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The effect of mesenchymal stem cells as co-culture in *in vitro* nuclear maturation of ovine oocytes

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This study compared the effects of ovine mesenchymal stem cells (MSCs) and ovine oviductal epithelial cells (OECs) as feeder cells in cell free culture systems (HEPES-modified tissue culture medium, TCM199) supplemented with polyvinyl alcohol (PVA) or fetal calf serum (FCS) on in vitro oocyte maturation and subsequent embryo development (IVM/IVC). Cumulus-oocyte complexes (COCs) were harvested from ovine ovaries and subjected to IVM in the above-mentioned culture media. After culture for 24 h, nuclear maturation of the oocytes was evaluated by 4, 6-diamino-2-phenylindole (DAPI) staining. After fertilization the presumptive zygotes were cultured under identical culture conditions and embryo development was evaluated. The percentage of oocytes at nuclear maturation (metaphase II) cultured in the MSC group was higher than for the IVM medium + PVA group (P<0.05), while between MSCs, OECs and IVM medium + FCS it was non-significant. The rates (%) of cleavage and the percentage of total blastocysts in MSCs and the IVM medium + FCS group were higher than for OECs and the IVM medium + PVA group (P<0.05). These rates were non-significant between MSCs and the IVM medium + FCS group or between OECs and the IVM medium + PVA group. The percentage of hatched blastocysts (%) was significantly increased in MSCs and the IVM medium + FCS group when compared to OECs and the IVM medium + PVA group (P<0.05). In conclusion, the effects of mesenchymal stem cells as co-culture on oocyte maturation and the successive embryo development in vitro are similar to those in the medium supplemented with FCS. This study suggests that co-culturing with mesenchymal stem cells may be a promising alternative to FCS-medium.

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Feeder cell lines (co-culture) such as somatic cells and embryo somatic cells have been applied in the development of mammalian pre-implantation embryos in vitro [Nematollahi-mahani *et al.* 2009, Orsi and Reischl 2007, Teklenburg and Macklon 2009]. The mechanisms of feeder cell lines are not completely clear. However, putative mechanisms have been suggested to explain the function of feeder cells in the development of embryos. It has been confirmed that feeder cells can act as medium detoxifiers and also provide many specific growth stimulators [Heidari *et al.* 2013]. Proliferating feeder cells produced bioactive factors found not only in IVC media. However, co-cultures were able to inhibit developmental blocks in most laboratory and domestic animals [Orsi and Reischl 2007].

Feeder cells have many advantages in embryonic development experiments, including an increased number of blastomeres [Smith *et al.* 1991], a high survival rate of post-thaw blastomeres [Tucker *et al.* 1995], lower apoptosis [Xu *et al.* 2000], a higher blastocyst rate [Joo *et al.* 2001], a higher implantation ratio [Wetzels *et al.* 1998] and a higher percentage of live births [Marcus and Brinsden 1996].

Despite of the above-mentioned benefits, there are controversial reports indicating that feeder cells had no improving effects on early embryogenesis [Hu *et al.* 1998, Tucker *et al.* 1995] or the subsequent clinical pregnancy rates [Hu *et al.* 1998]. Moreover, some reports indicated that co-culture has a harmful effect on embryo quality, such as discrepant intercellular contacts between the trophectoderm and inner cell mass, abnormal development of trophoblast apical microvilli, cytoplasmic vacuolation, hooded mitochondria, wide inter-cellular spaces and numerous cytoplasmic vesicles, phagosomes and lipid droplets [Shamsuddin and Rodriguez-Martinez 1994].

The embryotrophic properties of co-culture cells, such as oviductal epithelial cells (OECs) and mesenchymal stem cells (MSCs), have been thoroughly defined in human and animal embryos [Kervancioglu *et al.* 1997, Orsi and Reischl 2007, Park *et al.* 2010]. It has been demonstrated that adult mesenchymal stem cells can secrete a variety of cytokine and growth factors, such as MCP-1, VEGF-AEGF, FGF-2, IL-6, LIF, or TGF-ß [Li *et al.* 2011, Park *et al.* 2010]. Apparently, these factors improve meiotic maturation and the subsequent embryo development [Ling *et al.* 2008].

