**Differential Influence of Ovine Oviduct Ampullary and Isthmic Derived Epithelial Cells on
in vitro Early Embryo Development and Kinetic**

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Abstract

Several critical physiological procedures occurred in oviduct during early embryo development. It is now well documented that *in vivo* there exist a great cross talk between early embryos and oviductal epithelial cells. For many years, the use of somatic cells for coculture with embryos has been investigated to improve *in vitro* embryo production (IVEP). The objectives of this study was to investigate; i) whether ampullary and isthmic derived epithelial cells have different effects on IVEP, ii) if by using different segments (2 equal segments) of isthmus in a sequential coculture system (2×2 Days culture period) we could improve IVEP outcomes, and iii) whether the different coculture systems accelerate the kinetics of blastocyst development. Results of the present study confirmed the positive and highly specialized effects of isthmic compared to ampullary derived epithelial cells on *in vitro* early development of ovine embryos (45.42%±4.01 vs. 36.62%±4.75 expanded blastocysts, 18.26%±1.00 vs. 10.22%±2.75 hatched blastocysts). The usage of isthmic derived epithelial cells as feeder cells in a coculture system compare to ampullary led to a significant ($P < 0.05$) increase in the total cells number of blastocysts (290.88±68.76 vs. 190.22±55.65). The kinetics of blastocyst development showed a significant increase in coculture system by using isthmic epithelial cells as the feeder cells compare to ampullary derived epithelial cells (25.62%±3.05 vs. 12.42%±1.00 blastocysts at day 6). Our results showed that by using different segments of isthmus in a sequential coculture system as the feeder cells did not improve the IVEP parameters concerning development or kinetic. In conclusion, we found for the first time that oviductal epithelial cells play the highly specialized role *in vitro*, same as their *in vivo* counterparts, in terms of development rate and kinetic.

Keywords: Ovine, Oviduct, Coculture System, Isthmic Epithelial cells, Ampullary Epithelial Cells

Abbreviations

in vitro Embryo Production, IVEP; *in vitro* Maturation, IVM, *in vitro* Fertilization, IVF; *in vitro* Culture, IVC; Phosphate buffer saline, PBS; Polyvinylpyrrolidone, PVP; TCM, Tissue Culture Medium; Cumulus Oocyte Complex, COC; Uterus-Tubal-Junction, UTJ; Ampullary-Isthmic Junction, AIJ; Synthetic Oviduct Fluid, SOF; Sequential Coculture System, SCS; Simple Coculture System-I, SCS-I; Simple Coculture System-II, SCS-II; Zygotic Genome Activation, ZGA; Ovine Oviduct Epithelial Cell, OOEC; Ovine Oviduct Ampullary Epithelial Cell, OOAE; Ovine Oviduct Isthmic Epithelial Cell, OOIE; Tyrodes-Albumin-Lactate-Pyruvate, TALP

1. Introduction

In case of *in vivo* reproduction specific organs have been developed that allow the introduction of both gametes to each other during copulation. These organs support all the physiological demands of gametes and early embryo(s). Oviduct and uterus are the crucial organs in reproductive system. There had long been thought that the oviduct serves as a passive conduit for the gamete and embryo(s) in the early reproductive events. Numerous studies have performed during last two decades showed that the oviduct is involved in several important processes that are necessary for the appropriate early embryo development (Lee and Yeung, 2006; Coy et al., 2008; Avilés et al., 2010; Besenfelder et al., 2012). The crucial functions of oviduct during gametes storage, maturation, fertilization, and early embryo development were bypassed by *in vitro* maturation (IVM), *in vitro* fertilization (IVF), and *in vitro* culture (IVC). The suboptimum culture conditions affect the normal embryo development. Therefore, the quality of *in vitro* cultured gametes and embryo(s) are far from their counterparts derived from *in vivo* condition (Watson et al., 2004).

