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Equine bone marrow mesenchymal or amniotic epithelial stem cells as feeder in a model for the *in vitro* culture of bovine embryos

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Summary

Various studies have shown that the *in vitro* culture environment is one of the key determinants of the blastocyst output. In the present study we investigated the effects of co-culturing bovine embryos with equine bone marrow mesenchymal stem cells (BM-MSCs) or equine amniotic epithelial stem cells (AE-SCs) on *in vitro* blastocysts development. BM specimens were obtained aseptically from sternal aspirates of horses under local anaesthesia and the isolated cells were resuspended in Dulbecco Modified Earle's Medium supplemented with 10 ng/ml of basic fibroblast growth factor (bFGF). Amniotic membranes were obtained from fresh placentas and, to release the AE cells, amniotic fragments were incubated with 0.05% trypsin for 45 min. Separated AE cells were plated in standard culture medium containing 10 ng/ml epidermal growth factor (EGF). Seven hundred and five cumulus–oocyte complexes were used and, after IVM and IVF, cumulus-free presumptive zygotes were randomly transferred into one of three co-culture systems in which they were cultured up to day 7: (1) co-culture with cumulus cells (control); (2) co-culture with BM-MSCs; and (3) co-culture with AE-SCs. Statistical analyses were performed by ANOVA. Blastocyst developmental rates were significantly different ($p < 0.001$) between control, AE-SCs and BM-MSCs (respectively 35.45, 41.84 and 30.09%). In conclusion, the AE-SC monolayer create a more suitable microenvironment necessary for inducing local cell activation and proliferation of the growing embryos in comparison with BM-MSCs and cumulus cells. It can be suggested that these cells secrete biologically active substances, including signalling molecules and growth factors of epithelial nature, different to those of the BM cells of mesenchymal origin.

Keywords: Amniotic cells, Bovine embryos, Co-culture, Horse, Mesenchymal cells

Introduction

In the intervening five decades the ability to maintain the early mammalian embryo in culture has increased significantly, culminating in higher pregnancy rates post-transfer, but the reality of working *in vitro* is that embryos are exposed to several stresses they do not experience *in vivo*. A direct result of stress *in vitro* is compromised embryo physiology, gene expression and development (Gardner & Lane, 2005). Some researchers reported that the embryo development *ex vivo* is associated with developmental delay (Harlow & Quinn, 1982; Li *et al.*, 2007) and that the culture system and the composition of the medium can affect embryo quality (Abe *et al.*, 2002; Rizos *et al.*, 2002). In fact, while the innate quality of the oocyte is the major factor that determines the blastocyst yield, there is considerable evidence supporting the notion that the post-fertilization culture environment is critical in determining the quality of the blastocyst (Rizos *et al.*, 2002, 2003; Costa Pereira *et al.*, 2005; Lonergan, 2006). Embryo development *in vitro* is influenced by a number of factors, such as co-culture with somatic cells (Donnay *et al.*, 1997), addition of anti-oxidants to the culture medium (Ali *et al.*, 2003), oxygen tension (Van Soom *et al.*, 2002) and the number of embryos present in the culture drop (Hendriksen *et al.*, 1999; Gardner

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& Lane, 2000; Vajta *et al.*, 2000; Costa Pereira *et al.*, 2005).

However, it should be noted that, with the appropriate media and the appropriate laboratory systems, it is possible in the mouse model to obtain cleavage rates similar to that *in vivo* (Gardner & Lane, 2003). Furthermore, in human medicine, it is actually possible to establish 80% pregnancy rates with a donor oocyte model, indicating that, across several mammalian species, culture conditions can now maintain high rates of embryo viability (Gardner, 2008).

In the horse, the development of assisted reproductive technologies is relatively slow compared with other domestic species. Recently, several studies have attempted to improve the *in vitro* culture of embryos (IVC) in horse but, unlike other mammalian species, as ruminants and pigs, its efficiency remains very low. A comparison between the published reports on *in vivo* culture of ICSI early cleavage stage embryos in the oviducts of mares (Choi *et al.*, 2006a) or temporary recipient sheep (Galli & Lazzari, 2001; Galli *et al.*, 2000, 2002; Lazzari *et al.*, 2002) and *in vitro* culture in various culture media, clearly demonstrated that the *in vivo* environment supports high blastocyst development, approximately 36% of injected oocytes in both the mare and sheep oviducts (Galli *et al.*, 2007). *In vitro*, many different culture conditions have been reported for preimplantation development of ICSI fertilized horse oocytes, including defined media such as G1.2 (Choi *et al.*, 2001), DMEM-F12 and CZB (Choi *et al.*, 2006b) and modified SOF (Galli *et al.*, 2002). Earlier work evaluated co-culture with somatic cells including Vero cells (Dell'Aquila *et al.*, 1997), oviduct epithelial cells (Battut *et al.*, 1991), cumulus cells (Li *et al.*, 2001), granulosa cells (Rosati *et al.*, 2002) or culture in conditioned media (Choi *et al.*, 2001) but in most of these systems the blastocyst rates remained low, ranging from 4 to 16% (Galli *et al.*, 2007).