In view of the controversial reports on the advantages of co-culture systems compared with chemically defined medium in the production of mammalian embryos and the advent of MSCs as a new source of somatic cells in the co-culture system, this study was designed to compare the effects of MSCs and OECs as feeder cells in cell free culture (with and without fetal calf serum) systems on in vitro ovine oocyte maturation.

Material and methods

Preparation of oviductal epithelial cells as co-culture

The ovine oviducts were prepared in an abattoir, submerged in phosphate-buffered saline (PBS) and transported to the laboratory on ice within 2 h. Trypsin-EDTA solution (0.5% trypsin and 0.25% EDTA in PBS) was injected into the oviductal lumen and after 2-3 min. the lumen was squeezed with tissue forceps and its content was transferred into a conical tube. More trypsin-EDTA was added to the suspension and the cellular clumps were dispersed by pipetting. Trypsin was neutralized by TCM199 + 10% fetal calf serum (FCS) and then the suspension was centrifuged. After discarding the upper fluid, the pellet was resuspended in TCM199 + 10% FCS + NaHCO3 + Na pyrovate + penicillin/streptomycin. The pellet cells were cultured in 50 µl droplets of the above-mentioned medium at 39°C and 7% CO2. At 60-70% confluency and 2 h prior to oocyte culture the medium was refreshed with TCM199 without FCS.

Preparation of mesenchymal stem cells as co-culture

The mesenchymal stem cells were prepared from bone marrow of adult ewes. Femoral bone marrow was collected by a Jamshidi needle. Mononuclear cells were harvested by Ficoll separation of marrow cells. After separation of the cloudy corona and dilution with PBS the suspension was centrifuged in PBS. The cells were then incubated in a medium consisting of DMEM, 10% FCS, non-essential amino acids, NaHCO3, l-glutamine, and penicillin/streptomycin at a cell density of 5×10^6 cells/ml. The cells were cultured in 50 µl droplets at 39°C and 7% CO2 until achieving 40% confluency. The medium of droplets was refreshed with FCS-free TCM199 2 h prior to oocyte culture.

The mesenchymal stem cells were identified based on their morphologic or phenotypic characteristics and their capacity of multilineage differentiation into bone and fat. The osteogenic and adipogenic differentiation capacity of MSCs was confirmed using Alizarin-red and Oil red O staining, respectively, and those capacities were evaluated by the presence of osteogenic foci and intracellular accumulated lipid-rich vacuoles [Lee *et al.* 2004]. In addition to staining, one specific gene expression was also evaluated in differentiated cells as follows.

Molecular evaluation of differentiated mesenchymal stem cells

The reverse transcriptase-PCR analysis was performed to assess expression of Osteocalsin and LPL genes as osteocyte and adipocyte related genes in differentiated cell lineages (one gene for each cell lineage). Total RNA was extracted using the RNA Extraction Kit (Rima zol; Sinaclon Bioscience, Karaj, Iran) according to the manufacturer's instructions. Total RNA was treated with RNase-free DNase (Sinaclon Bioscience, Karaj, Iran) to avoid amplification of contaminating genomic DNA. RNA was evaluated by agarose gel electrophoresis. The amount and quality of RNA were determined by spectrophotometry. Only RNA of sufficient purity, having an

absorbance ratio (A260/280) greater than 1.9, was considered for synthesis of cDNA. To prepare cDNA, reverse transcription was performed according to Mirshokraei *et al*. [2013]. The PCR reactions were run using the following primer sequences: Osteocalsin (296 bp; Accession No. X51700.1), 5'-AGAACCCCCATGCTGCTCG-3' and 5'-GACTGGGCCGTAGAAGCGC-3'; lipoprotein lipase (LPL) (489 bp; Accession No. X68308.1), 5'-TTGACGGTGACAGGAATGTA-3' and 5'-ACATCCTGGTTGGAAAGTGC-3'. The amplification profile for Oscalsin and LPL genes was as follows: initial denaturation at 95°C for 5 min, 40 cycles with denaturation at 95°C (30 s), annealing at 55°C (30 s), and extension at 72°C (45 s). PCR products were analyzed in 1.5% agarose gel, stained with ethidium bromide and visualized by Uvitec gel documentation.