Some studies showed that the oviductal fluid vary quantitatively and qualitatively during the oestrous cycle (Hunter, 2005; Smith, 2012). On the other hand, electron probe analysis revealed that there are differences in element composition between ampullary and isthmic fluid regardless of the stage of oestrous cycle (Roblero et al., 1976). In addition, histological studies have shown regional variations in ultrastructural features of the secretory cells in the oviductal epithelium (Abe, 1996). Regional variations have been found in the number of putative secretory granules in the oviductal secretory cells (Abe, 1996). Histochemical and immunocytochemical studies have also demonstrated regional differences in the localization of various proteins and growth factors in the oviductal epithelium (Abe, 1996).

Several studies have been conducted to evaluate various types of conditions in order to improve embryo development rate and quality *in vitro* (Smith, 2012). The first mammalian embryo culture systems used in domestic animal biotechnology were dependent upon coculture with helper cells in order to achieve embryo transfer (Smith, 2012). Several studies have indicated that embryo coculture systems improve embryo quality and result in better pregnancy rates than conventional culture systems. Coculture systems have been proposed to exert their effect through two different mechanisms: removal of toxic compounds and/or secretion of embryotrophic factors. Therefore, coculture as a practical system would be the best approach to ensure that all the needed proteins, growth factors, and other unknown elements are provided for early embryo development (Smith, 2012).

Early embryos displaced through the isthmic region of oviduct with a constant speed (Abe and Oikawa, 1991; Abe 1996). *In vivo* ovine early embryos are arrived to the uterus horn at day 4 post fertilization. Anatomical and histological data showed that 1/3 of oviductal length is belonging to isthmus segment (Abe and Oikawa, 1991; Abe, 1996). In recent years there are

growing evidence regarding a dialogue between early embryos and oviductal epithelial cells *in vivo* (Avilés et al., 2010). The proved cross talk between early embryo and isthmic epithelial cells and the constant speed of embryo displacement through isthmus led us to assume that different sub-domains of isthmic region can differentially affect ovine IVEP.

Transcription of the embryonic genome (zygotic genome activation, ZGA) occurs after fertilization and is critical for normal embryonic development. The initiation of ZGA varies depending on the species. In mouse, ZGA is observed as early as the S/G2 phase in the male pronucleus of the 1-cell zygote and becomes robust at the 2-cell stage, in human and pig the ZGA occur at 4–8- cell stage; while in cow and sheep it is delayed until the 8–16-cell stage (Li et al., 2013). When the ZGA occurred in sheep the early embryo displaced from ampullary-isthmic-junction (AIJ) to the middle of isthmic segment. In other words, the ZGA occurred during the first 2 days post insemination. Considering these facts it appears that the first half of isthmic region would be the exact site for ZGA. Therefore, a sequential coculture system that can reproduce an *in vitro* condition similar to its *in vivo* counterparts may increase the developmental rate and kinetic.

The purpose of the four different groups in this study was then to evaluate ovine IVEP outcomes and kinetics of blastocysts development in different schemes of coculturing conditions by using ovine oviduct ampullary (OOAE) and ovine oviduct isthmic derived epithelial (OOIE) cells. Furthermore, the present study aimed to reveal if different segments of oviduct (ampullary vs. isthmic) has any specialized role in early embryo development.

2. Materials and Methods

All the Chemicals and Reagents were purchased from Sigma (Germany), unless otherwise stated.
The biological materials were obtained from local abattoir, Karaj, Iran.

2.1. Preparation of Ovine Oviduct Epithelial Cells (OOECs)

OOECs were recovered as describe by Rottmayer et al. (2006) with some modifications. The reproductive tract was recovered after animal slaughter. All the connective tissues were trimmed from the selected oviduct. Before washing in phosphate buffer saline without $\text{Ca}^{2+}/\text{Mg}^{2+}$ (PBS-), each end of oviduct was tightly wrapped by a sterile surgical suture. Then the oviducts dipped in 70% ethanol before being transported on ice to the laboratory in PBS- supplemented with 2% penicillin–streptomycin solution. Following arrival to laboratory oviducts were dipped again in ethanol and washed in HEPES buffered TCM-199 under a laminar flow hood. The desired segments of ampulla and isthmus gently squeezed in a stripping motion with forceps to obtain OOECs. Recognition of different segments of oviducts was performed by considering the variations in oviduct diameter. Ampullary segment of oviduct has greater diameter compare to isthmus region. A yellowish paste that was contained OOECs was collected in culture medium TCM-199 supplemented with 2% estrous cow serum (ECS) and 0.25 mg/mL gentamicin. The cell suspension was pipetted 10-15 times with a 1000 μL filter tip (JET Biofil, Guangzhou, China). Then the cell suspension was being passed 5 times through a tuberculin 1 mL syringe was attached by 22 gauge needle. Following the washing procedure the cells suspension were evaluated under stereo microscope for the size of cells clusters. The two segments of isthmus was dissected and the epithelial cells were recovered at IVF day. Then 60 moving cells clusters of each specified segments seeded to a drop of 250 μL culture medium in a four-well plate separately. The cultured epithelial cells clusters were lived for the next 5 days. Then 30 moving

