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The effects of oviductal cell co-culture and different gas mixtures on the development of bovine embryos in vitro

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Abstract: The objective of the current study was to investigate the effects of the addition of oviduct co-culture to synthetic oviduct fluid (SOF) and Ménézo B2 media used in an in vitro study of cows, and the effects of different gas atmospheres (5% CO_2 or 5% CO_2, 5% O_2, and 90% N_2) on in vitro cultures. Oocytes obtained through aspiration from the ovaries of slaughtered Holstein cows were washed and cultured within a TCM-199 maturation medium at 38.8 °C for 23 h. Then the matured oocytes and thawed semen, prepared in accordance with the swim-up method, were incubated for fertilization within in vitro fertilization (IVF)-Tyrode's albumin lactate pyruvate (TALP) medium with an atmosphere including the combination of 5% CO_2, 5% O_2, and 90% N_2 gas, at 38.8 °C for 18-24 h. Fertilized oocytes were distributed into 3 main groups: Group I: B2 (5% CO_2), Group II: B2 medium (5% CO_2, 5% O_2, and 90% N_2), and Group III: SOF (5% CO_2, 5% O_2, and 90% N_2), and each group was divided into 2 subgroups, with and without oviduct co-culture cells, and were cultured for 9 days. The percentage of embryos that reached the blastocyst stage was 34.0%, 20.0%, and 32.3% in the co-culture group and 26.7% and 23.3% in the without co-culture group, respectively. Group III showed the highest development of expanded blastocyst stage ($P < 0.01$). In conclusion, the addition of co-culture to B2 (5% CO_2 atmosphere) and SOF (5% CO_2, 5% O_2, and 90% N_2 atmosphere) media increased the rate of transferable embryos.

Key words: Cattle, embryo, oviduct co-culture, in vitro, gas mixture

Introduction

The effects of in vitro maturation media on transferable embryos are not completely known. Despite extensive research in recent years, limited progress has been achieved with in vitro embryo production (1,2). Different media can include ions and energy substances at a wide range of concentrations. It has been reported that energy consumption and cellular adjustments can be required due to possible alterations in osmolarity and pH during in vitro culture (3). Various cell cultures and media (SOF, Cr1, B2, TCM-199 etc.) are used for the improvement of embryo development during in vitro culture studies (4-6).

Embryos require specific nutritional substances at various phases of embryonic development (7-

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9). Oviduct cells and high potassium concentration within the female genital tract play important roles in embryonic development (7,10). Therefore, oviduct cell cultures are widely used during in vitro culture studies (11,12). Although the oviduct cell co-culture supports the development of the embryo, it does not yield the same result in every medium, and it sometimes negatively affects embryo development, owing to the gas combination used (1,7,13). Today, many researchers widely use synthetic oviduct fluid (SOF) media during in vitro culture studies, which has similar characteristics to the oviduct (10,12,14). Many researchers report that the use of oviduct co-culture improves in vitro embryo development (11,12,15,16). Use of a co-culture passively influences the development of embryos at an early development stage, reducing the negative effects of toxic substances within the culture media (7,17). Moore and Bondiolo (18) used amino acids, bovine serum albumin (BSA), and co-culture cells instead of serum within the culture media and reported that 50% of the divided embryos had reached the morula phase on day 7 of the culture. They further mentioned that use of small quantities of fetal calf serum (FCS) and estrus cow serum (OCS) improved the development of differentiated cells, with consequently better cellular release of embryotrophic factors and embryo development (5,8,14,19). Rief et al. (15) reported that the SOF culture medium improved the development of oviduct cells within the co-culture. Therefore, more research is required for more efficient cultures, co-cultures (4,5), and gas mixtures (20) for in vitro bovine embryo production.

The objective of the present study was to determine the effects of different gas atmospheres and oviduct co-culture on the development capacity of developing embryos within SOF and Ménézo B2 (B2) media.

Materials and methods

Oocyte obtaining and maturation

Bovine ovaries from slaughtered Holstein cows were placed in a phosphate buffer solution containing 5% FCS at 35 °C and delivered to the laboratory within 2-3 h. Oocytes were obtained from the ovaries through aspiration. Oocytes with uniform zona, compact vitellus, and with 3-4 layers of cumulus oophorus cells were selected for maturation. The selected oocytes were washed 3 times with TCM-199 medium containing lactate, pyruvate, FCS, 50 µg/mL gentamicin sulfate, and 1 mM L-glutamine. They were then washed 2 more times with Hepes TCM-199 maturation medium containing 1 mM L-glutamine, 0.2 mM of sodium pyruvate, 50 µg/mL gentamicin sulfate, 24 IU/mL luteinizing hormone (LH), and 10% FCS. The washed oocytes were transferred to 4-chambered culture flasks containing 700 µL TCM-199 maturation medium and were incubated at 38.8 °C for 23 h in a gas mixture of 5% CO_2, 5% O_2, and 90% N_2. The procedure was repeated 7 times.