In some mammalian species, somatic cell co-cultures improved the *in vitro* development of embryos (White *et al.*, 1989; Rexroad & Powell, 1993; Park *et al.*, 2000; Li *et al.*, 2001) probably, by means of secreted embryo-trophic factors during co-culture. In this view, the co-culture with mouse embryonic fibroblasts improved the development of bovine and ovine embryos (Rexroad & Powell, 1993; Park *et al.*, 2000). Embryonic fibroblast cells may secrete various factors that enhance embryonic development (Park *et al.*, 2000; Hatoya *et al.*, 2006).

The effect of co-culturing with amniotic epithelial stem cells (AE-SCs) or bone marrow mesenchymal stem cells (BM-MSCs), on IVC of horse embryos or other species, has not yet been studied. In the present study, we investigated the feasibility of using horse AE-SCs and BM-MSCs as feeder to support embryo development *in vitro*, employing the bovine embryo as a model to standardize a protocol for the culture of equine embryos.

Materials and methods

Chemicals, unless otherwise indicated, were purchased from Sigma Aldrich, Milano, Italy.

Isolation and culture of amniotic epithelial stem cells (AE-SCs)

From three samples of allantoamnion retrieved at delivery, each amniotic membrane was stripped from the overlying allantois and, for isolation of the epithelial cells, it was digested with 0.05% trypsin for 45 min at 37 °C. Finally, the cells were washed in high glucose Dulbecco's Modified Eagle's Medium (DMEM-HG, CELBIO Euroclone) plus 10% fetal calf serum (FCS), centrifuged at 300 *g* for 10 min and counted before plating.

Isolated cells were plated on 25 cm^2 flask in DMEM-HG supplemented with 10% FCS, 100 U/ml penicillin, 100 μg/ml streptomycin, 0.25 μg/ml amphotericin B, 2 mM/l L-glutamine and 10 ng/ml of epidermal growth factor (EGF).

Amniotic stem cells were previously characterized as published in Lange Consiglio *et al.* (2010).

Isolation and culture of bone marrow mesenchymal stem cells (BM-MSCs)

Bone marrow samples were collected using sterile conditions from three horses under sedation (mean age of 7 years old).

Bone marrow aspirates were obtained from the sternum by using a Jamshidi biopsy needle (10 cm; 11G) into 12500 UI/ml heparin preloaded 60 ml syringes. The total bone marrow volume collected from each horse was about 30 ml. MSCs isolation from bone marrow consisted of layering over Histopaque™ 1.080, centrifuging for 20 min at 400 *g* at 4 °C, collecting the cell buffy coat, washing two times in phosphate-buffered saline (PBS) and plating mononucleated cells in DMEM-HG supplemented with 10% FCS, 100 U/ml penicillin, 100 μg/ml streptomycin, 0.25 μg/ml amphotericin B, 2 mM L-glutamine and 10 ng/ml of basic fibroblast growth factor (bFGF).

Bone marrow stem cells were previously characterized as published in Cremonesi *et al.* (2008).

Isolation and culture of cumulus cells (control)

Cumulus cells remaining attached to the plastic culture dishes after *in vitro* maturation (IVM) of bovine cumulus–oocyte complexes were cultured at 38.5 °C,

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5% CO_2 in air until starting of the *in vitro* embryo culture, when the IVM medium was replaced with the embryo culture medium.

Preparation of feeder layers

The primary AE-SCs and BM-MSCs were cultured until confluent and thereafter, proliferated through two subsequent passages. These cells were then frozen at −80 °C using 10% of dimethylsulfoxide (DMSO). Frozen cells were thawed in water at 37 °C and re-seeded at a density of 1.0×10^4 cells per 100 µl droplet in IVC plates. The AE-SCs and BM-MSCs were plated 1 day before IVM and formed monolayer within 3 days of preparation, while the cumulus cells were ready 42 h after the beginning of the IVM.