clusters were selected and transferred to 250 μ L drops of IVC medium. This procedure was done at least 6 h prior to IVC.

2.2. Ovary and Oocyte Recovery

Ovaries were obtained immediately from slaughtered animals. Following removal, ovaries were placed in an insulate container filled with physiologic saline solution (0.9% NaCl) supplemented with penicillin-streptomycin (100 μ g/mL Gibco), at 37 °C. The ovaries were transported to the laboratory within 2 h. The ovarian follicles were aspirated using an aspiration pump (MEDAP, Sekretrauger P7040; Germany), fitted with a disposable vacuum line (length 35 cm, internal diameter of 3 mm) and set at flow rates of 10 mL H₂O/min – with a disposable 20 gauge needle attached.

2.3. Oocyte *in vitro* Maturation

The medium used for IVM was TCM199 supplemented with 10% heat-inactivated fetal calf serum (FCS), 0.2 mM sodium pyruvate, 5 μ g/mL gentamycin, 10 μ g/mL FSH and 1 μ g/mL estradiol. 30 COCs were cultured in the drops of pre-warmed culture medium, covered with mineral oil for 24 h, at 38.5 °C in 5% CO₂ in air and 95% humidity.

2.4. Sperm Preparation, IVF, IVC, and Staining Procedure

In the present study, the initiation of IVF (introduction of sperm to IVF medium containing oocytes; day 0) was termed “insemination.” In brief, immediately following animal slaughter the testis (n=145) of Lory-Bakhtiary 2-3 year old rams (n=110) were removed and placed in a cool box set at 5°C and transported to the laboratory. At the laboratory the testis was washed three times in phosphate buffer saline (PBS) supplemented with 100 μ L/mL gentamicine at 5°C. All

the blood and connective tissues were removed aseptically at cold room, set at 5°C. For sperm recovery 1 mL tuberculin syringe attached with 22 gauge needle was inserted in to the Vas deference. The content of Vas deference was aspirated gently and the recovered spermatozoa were diluted 1:100 in Sperm-TALP. The samples were stored at refrigerator at 5°C for 24 h. At the day of use (day 0; IVF) progressive motility, and morphologic criteria for spermatozoa samples were evaluated under microscope. Samples with more than 60% progressive motility which had normal appearance were selected for IVF. Motile spermatozoa were separated from the samples using percoll gradient (45% over 90%). The fertilization medium included 12 mM KCL, 25 mM NaHCO₃, 90 mM NaCl, 0.5 mM NaH₂PO₄, 0.5 mM MgSO₄, 10 mM sodium lactate, 3 mg/mL bovine serum albumin (BSA; fatty acid free), 50 µg/mL gentamicin. At least 15 minutes prior to insemination oocytes were transferred to fertilization drops. At the time of insemination a portion (10 µL) of the preincubated spermatozoa was introduced into 90 µL of fertilization medium containing about 20 oocytes. The sperm concentration was adjusted to 1×10^6 /mL. Co-incubation of gametes was carried out for 18 h, at 38.5 °C, 5% CO₂ and 5% O₂. After co-incubation, spermatozoa attached to the ZP were removed from oocytes by gentle pipetting. The fertilized oocytes were transferred to synthetic oviduct fluid (SOF) as the IVC medium, which was subjected to addition of 30 moving groups of isthmic OR ampullary epithelial cells (Dadashpour Davachi et al., 2015); the embryo density was 1:4. Fifty embryos were cultured in a drop of 200 µL. At day 6, 7 and 8 after insemination the rate of embryo development to blastocyst stage was recorded.