Preparation of ovine oocytes

Ovaries were collected from ewes at a local abattoir and transported to the laboratory in normal saline at the temperature of 30-35°C within 2 h. Ovaries were washed with warmed saline (37°C) and follicles with 2-6 mm diameters were aspirated. The collected follicles were released in pre-incubated HEPES-TCM199 + penicillin + streptomycin + heparin. The oocytes with at least 3 layers of cumulus cells (COCs), a uniform granulated cytoplasm and a homogenous distribution of lipid droplets in the cytoplasm were selected for this experiment.

Assessment of nuclear stage after maturation period of oocytes in different culture media

In order to evaluate the effect of different culture systems on the in vitro oocyte maturation (IVM) the COCs were randomly allocated into culture systems as follows: (1) IVM medium (TCM199 + NaHCO3 + Na Pyrovate + Cysteamine + 17β-estradiol + HCG + FSH + penicillin/streptomycin) with (1) polyvinyl alcohol (PVA), (2) IVM medium with 10% FCS, (3) Co-culturing with MSCs, and (4) Co-culturing with OECs. For IVM, 10-15 COCs were transferred into each droplet (50 μl) of the above-mentioned mediums in the experimental groups, layered with sterile mineral oil, and cultured in 7% CO2 in air at 39°C with 100% humidity for 24 h.

After the oocyte maturation period in the above-mentioned culture media, COCs were denuded by vortexing and then fixed in 2.5% (w/v) glutaraldehyde. The nuclear status of the oocytes was determined by 4, 6-diamino-2-phenylindole (DAPI) staining as described by Shirazi *et al*. [2010]. Oocytes were classified as the germinal vesicle (GV), germinal vesicle breakdown (GVBD), metaphase I (MI), or metaphase II (MII) stages of the maturation process.

The matured oocytes in the experimental groups were exposed to motile spermatozoa (5-10 × 10^3 sperm / oocyte) obtained from fresh semen by the swim-up procedure. Fertilization was carried out in IVF-SOF medium [Heidari *et al.* 2013] at 39°C for 22–24 h in the maximum humidified air atmosphere with 7% CO_2. After fertilization (Day 0) the presumptive zygotes were mechanically denuded of their cumulus cells and randomly allocated into IVC-SOF medium [Heidari *et al.* 2013].

During IVC, the embryos (10 embryos/50 µl drop) were cultured at 39°C in the maximum humidity atmosphere of 7% CO_2, 5% O_2 and 88% N_2. The culture medium was refreshed on days 3 and 5 with IVC-SOF + 10% Charcoal strip FCS. Embryonic development was evaluated on days 3, 5, 6, 7, and 8. Cleavage rate was recorded on day 3 and the embryos were assessed for the morphological development to blastocyst and hatched blastocyst until day 8. The efficiency of matured oocytes in the different culture systems was evaluated with their formation and development into embryos. This evaluation was conducted by comparing cleavage rates, blastocyst, expanded blastocyst and hatched blastocyst percentages.

All experiments were replicated at least three times. Empirical oocyte distribution within the different categories was examined using chi-square statistics.

