Original Article

Differential Effect of Medium on the Ratio of ICM/TE of Bovine Embryos in a Co-culture System

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Abstract

Background: This study was undertaken to investigate the efficiency of two different embryo somatic cell co-culture conditions, tissue culture medium 199 (TCM199)–vero cells and Menezo B2 (B2)-vero cells, for the *in vitro* developmental quantity and quality of bovine embryos.

Materials and Methods: Bovine oocytes were allowed to mature and subsequently undergo fertilization *in vitro*. Their presumptive zygotes were cultured in either TCM199 or B2 culture media in conjunction with vero cells for up to nine days. The culture media were refreshed every two days and the proportion of embryos that cleaved and further developed to the morula and blastocyst (early, expand and hatched) stages were recorded. Hatched blastocysts underwent differential staining in order to determine the numbers of inner cell mass (ICM) and tropho ectoderm (TE) and total cell number (TCN).

Results: Of the two groups, no significant difference was seen between the proportions of the presumptive zygotes cleaved, those which developed to 8-16 cells, morula and reached days 7or 8 blastocyst stage or hatched. However, the values for TCN and TE of the TCM199-vero embryos were significantly greater than those of B2-vero embryos. The values for ICM/TCN and ICM/TE were significantly greater in the B2-vero group versus the TCM199-vero group.

Conclusion: Both TCM199 and B2 culture media in conjunction with vero cells were of the same efficiency when used for *in vitro* development of bovine presumptive zygotes. However, TCM199 was superior in providing embryos with more embryo cell numbers, whereas B2 medium was superior in providing embryos with greater ICM/TE and ICM/TCN ratios.

Keywords: *In Vitro* Fertilization, Embryo Culture Techniques, Co-culture

Introduction

In vitro embryo production (IVP) is a reproductive biotechnology with great potential for both medical and agricultural purposes. However the overall credibility of the IVP system, as measured by the percentages of embryos produced per the numbers of *in vitro* cultured oocytes as well as the percentages of live offspring from the numbers of embryos transferred have lagged behind those of in vivo counterparts (1-3). Although the exact mechanism(s) of this discrepancy is not completely understood, it seems that the culture condition in which the early embryos develop has a remarkable effect on the developmental competence of the cultured embryos (4, 5).

In vivo, cleavage stage embryos develop within the oviduct which contains a highly dynamic environment due to the contraction of the oviduct and differential secretions in various parts of this structure (6). *In vitro*, the basis of the embryo culture medium is designed considering the compositional analysis of the oviductal fluid. However, the absence of the oviductal cells adversely affects the pattern of embryo interaction with its surrounding environment; a situation which seems to be one important reason for culture medium inefficiency. Therefore, some studies have proposed the co-culture of embryos

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with oviductal epithelial cells to overcome this problem (7-9).
Given the difficulties regarding isolation of oviductal epithelial cells, in addition to experimental inconsistency and possible infectious hazards, numerous studies have attempted to substitute oviductal epithelial cells with different types of somatic cells, including cumulus cells (10, 11), granulosa cells (12), and buffalo rat liver cells (13, 14). Among the somatic cell used for co-culture system, the African green monkey (Ceropithiops aethiops) kidney epithelial cells or vero cell lineage is of interest due to a number of advantages, including a common embryonic origin (mesoderm) with the early embryo, lack of heterogeneity and ease of proliferation in the culture condition (15, 16).
However, different types of culture medium have differential effects on both the profile and extent of trophic factors secreted by somatic cells cultured *in vitro* (10, 13). Accordingly, despite the large number of studies performed on the usefulness of somatic cells for *in vitro* embryonic development, there is no conclusive report about the effect of culture medium-somatic cell interactions on the efficiency of *in vitro* embryo production. These studies are essential to address the most suitable embryo culture medium for matched somatic cells.
Tissue culture medium 199 (TCM199 - Gibco) and B2 medium (CCD, Paris, France) are the two commercially available culture media routinely used for *in vitro* culture of mammalian embryos (17, 18). Therefore, the aim of this study is to compare the efficiency of TCM199 or B2 media with vero cells on preimplantation development of bovine embryos.