For examining the IVC results, 7-8 days post insemination embryos were removed from culture medium and washed twice in phosphate buffer saline containing 1 mg/mL polyvinylpyrrolidone (PBS-PVP). Then embryos were fixed in 100 µL drop of paraformaldehyde solution [4% (w/v)

in PBS, pH 7.4], at room temperature. At the final step, embryos were removed from fixation drop and washed 3 times in PBS-PVP. Then all the embryos were stained for 15 minutes in 1 µg/mL Hoechst 33342. Finally, the embryos were evaluated under a fluorescent microscope with a UV filter. The scoring system used to evaluate the embryo quality was similar to that suggested by Saadeldin et al. (2011).

2.5. Experimental Design

In this study, 4 different groups were designed to evaluate the possible effects of the treatments. In each group 5 replicates had been considered. Table 1 shows the different groups design. Group 1: the isthmic region divided in to 2 equal segments. The first 4 days *in vitro* culture of presumptive zygotes divided in to 2×2 days coculture periods. At the first 2 days the first half of isthmic region was dissected. The epithelial cells were recovered as described in section 2.1. At the end of the first 2 days of coculture the second half of isthmic region was dissected and the epithelial cells was recovered and used for the second part of coculture (Zygotes number = 102). Group 2: the presumptive zygotes were cultured in a coculture system for 4 days. The feeder cells used for this part of the study were recovered from the whole isthmic segment (Zygotes number = 102). Group 3: the presumptive zygotes were cultured in a coculture system for 4 days. The feeder cells used for this part of the study were recovered from the whole ampullary segment (Zygotes number = 107). At the end of these 4 days, in all cases studied, the zygotes were removed from the drops of coculture and put in the fresh synthetic oviduct fluid for the rest 3.5 days of IVC. Group 4: as a control group presumptive zygotes were simply cultured in SOF for 7-8 days after IVF (Zygotes number = 97).

2.6. Statistical Analysis

Data are presented as mean $\pm$ SEM, and all percentages were modeled according to the binomial model of variables and arcsine transformation to achieve normal distribution. The variables in all of the groups were analyzed by one-way ANOVA. When ANOVAs revealed a significant effect, values were compared by the Tukey test. P value < 0.05 were taken to denote statistical significance.

3. Results

3.1. Developmental Competence and Kinetics

Different embryo culture systems were evaluated during this study. Embryo competence was determined as cleavage rate or blastocyst rate and total cell count per blastocyst at different periods following insemination (p. i.) (Tables 2 and 3).

The cleavage rate of SCS (90.23 $\pm$ 4.05), SCS-I (90.00 $\pm$ 3.01), and SCS-II (91.32 $\pm$ 4.15) groups were significantly higher than control (81.17 $\pm$ 4.00). The expanded (45.42 $\pm$ 4.01) and hatched blastocyst (18.26 $\pm$ 1.00) rate were increased in the SCS-I group compare with other culture systems (P<0.05) (Table 2).

At day 6 of development, blastocyst rate (25.62 $\pm$ 3.05) was significantly increased in SCS-I compare to other culture systems. The rate of blastocyst development was similar in SCS (10.22 $\pm$ 2.75) and SCS-II (12.42 $\pm$ 1.00) groups. However, at day 7 and 8 of development, blastocyst rates from SCS-I and SCS-II were significantly higher than control and SCS groups. However, the rate of blastocyst development for the SCS-I (45.42 $\pm$ 4.01) culture system was still significantly higher than SCS-II (36.62 $\pm$ 4.75) (Table 3).

The total cell count per blastocyst was significantly higher for SCS-I (290.88 ± 68.76) compared to other treatments (130.23 ± 41.23 , 190.22 ± 55.65 and 128.16 ± 43.15 respectively for SCS, SCS-II, and Control) (Table 3). Interestingly there was no significant difference for blastocyst cell count in SCS and control group (Table 3).