In vitro production of bovine embryos

Cumulus–oocyte complexes (COCs) were collected from ovaries obtained from an abattoir by aspirating follicles 2–8 mm in diameter and washing them twice in preincubated (38.5 °C, 5% CO_2 in air) TCM 199-HEPES buffered culture medium supplemented with 10% FCS.

IVM was performed for 24 h in TCM199 Earle's Salt medium supplemented with 10% FCS, 5 µg/ml luteinizing hormone (LH) (Lutropin, Bioniche), 0.5 µg/ml follicle stimulating hormone (FSH) (Folltropin, Bioniche), 0.2 mM sodium pyruvate, 10 µg/ml gentamycin and 1 mg/ml estradiol 17β. Cultures were in 100 µl droplets (up to 20 oocytes/droplet) of the medium under oil, at 38.5 °C in 5% CO_2.

In vitro fertilization (IVF) was performed in TALP medium containing 2 mM penicillamine, 1 mM hypotaurine, 250 mM epinephrine and 20 µg/ml heparin. Frozen–thawed semen (10^6 spermatozoa/ml) was prepared by Percoll gradient (Amersham Pharmacia Biotec). In a 15 ml conic tube, 1 ml Percoll 90% was added followed by 1 ml Percoll 45%. Semen was thawed at 37 °C for 30 s, placed on the top of the Percoll gradient and centrifuged for 10–12 min at 650 *g*. After removal of the supernatant, 4 ml Tyrode's-albumin-lactate-pyruvate (TALP) medium were added and the sample centrifuged again for 2 min at 200 *g* to remove excess Percoll.

Semen was coincubated with matured oocytes for 18 h at 38.5 °C in 5% CO_2. At the end of gametes co-culture, the cumulus cells were completely removed and cumulus-free presumptive zygotes were randomly transferred into one of three co-culture systems in which they were cultured up to day 7: (1) co-culture with cumulus cells (control); (2) co-culture with BM-MSCs; and (3) co-culture with AE-SCs. For all the feeders under experiments, the embryo culture medium was TCM199 Earle's Salt medium plus 10% FCS, 0.2 mM pyruvate and 10 µg/ml gentamycin at 38.5 °C in 5% CO_2. Medium was changed every 48 h throughout the culture period.

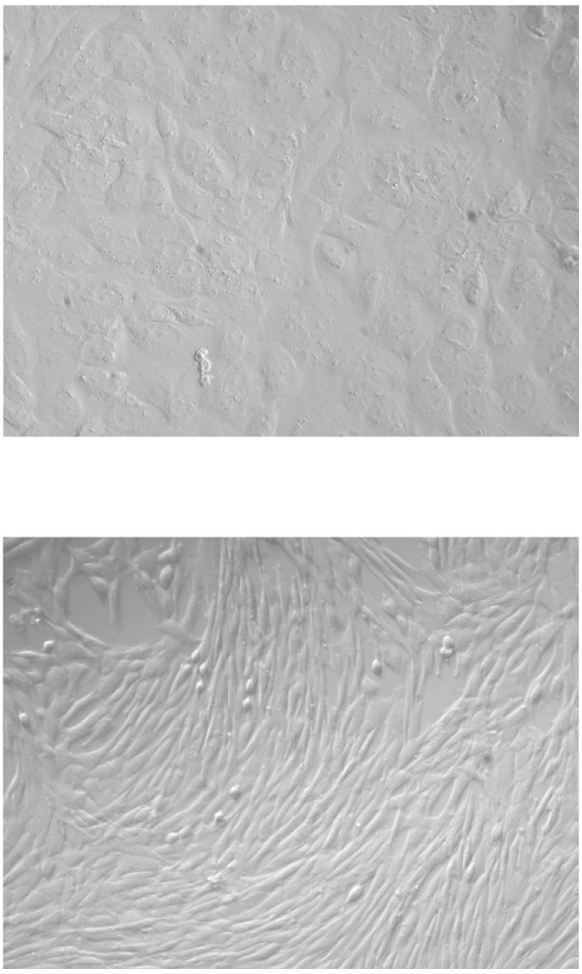

Figure 1 Monolayer of amniotic epithelial stem cells (*a*) and of bone marrow stem cells (*b*). Magnification ×10.

Statistical analysis

The data were analyzed by ANOVA using GraphPad Instat 3.00 for Windows (GraphPad Software, Inc.).

Results and Discussion

Isolated cells readily attached to plastic culture dishes. AE-SCs displayed typical cuboidal morphology (Fig. 1*a*) while BM-SCs were fibroblast-like (Fig. 1*b*). A monolayer of cumulus cells developed progressively in the culture drop.