Results and discussion

The osteogenic and adipogenic differentiation capacities of MSCs were confirmed by specific staining and detection of related markers (Osteocalsin and LPL transcripts) presented in Figures 1 and 2. The effects of MSCs, OECs and cell free culture (with

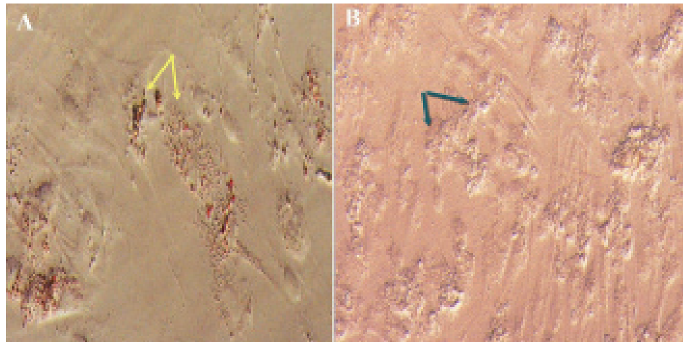

Fig. 1. Differentiation potential of mesenchymal stem cell. (A, B) Results of Oil red O and Alizarin-red staining in cell cultures growing in adipogenic and osteogenic medium, respectively. Intracellular accumulated lipid-rich vacuoles (arrows in Fig. A) and osteogenic foci (arrows in Fig. B).

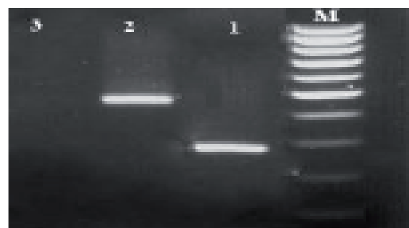

Fig. 2. Gel electrophoresis of semi-quantitative RT-PCR to determine differentiation potential of mesenchymal stem cell. Detection of Osteocalsin (band 1, 296 bp) and lipoprotein lipase (band 2, 489 bp) in cell cultures growing in osteogenic and adipogenic medium, respectively. Mesenchymal stem cell (3) as negative control. M, marker (100 bp).

Table 1. Nuclear maturation status of sheep oocytes under four different maturation conditions

Experimental groups	Number of cultured oocytes	Nuclear division stage			
		GV n (%)	GVBD n (%)	MI n (%)	MII n (%)
IVM medium + PVA	62	5 (8.0)	4 (6.5)	15 (24.2)	38 (61.3)[a]
IVM medium + FCS	53	1 (1.9)	5 (9.4)	5 (9.4)	42 (79.3)[ab]
MSCs	59	0 (0.0)	3 (5.0)	9 (15.3)	47 (79.7)[b]
OECs	60	0 (0.0)	6 (10.0)	12 (20.0)	42 (70.0)[ab]

GV – germinal vesicle; GVBD – germinal vesicle breakdown; MI – metaphase I; MII – metaphase II; MSCs – mesenchymal stem cells; OECs– oviductal epithelial cells; IVM – *in vitro* maturation; FCS – fetal calf serum; PVA – polyvinyl alcohol; n – number;
[ab]In the same column numbers with different superscript differ significantly at P<0.05.

Table 2. Development of embryos derived from oocytes matured under four different maturation conditions

Group	Oocytes no.	Cleavage n (%)	Total blastocyst n (%)	Blastocyst n (%)	Expanded blastocyst n (%)	Hatched blastocyst n (%)
IVM medium + PVA	147	86 (58.5)[a]	13 (8.8)[a]	6 (46.2)	7 (53.9)	0 (0.0)[a]
IVM medium + FCS	153	132 (86.3)[b]	49 (32.0)[b]	13 (26.5)	14 (28.6)	22 (44.9)[b]
MSCs	184	143 (77.7)[b]	41 (22.3)[b]	9 (21.9)	14 (34.2)	18 (43.9)[b]
OECs	185	126 (68.1)[a]	21(11.4)[a]	5 (23.8)	9 (42.9)	7 (33.3)[c]

MSCs, mesenchymal stem cells; OECs, oviductal epithelial cells; IVM, in vitro maturation; FCS, fetal calf serum; PVA, polyvinyl alcohol; n, number.
[a b]Numbers with different superscript letters in the same column differ significantly (P<0.05).