4. Discussion

Creation of the appropriate *in vitro* culture condition is the main goal for animal embryo biotechnologies. To our current knowledge, it is well documented that oviduct epithelial cells coculture led to production of better quality embryos in terms of blastocyst rate and morphology (Cordova et al., 2014). However, the possible specialized role of ampullary and isthmic derived epithelial cells and the sequential coculture systems, which can reproduce the embryo displacement, on embryo developmental rates and kinetics has still not been addressed yet. The present study investigated the embryo developmental rate and kinetic following simple culture as the control group compared with specialized coculture system and sequential coculture of IVP embryos during the first 4 days. Several types of coculture conditions have already been studied, including the use of somatic cells in coculture or media conditioned by these cells in order to achieve better embryo development rates.

The results of the present study indicate that the optimum feeder cells providing the best coculture condition required for proper embryo development would be the isthmus derived epithelial cells. However, this development depends not only on the kind of epithelial cells but also on the constant culture condition. Moreover, the results revealed that changing the epithelial cells in a coculture system after a period of 2 days of coculture in order to mimic the *in vivo* embryo displacement has several deleterious effects on embryo developmental rate and kinetics.

Therefore, different segments of isthmic region has no beneficial effects in terms of embryo development rate and kinetic.

At Day 6 p.i., when SCS, SCS-I and SCS-II were compared to control group, higher development rates were found ($P < 0.05$) for SCS-I. In contrast to the present research, a study conducted by Lonergan et al. (2003) showed that there were no differences in cleavage or developmental rates between *in vivo* and *in vitro* treatments at Day 6. In the present study the percentages of cleaved zygotes showed no significant differences between SCS, SCS-I, and SCS-II, but this parameter was significantly reduced for control group. The present study recorded a significant difference in developmental rate from Day 7 to Day 8, for the SCS-I compared to SCS, SCS-II, and control. At Day 8, no differences were recorded with regard to hatched and expanded blastocysts between SCS and control group. As stated above, no differences were observed with regard to cleavage rate between all the coculture systems. This could be explained as the general benefits of the coculture systems. In other words, it can be stated that both ampullary and isthmic derived epithelial cells can fully support the cleavage stage compare to control.

In terms of morula percentages, there were no significant differences between SCS and SCS-I, while these rate was significantly higher than the recorded results in SCS-II. However, morula rate was significantly higher compared with control group. When comparing the percentages of zygotes developed to morula, the specialized effects of isthmic derived epithelial cells were revealed compared with ampullary epithelial cells. In fact, it can be stated that the embryonic genome activation is highly dependent on the specified cross talk between embryo and isthmic derived epithelial cells. It is suggested that a specialized protein / growth factor could be produced by isthmic derived epithelial cells in response to the presence of embryo. This factor

may be produced at a basal level by both ampullary and isthmic derived epithelial cells; however, the optimum amount will only be produced when embryos are cocultured with isthmic derived epithelial cells. It can be stated that in SCS, embryos are exposed to osmotic and physical stress due to changing the epithelial cells and culture drops. The detrimental effects of this stress could be duplicated, when considering that the renewal of culture drops led to depletion of secreted proteins and growth factors from isthmic epithelial cells to the culture environment. Cordova et al. (2014) reported lower bovine blastocyst rate at Day 8 of coculture in relation to the results of the present study (30% vs 45%). Furthermore, embryo development rate and kinetic were extremely increased in SCS-I group in relation to the results reported in bovine (Thibodeaux et al., 1992), in Human (Kattal et al., 2008), and Sheep (Heyman et al., 1987). Results of the present study may indicate that during the period of the first 4 days of *in vitro* development, the isthmic derived epithelial cells provide almost all the necessary embryotrophic factors for the embryos to develop properly during their first stages of development and to pass the developmental block occurring mostly during in vitro conditions at the moment of embryonic genome activation. The production of this embryotrophic factors from ampullary derived epithelial cells in a coculture system did not stand at the optimum level to fully support early embryo development compare with isthmus coculture group. One of the possible explanations for the greater results in coculture groups compared with control group would be the ability of feeder cells to decrease the percentage of oxygen in coculture system.