Fertilization and in vitro culture of oocytes

At the end of maturation, oocytes showing cumulus expansion were washed 2 times in the semen-Tyrode's albumin lactate pyruvate (TALP) medium for fertilization. The matured oocytes were then transferred to 4-chambered fertilization flasks including 400 µL of in vitro fertilization (IVF)-TALP medium. Frozen-thawed Holstein bull semen was used for fertilization. Frozen-thawed bull semen was separated by swim-up using sperm-TALP medium and the concentration of the semen was adjusted to 1×10^6 per mL (16). The matured oocytes were transferred into the fertilization flasks; each cell included 20-25 matured oocytes. The oocytes and spermatozoa were incubated for fertilization at 38.8 °C for 18-24 h under an atmospheric mixture of 5% CO_2, 5% O_2, and 90% N_2. At the end of the fertilization, embryos considered to be fertilized were transferred into tubes containing 1 mL TCM 199 washing medium in order to eliminate the surrounding cumulus cells and spermatozoa. The tubes were then subjected to mixing by means of vortex for 75 s and the embryos were washed 3 times. The washed embryos were distributed into 3 main culture groups: Group I: the fertilized oocytes were cultured in the B2 medium in a 5% CO_2 gaseous atmosphere; Group II: the fertilized oocytes were cultured B2 medium in 5% CO_2, 5% O_2, and 90% N_2 gas mixtures; and Group III: the fertilized oocytes were cultured in SOF medium in 5% CO_2, 5% O_2, and 90% N_2 gas mixtures. For each main group, 2 culture flasks were prepared for each culture. Each one of

these was cultured without the addition of co-culture (subgroups IA, IIA, and IIIA), while the others were cultured with the addition of the oviduct co-culture (subgroups IB, IIB, and IIIB). As a result, 6 subgroups were formed. The culture drops (100 µL) were prepared and covered with mineral oil. Each group was exposed to the corresponding gas atmosphere for 1 h.

Preparation of oviduct cells for co-cultures

Bovine oviducts that had been delivered to the laboratory at the same time as the ovaries were eliminated from surrounding connective, fat, and fascia tissues by washing with sterile phosphate buffered saline 3 times, without injuring the channel integrity. The channel epithelial cells were then transferred into 5 mL TCM-199 washing medium by means of compression, using a pair of forceps throughout the channel, beginning from the region of the uterotubal junction. The cells were centrifuged at 3000 × *g* for 5 min; the upper liquid portion was removed and new washing medium was added. After repeating the centrifugation and washing procedures 3 times, the upper liquid portion was removed and the epithelial cell palette at the bottom was obtained and transferred into 80 cm^2 culture flasks that included 20 mL B2 medium. The flask, including the oviduct cells, was incubated in an incubator containing 5% CO_2 gas for 48 h. At the end of the culture, 2 separate 5 mL samples were obtained from the suspension, including oviduct epithelial cells, and introduced into 2 tubes, and the tubes were centrifuged at 3000 × *g* for 2 min. The oviduct cells were centrifuged and washed 2 more times; the cell palette at the bottom of the suspension was obtained and transferred to small petri dishes including 2 mL SOF and B2 media.

Embryo culture

The embryos were inoculated, each culture drop containing 20-25 embryos. Added to each drop of every co-culture subgroup (groups IB, IIB, and IIIB) was 0.5 µL of co-culture suspension. The embryos were cultured in an incubator at 38.8 °C including different rates of gas combinations prepared corresponding to each of the groups, for 9 days. The chi-square test was used for statistical analyses.

Results

The results obtained are presented in the Table.

Table. Effects of oviductal cell co-culture and different in vitro culture systems on cleavage and development of bovine IVF embryos.

Groups	Total oocytes	Total cleaved oocyte	Total early blastocyst-blastocyst	Expanded blastocyst-hatching
Group IA B2, 5% CO_2 without cells	172	107/172 (62.2%)^{a**}	30/107 (28.0%)^{ab}	0.0 (0%)^{d}
Group IB (B2, 5% CO_2 with cells)	172	120/172 (69.8%)^{ab}	41/120 (34.2%)^{b*}	15 (12.5%)^{ef}
Group IIA B2, 5% O_2, 5% CO_2, and 90% N_2 without cells	182	131/182 (72.0%)^{ab}	35/131 (26.7%)^{ab}	1 (0.8%)^{d}
Group IIB B2, 5% O_2 and 5% CO_2 with cells	198	155/198 (78.3%)^{bc**}	31/155 (20.0%)^{a*}	14 (9.0%)^{e}
Group IIIA SOF, 5% O_2 and 5% CO_2 without cells	199	146/199 (73.4%)^{bc}	34/146 (23.3%)^{ac}	11 (7.5%)^{e}
Group IIIB SOF, 5% O_2 and 5% CO_2 with cells	216	158/216 (73.1%)^{bc}	51 (32.3%)^{bc}	26 (16.5%)^{f}

^{abc}: Rates with different letters in the same column are statistically significant in bovine IVF embryos ($P < 0.05$, def: $P < 0.01$, *: $P < 0.01$, and **: $P < 0.001$).

