ovine oocytes (p<0.05). Results of the study can be compared to the studied conducted by (4, 10, 12). Kang *et al*., (2016) in that they maneuvered on the positive use of the treatment of porcine oocytes with the flavonoid quercetin, which had a significant effect on embryonic development and simultaneously resulted in reduction of ROS generation by increasing intracellular GSH levels at low concentrations, while high concentrations was found to be detrimental (4). Yu *et al*.'s, (2014) research indicated that the improvement of the quality of H_2O_2 -treated mouse embryos by exposing quercetin in the medium can be obtained by increased blastocyst formation, reduced apoptosis and fewer incidences of fragmentation as well as developmental retardation (6). Such development of preimplantation embryos caused by quercetin may be related to the antioxidant actions of quercetin in reducing ROS levels in zygotes. In this regard, in a study done by (4), researchers concluded that quercetin at 50 µg/mL inhibited the production of progesterone by granulosa cells, altered estradiol-17ß production, and interfered with angiogenesis by inhibiting vascular endothelial growth factor production, all of which proved the negative influence of quercetin on ovarian physiology. Kang *et al*. (2013) also put forward that the reduction of oocyte maturation and blastocyst formation rates as a result of the above-mentioned concentration of quercetin (i.e. 50 µg/mL) may be originated from unresponsive signaling to oocytes and embryos or direct embryo toxicity because of excessive levels of flavonoids (12).

The present study used GSE as natural antioxidant in maturation medium of ovine oocytes. The rate of cleavage, morula and blastocyst improved through increasing the concentration of the extract. However, no significant differences were observed between different concentrations of GSE and control. Previous research also proved the beneficial effect of herbal extracts such as green tea, saffron and Papaver rhoeas on IVM and blastocyst formation in different species (5, 23-25), however, to the best of the researcher's knowledge, findings of the present study seems to be the first investigation of demonstrating the effect of GSE in maturation medium of ovine oocytes. Grape seeds containing 60-70% phenolic compounds and catechin, epicatechin and procyanidin were the major antioxidants (9).

To conclude, the significant effect on blastocyst rate was observed although the addition GSE at 800 µg/mL was of great importance. However, the best recovery rate of blastocyst was obtained in presence of quercetin. Findings suggested the need for further studies on special molecules derived from GSE.

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Interest conflict

Authors declare that there is no conflict of interest for this study.

Authors' contributions

MK was involved in the experimental design, data collection and data analysis. MZ was involved in study design, data analysis, data interpretation and manuscript editing. MRS, MM, HO was involved in study design and data interpretation. All authors have read and appro-

ved the final manuscript.

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