Total cell number of blastocysts in SCS-I dramatically increased compare to other coculture systems and control group. This finding suggests that isthmic derived epithelial cells can fully support mitosis activity in blastocysts. However, total cell count was similar in SCS and control

groups; it is suggesting that isthmic epithelial cells can only exert their beneficial effects on early embryo development in a constant culture condition.

In conclusion, the present study confirmed the specialized role of isthmic derived epithelial cells on early embryo development as its *in vivo* counterparts. We showed that the presence of isthmic derived epithelial cells during the first 4 days of IVC (SCS-I) had greater effects on early embryo development compared with ampullary coculture system (SCS-II) and the new sequential coculture system (SCS).

Competing interest

The authors declare that they have no competing interests.

Authors' contributions

SD participated in laboratory practices. AZS and HK proof reading the manuscript, conceived of the study, and financial support for the research project. MZ participated in preparation of the manuscript. The study concept and creating the idea of the study was conducted by NDD, also NDD participated in the design of the study, writing the manuscript, final approval of the version to be published, and managing the IVF laboratory during the 2 years of the research. All authors read and approved the final manuscript.

Acknowledgments

We are thankful to Helia Shamsi, Payam Hatami, and Abas Godarzi for their assistance during the laboratory procedure. Also the authors would like to acknowledge the financial support of Iran National Science Foundation under grant number: 93020720.

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Table 1. The coculture system design are presented in this table

Culture System [*]	Feeder Cells	
SCS	Isthmus Derived Epithelial Cells	
	Desired Segment	
	First Half	Second Half
	Duration of Coculture (day)	
	2	2
SCS-I	Feeder Cells	
	Isthmus Derived Epithelial Cells	
	Desired Segment	
	Epithelial Cells Recovered from Entire of Isthmus	
	Duration of Coculture (day)	
	4	
SCS-II	Feeder Cells	
	Ampulla Derived Epithelial Cells	
	Desired Segment	
	Epithelial Cells Recovered from Entire of Ampulla	
	Duration of Coculture (day)	
	4	
Control	No Feeder Cell; zygotes were simply cultured in Synthetic Oviduct Fluid F for 8 days after IVF	

^{*} Sequential Coculture System, SCS; Simple Coculture System-I, SCS-I; Simple Coculture System-II, SCS-II

Table 2. Effect of Culture System on the Early Embryo Development

Culture System	Zygotes (n)	Cleaved zygotes % (n)	Morula % (n)	Blastocyst % (n)	
				Expanded	Hatched
SCS	102	90.23±4.05 (92) ^a	75.77±4.89 (78) ^a	27.42±4.01 (27) ^a	6.02±0.05 (6) ^a
SCS-I	102	90.00 ±3.01 (92) ^a	73.65±3.69 (76) ^a	45.42±4.01 (47) ^b	18.26±1.00 (19) ^b
SCS-II	107	91.32±4.15 (97) ^a	69.32±2.05 (74) ^b	36.62±4.75 (39) ^c	10.22±2.75 (11) ^c
Control	97	81.17±4.00 (79) ^b	59.12±3.05 (57) ^c	29.32±2.22 (28) ^a	5.02±0.75 (5) ^a

Different superscript in the same column differ significantly (P<0.05)

Table 3. Effect of Culture System on Blastocyst Development Kinetics

Culture System	Zygotes (n)	Blastocyst at day 6 % (n)	Blastocyst at day 7 % (n)	Blastocyst at day 8 % (n)	Total Cell Number (mean±SEM)
SCS	102	10.22±2.75 (10) a	18.32±1.98 (18) a	27.42±4.01 (27) a	130.23±41.23 ^a
SCS-I	102	25.62±3.05 (26) b	40.62±2.98 (41) b	45.42±4.01 (47) b	290.88±68.76 ^b
SCS-II	107	12.42±1.00 (13) a	30.52±2.75 (32) c	36.62±4.75 (39) c	190.22±55.65 ^c
Control	97	6.82±1.05 (7) c	22.52±2.65 (22) a	29.32±2.22 (28) a	128.16±43.15 ^a

Different superscript in the same column differ significantly (P<0.05)