Materials and Methods

Unless otherwise specified, chemicals and media were obtained from Sigma (St. Louis, MO, USA) and Gibco (Life Technologies, Rockville, MD, USA) companies, respectively.
This study received the approval of the Ethical Committee of Royan institute.

Oocyte collection, in vitro maturation and in vitro fertilization

These procedures were carried out as described previously (19). In brief, ovaries were obtained from cattle and transported in warm saline (35°C) to the laboratory. After thorough washing, the contents of transparent antral follicles (2 to 8 mm in diameter) were aspirated using an 18 gauge needle attached to a vacuum pump (80 mmHg pressure) and deposited into 10 ml conical tubes for sedimentation. The aspirant was then depleted into 12 cm petri dishes and with a stereomicroscope, cumulus oocyte complexes (COCs) which contained homogenous cytoplasms and at least three surrounding cumulus cells were collected. COCs were thoroughly washed in 200 µl droplets of Hepes buffered TCM199 plus 10% fetal calf serum (FCS) and once in 100 µl droplets of maturation medium. Subsequently, groups of 8-10 COCs were cultured in 50µl droplets of maturation medium in the presence of vero cell monolayers. The maturation medium was composed of TCM199 supplemented with 10% FCS, 10µg/ml luteinizing hormone (LH), 10µg/ml follicle stimulating hormone (FSH) and 1µg/ml 17-βestradiol. COCs were incubated at 39°C, 5% CO_2 and maximum humidity for 24 hours. Following *in vitro* maturation, COCs with expanded and modified cumulus cells were selected and washed three times in fertilization medium. Groups of 40-50 COCs were then allocated in 200 µl droplets of fertilization medium. Two 0.25 ml straws which contained the semen of two bulls with proven fertility were quickly thawed at 37°C and the contents of the straws were centrifuged at 1200 rpm for 10 minutes. Motile spermatozoa were obtained by the wim-up procedure as described by Parrish et al. (20). Washed and prepared spermatozoa were then loaded into the fertilization droplets at a final concentration of 1×10^6/ml. After co-incubation for 18-20 hours at 39°C in 5% CO_2, oocytes were removed from the spermatozoa and cumulus cells, and washed twice with *in vitro* culture medium (IVC) to prepare them for *in vitro* culturing.

Vero cell preparation, in vitro culture and experimental design

Three frozen cryovials of an established vero cell line were obtained from Royan Institute (www.royaninstitute.org) and used for the entire study as described elsewhere (19). In brief, after the cryovials were thawed, the washed and centrifuged cells at a concentration of 1×10^6/ml were cultured in 3 cm^2 culture dishes (Falcon) which contained DMEM medium supplemented with 10% FCS at a temperature of 38.5°C and 5% CO_2 in humidified air. The culture medium was refreshed each two days. The confluent dishes were trypsinised (0.25% trypsin), and the detached and singled cells were either sub-cultured (to sustain the reserve cell source) or used for monolayer preparation. For the latter purpose, cells were diluted in the appropriate amount of

either B2 or TCM199 plus 10% FCS to make a final concentration of 2×10^5/ml. Embryos were cultured in micro droplets (50 µl), covered with mineral oil and incubated at 38.5°C, and 5% CO_2 in humidified air. After 24 hours, the medium was replaced with fresh medium and preincubated for at least 2 hours prior to embryo culturing. For *in vitro* culture, presumptive zygotes were randomly allocated in TCM199-vero or B2-vero culture conditions in 5% CO_2, 5% O_2, and humidified air. Embryos were refreshed into new dishes each two days where, concurrently the numbers of embryos that cleaved and developed into 8-16 cell, morula, blastocyst and hatched blastocysts stages were recorded.

Differential embryo staining for quality assessment

In order to determine the total cell number (TCN) and the sole number of cells allocated in the sites of inner mass (ICM) and trophectoderm (TE), the hatched blastocysts in both groups were assigned to differential staining as described by Hosseini et al. (21) with minor modifications. In brief, hatched blastocysts were incubated in 500 µl of 1% triton X-100 and 100 µg/ml propidium iodide (solution 1) for up to 30 seconds, depending on the size of the embryos, and then immediately transferred into a 500 µl solution of 100% ethanol plus 25 µg/ml Hoechst 33258 (solution 2). Care was taken to carry the minimum amount of solution 1 when the embryos were transferred into solution 2. The embryos were extensively washed in solution 2 to remove any trace amounts of solution 1. Samples were then stored in solution at 4°C overnight. Fixed and stained embryos were subsequently mounted onto a glass slide in one drop of glycerol, gently flattened with a cover slip and visualized for cell counting on a fluorescence microscope (excitation filter 460 nm for blue and 560 nm for red). TE cells were visualized as blue and ICM as pink to red. TCN was calculated by counting the numbers of both ICM and TE.