Discussion

Embryos utilize chemical substances within culture media at different rates according to the phase of their development (7,19). It is known that embryos at the cleavage stage require different gas atmospheres, energy substrate depending on the chemical composition of the medium, and co-culture (7,16,21). It was observed that for the B2 medium with or without the co-culture, the gas combination of the culture atmosphere did not affect the cleavage, expansion, or hatching rates of the oocytes ($P > 0.05$). Furthermore, for the B2 media without the co-culture (groups IA and IIA), their development at the early-blastocyst and blastocyst phases was not affected by the gas ratios; however, this was not the case for the co-culture combined groups (groups IB and IIB; 34.2% and 20.0%, respectively, $P < 0.01$). These results show that the gas combination had a significant effect on development at the early-blastocyst and blastocyst phases, regardless of the use of the same medium and the same culture environment. Embryonic development can be negatively affected in cases of high oxygen consumption by co-cultures used in the B2 medium, and when there is insufficient oxygen in the medium (7,16,19). Similarly, it has been reported that the positive effects of co-culture cells on the embryos can be reversed to negative effects when a suitable culture medium is not provided for the embryos (7,15,16).

In this study, the rates of embryo development within the B2 and SOF media excluding co-culture, with the same gas combination, at the early-blastocyst and blastocyst phases were similar (groups IIA and IIIA), while the rates of embryo development at the expanded blastocyst-hatching phase were significantly higher in the SOF medium (0.8% and 7.5%, respectively, $P < 0.01$). With the addition of co-culture to the medium (groups IIB and IIIB), the rates of both the embryo development at the early-blastocyst and blastocyst phase (20.0% and 32.3%, $P < 0.01$) and the expanded-blastocyst-hatching phase (9.0% and 16.5%, $P < 0.05$) in the SOF medium were significantly higher, compared to those of the B2 medium (Table). It has been reported that the media include ions and energy substances in various concentrations, and that the embryos consume these substances and the embryonic wastes alter the media osmolarity and pH (3). The co-culture used in the SOF medium, assumed to carry similar characteristics to oviduct composition, eliminates the toxic and inhibitory effects of metabolic wastes of the embryos and improves embryonic development by releasing embryotrophic factors (7,16,22). However, little is known about the way the oviduct co-culture affects embryo development. Various researchers have reported very different suggestions with respect to obtained results (3,23,24). While some researchers advocate that the addition of an oviduct co-culture to SOF medium to improve embryo development is not necessary, some others suggest that a co-culture addition has positive effects on embryo development (15,24). In the present study, the rates of embryo development at the early-blastocyst and blastocyst phases of the co-culture-treated and untreated subgroups of each of the main groups (I, II, and III) were found to be similar. However, the rates of embryo development at the expanded-blastocyst and hatching phase in the co-culture-added subgroups were significantly higher than those of the co-culture-excluded subgroups ($P < 0.001$). These data show that the addition of co-culture to the medium increases the rates of embryo development at the expanded-blastocyst and hatching phases.

When considering the results of the co-culture addition to the B2 medium and incubations with different gas atmospheres in general, it can be seen that the highest rates of embryo development at the early-blastocyst, blastocyst, and expanded-blastocyst-hatching phases were obtained in the IB group. When evaluating the results of B2 and SOF use with the same gas atmospheres (5% O_2, 5% CO_2, and 90% N_2), it can be seen that the highest rates of embryo development at the early blastocyst, blastocyst, and expanded-blastocyst-hatching phases were obtained in the IIIB group (Table). These data show that the rates of transferable embryos in cows would be positively affected when: a) a B2 medium is used with 5% CO_2 gas atmosphere along with the addition of a co-culture, and (b) when SOF is used instead of the B2 medium, with a 5% O_2, 5% CO_2, and 90% N_2 gas combination, with the addition of the co-culture (Table). It has been reported that higher pregnancy rates are obtained as a result of the transfer of embryos obtained using co-culture, which is consistent with the aforementioned suggestion (8).

In summary, we conclude from this study that the addition of co-culture to B2 and SOF media, and the use of 5% CO_2 atmosphere in the in vitro culture will increase the rate of transferable embryos.

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