PVA or fetal calf serum) systems on ovine oocyte maturation were compared in Table 1. Differences between the oocyte groups in terms of percentages of nuclear division stages of GV, GVBD and MI were non-significant (P>0.05). The percentage of oocytes in the MII stage was significantly higher in the MSCs group than the IVM medium + PVA group (P<0.05), while between MSCs, OECs and IVM medium + FCS it was non-significant (P>0.05) (Tab. 1). The matured oocytes in the four culture systems were fertilized and their embryo development was investigated (Tab. 2). The percentages (%) of cleavage and total blastocysts in MSCs and the IVM medium + FCS group were higher than in OECs and the IVM medium + PVA group (P<0.05). These rates were not significant between MSCs and the IVM medium + FCS group or between OECs and the IVM medium + PVA group (P>0.05). The percentages (%) of blastocysts and expanded blastocysts in MSCs and the IVM medium + FCS group were also higher than OECs and IVM medium + PVA groups, although they were non-significant (P>0.05). The percentage of hatched blastocysts (%) was significantly

increased in MSCs and the IVM medium + FCS group when compared to OECs and the IVM medium + PVA group (P<0.05). This rate in the IVM medium + PVA group was lower than in the other groups (P<0.05) (Tab. 2).

It has been confirmed that successful and reliable oocyte maturation would positively influence the efficiency of preimplantation embryonic development and subsequent fetal development [Sagirkaya *et al.* 2007]. In this study we cultured oocytes in four different media and confirmed again that not only some of the above-mentioned media had beneficial effects on oocyte maturation, but they also could improve the subsequent embryo development. In the present study the oocyte maturation in the serum-supplemented medium was considerably better than in free-serum medium or co-culture with oviductal epithelial cells. Application of serum as a protein source in culture media is common and its beneficial effects during oocyte maturation and embryonic development have been confirmed [Gordon 2003]. On the other hand, serum-supported media induced some harmful alterations in embryo ultrastructure and such abnormalities such as impaired compaction, abnormal blastulation, the large calf syndrome, aberrant mRNA expression profiles and greater incidences of stillbirths and deaths after birth have been observed [Abe *et al.* 1999, Holm *et al.* 2002, Wrenzycki *et al.* 2004]. In addition, undefined natural sera, e.g. fetal calf serum and estrus cow serum, might be carriers of zoonotic pathogens; thus, this type of protein sources has recently been avoided in the human IVP system [Sagirkaya *et al.* 2007]. For these reasons, there has been a trend to find a replacement for serum.

In the present study the effects on ovine oocyte maturation of mesenchymal stem cells and oviductal epithelial cells applied as feeder cells were compared with those of a serum-supplemented medium. This comparison confirmed that mesenchymal stem cells could be a satisfactory substitute of natural serum. In addition, supplementary evidence has been provided by cleavage rates, overall blastocyst and hatched blastocyst yields as indirect effects of these media. It must be stressed that the effect of oviductal epithelial cells was similar to that of mesenchymal stem cells in the case of oocyte maturation (MII stage), but its indirect effects on blastocyst developing stages were not comparable. In one study direct effects of mesenchymal stem cells, embryonic fibroblast cells and oviductal epithelial cells on the cleavage rate and blastocyst development were compared, with more positive effects found for mesenchymal stem cells during the early cleavage stages [Heidari *et al.* 2013]. As it has been noted above, mesenchymal stem cells release several trophic factors including cytokines and growth factors [Caplan and Dennis 2006, Park *et al.* 2010]. The trophic effects of these bioactive factors in the follicular growth and in vitro maturation of mouse oocytes have been shown [Ling *et al.* 2008]. In addition, it has been reported that mesenchymal stem cells can express high levels of anti-apoptotic signal molecules such as the X-linked inhibitor of apoptosis protein, Bcl-xL, Bcl-2, and heat shock protein-32. However, these advantages of mesenchymal stem cells could only partly justify their superiority in oocyte maturation and embryo development.

In conclusion, the effects of mesenchymal stem cells as co-culture on the oocyte maturation and the successive in vitro embryo development are similar to those of medium supplemented with FCS. This study suggests that co-culturing with mesenchymal stem cells may be a promising alternative to FCS-medium.

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