Statistical analysis

The analysis of variance (ANOVA) procedure was used for data analysis. The mean of treatments were compared with Duncan's multiple range test at a 0.05% probability level. Chi-square test was also used for comparison between different treatments with regards to differential staining.

Results

From 450 abattoir-derived ovaries, 3500 COCs were selected for *in vitro* maturation. There were 3028 oocytes subjected to *in vitro* fertilization which were further cultured up to nine days. Table 1 indicates the overall results of *in vitro* embryo development of the presumptive zygotes cultured upon the monolayer of vero cells in either TCM199 or B2 medium. As depicted in this table, the cleavage rates of the TCM199 and B2 groups were 76.9% and 81.2%, respectively which were insignificantly in favor of the B2 group ($p>0.05$). The percentages of embryos that attained the 8-16 cell stage were 54.1% for TCM199 and 67.1% for B2 culture systems, again insignificantly in favor of the B2 medium ($p<0.05$). The ratios of TCM199 and B2 groups of embryos that further developed into morula (42.5% vs. 39.3%) and days 7 (23.8% vs. 25.7%), 8 (30.3% vs. 29.0%) and 9 (31.7% vs. 27.7%) blastocysts were not statistically significant ($p> 0.05$).

The overall percentages of hatching were 40.3% and 45.7% for the TCM199 and B2 embryo culture media which were not significant ($p>0.05$).

Differential staining of the embryos (Table 1 and Fig 1) indicated that the TCN of blastocysts that developed in the TCM199 group (338.5) were significantly greater than the B2 group (263), ($p<0.05$). Moreover, although the ICM rates of both groups were not significantly different; the TE mean number of the TCM199 group was 266.75 which were significantly greater than the B2 group (189.5).

Table 1: In vitro developmental competence of bovine presumptive zygotes cultured in TCM199-vero cells and B2-vero cells

Proportion of presumptive zygotes developed to blastocyst stage								
					Blastocyst			
Culture conditions	**Oocyte (n)**	**Cleaved**	**8-16 cells**	**Morula**	**Day 7**	**Day 8**	**Day 9**	**Hatched**
TCM199-vero cells	1528	76.9	54.1	42.5	23.8	30.3	31.7	40.3
B2-vero cells	1500	81.2	67.1	39.3	25.7	29.0	27.7	45.7

There was no significant difference ($p\leq0.05$) between the proportions of different bovine preimplantaion embryos cultured in TCM199-vero cells or B2-vero.

Consequently, the ratios of ICM/TCN and ICM/TE in the TCM199 group were 18.83% and 23.89% which were significantly lower than the related rates of the B2 group (27.9% and 38.78%, respectively).

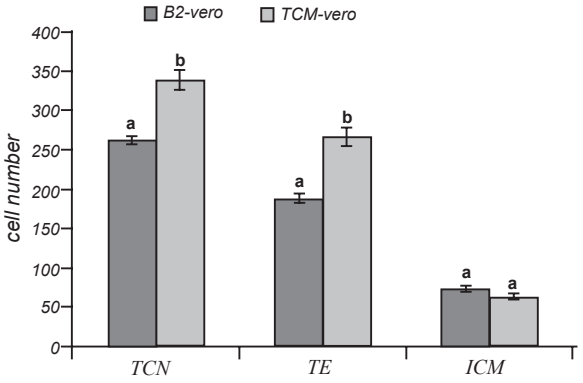

Fig 1: Assessment of the cell numbers of hatched blastocysts developed in TCM199-vero cell and B2-vero cell culture conditions. Different letters within the same column shows significant differences among the groups (p≤0.05).

Table 2: Quality assessment of hatched blastocysts developed in TCM199-vero cell and B2-vero cell culture conditions.

	TCM199-vero cells	B2-vero cells
ICM/TCN (%)	18.83 [a]	27.9 [b]
ICM/TE (%)	23.89 [a]	38.78 [b]

TCN , ICM and TE represents the total cell number, inner cell mass and trophoectoderm respectively.(a, b) different letters within the same row shows significant differences among the groups (p≤0.05).

Discussion

Embryo somatic cell co-culture, a conventional state-of-the art assisted reproduction technique, involves the placement of the embryos on a layer of cells. These cells have been preferentially extracted from the genital tract to create a more natural environment for embryonic development *in vitro*, thereby enhancing their further competency for both *in vitro* and in vivo development (19, 22). With regards to human assisted reproduction clinics, this unique approach not only has been traditionally devised and applied, however, more recently there is a new trend to utilize autologus or even heterologous co-culture systems, particularly for "poor prognosis" patients (23).

The results of this study indicated that when cultured over a vero cell monolayer, the overall potency of bovine presumptive zygotes to cleave and further develop until the blastocyst stage was not significantly different between the TCM199-vero and B2-vero groups. Notably, despite some minor differences between the rates of development, the overall percentages of embryo development were similar, irrespective of the type of media used. In a similar experimental design, Farin and co-workers assessed the developmental potential of embryos cultured in either TCM199 or B2 cultured medium using buffalo rat live cells (BRL) (17). In contrast with our results, they determined that the B2-BRL culture condition was significantly more advantageous when compared to TCM199. Therefore, two rationales exist for this discrepancy:

a) the type of cells established for co-culturing in these two studies were different; BRL (17) and vero cells (our study), and b) all cultures in the Farin et al. study were left undisturbed until day eight, whereas in our study the embryos were replated onto newly prepared dishes every two days. Thus, based on available reports in addition to our previous experiences, somatic cells in a continuous culture consume available nutrient elements and deplete the media of a wide range of substrates necessary for normal embryo development (19). Moreover, it is reported that stressful conditions such as trypsinisation and medium refreshment stimulate vero cells to release proteins beneficial for embryo development. Therefore, the presence of vero cells and the method of embryo culture employed in this study may have some advantages of B2 medium over TCM199.

Differential staining of the embryos indicated that the TCN of blastocysts which developed in the TCM199 group (338.5) was significantly greater than the B2 group (263), ($p<0.05$). Moreover, although the ICM rates of both groups were not significantly different; the TE mean number of the TCM199 group (266.7) was significantly greater than the B2 group (189.5). Consequently, the ratios of ICM/TCN and ICM/TE in the TCM199 group (18.83% and 23.89%, respectively) were significantly lower than the related rates of the B2 group (27.9% and 38.8%, respectively). TCN have been suggested as an important criterion to determine embryonic capacity for post-implantation embryo development (24). Accordingly, the results of Table 2 clearly indicate that development in TCM199 significantly increased the proliferation rate of the blastomeres of the embryos when compared with B2 medium.

AS shown in table 2, while the TE number of the blastocysts is greatly higher in TCM199 media relative to B2 media, the ICM numbers of the two groups are not significantly different.

ICM is the sole source of cell that participates

in the embryonic tissue formation while TE participates in placentation, it is unclear if having a larger ratio of TE to ICM is favorable for future embryonic and fetal development or not.
Although the beneficial effects of embryo somatic cell co-culture have been reported (19), the exact mechanism by which feeder cells support embryo development is not completely understood. It is possible that somatic helper cells may either remove potentially toxic factors that are present or produced in the culture medium, or secrete a wide range of trophic factors such as leukemia inhibitory factor (LIF) and growth factors.

Conclusion

In conclusion, the results of this study indicate that the use of vero cells as helper cells does not show a significant difference in the total number (proportion) of cleaved embryos that develope to both the advanced morula stages and blastocysts in either B2 or TCM media.
The results indicate that the number of cells per embryo at the blastocyst stage had a greater TCN for embryos developed in TCM199 medium relative to B2 medium. However, the ratio of ICM/TE and ICM/TCN of the embryos grown in B2 medium were significantly greater that those grown in TCM199 medium. The results of this study indicate that further studies are required in order to research the observed effects on post-implantation development.

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