Seven hundred and five cumulus–oocyte complexes with a homogeneous cytoplasm and two or more layers of cumulus cells were used.

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Data of three replicates are presented in Tables 1 and 2.

Rates of cleavage were similar but blastocysts developmental rates were significantly different ($p < 0.001$) between control, AE-SCs and BM-MSCs. The co-culture with AE-SCs gave a significantly higher percentage of blastocysts ($p < 0.001$).

Co-culture of oocytes and embryos with various types of cells has been widely used to improve development of bovine and ovine *in vitro* derived embryos (Rexroad & Powell, 1993; Duszewska *et al.*, 2000). It has been established that somatic cells, as cumulus cells, mainly have a beneficial effect on the development of bovine embryos because of the secretion of embryo-trophic factors (Donnay *et al.*, 1997; Geshi *et al.*, 1999).

The influence of co-culture with fetal fibroblast cells was studied by Li *et al.* (2001) on *in vitro* maturation of equine oocytes and by Hatoya *et al.* (2006) on IVM, IVF and IVC of canine oocytes. Signficantly higher proportions of blastocysts were produced from fetal fibroblast cells co-culture group than from the groups of control oocytes, demonstrating a beneficial influence of co-culture with fetal fibroblasts for nuclear and cytoplasmic maturation of oocytes *in vitro*.

These fibroblast cells secreted several cytokines, (e.g. leukemia inhibitory factor, steel factor and basic fibroblast growth factor (bFGF) (Dolci *et al.*, 1991; Matsui *et al.*, 1991; Cheng *et al.*, 1994; Xie *et al.*, 2004), and growth factors that affected meiotic maturation of oocytes *in vitro* (Hatoya *et al.*, 2006).

In the present study, we examined the effects of co-culturing bovine embryos with horse AE-SCs and BM-MSCs on *in vitro* development. We have previously studied the characteristic of stemness of AE-SCs and BM-MSCs used in this work as feeders to culture embryos, and we have showed that these cells express mesenchymal stem cells mRNA markers (CD29, CD105, CD44), are negative for CD34, express embryonic marker as Oct-4, Sox 2 and SSEA-4 and are plastic because they are able to differentiate into osteogenic and adipogenic lines (Cremonesi *et al.*, 2008, 2009; Lange Consiglio *et al.*, 2010). Moreover, AE-SCs are negative for the major histocompatibility complex (MHC) type II until fifth passages (data not shown).

Our results showed that the co-culture with AE-SCs gave a significantly higher percentage of blastocysts compared with control and BM-MSCs feeders. It's possible that AE-SCs monolayer create a more suitable microenvironment necessary for inducing local cell activation and proliferation of the growing embryos in comparison with BM-MSCs and cumulus cells used as control.

Koizumi *et al.* (2000) studied human amniotic epithelial cells and their gene expression and reported

Table 1 Rate of different stage of development of blastocysts during culture on different feeders cells in three replicates

Feeder	Rep. (*n*)	Oocytes (*n*)	Cleavage (%)	Morula (%)	Early Blast. (%)	Blast. (%)	Exp. Blast. (%)	Hatc. Blast. (%)
CTR	1	80	65.00	50.00	43.75	33.75	27.5	22.5
	2	78	75.64	56.41	42.31	35.90	33.33	26.92
	3	79	67.09	53.16	44.30	36.71	32.91	25.32
AE-SCs	1	78	65.38	46.15	42.31	39.74	35.90	32.05
	2	78	64.10	52.56	46.15	41.03	35.90	33.33
	3	76	67.11	52.63	48.68	44.74	39.47	34.21
BM-MSCs	1	78	65.38	44.87	37.18	28.21	24.36	19.23
	2	80	63.75	42.50	33.75	30.00	22.5	13.75
	3	78	67.95	43.59	35.90	32.05	20.51	15.38

Blast. = Blastocysts; Exp = Expanded Blastocysts; Hatc = Hatched Blastocycts, Rep. = Replicates.

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Table 2 Rate of cleavage and blastocysts on different feeders

Feeder	Cleavage (%)	Blastocysts (%)
CTR	69.24 ± 5.64^{a}	35.45 ± 1.53^{a}
AE-SCs	65.53 ± 1.51^{a}	41.84 ± 2.60^{b}
BM-MSCs	65.69 ± 2.12^{a}	30.09 ± 1.92^{c}

$^{a-c}$Values with different superscripts within same column are significantly ($p < 0.001$) different.

that they excrete or maintain on the membrane surface many proteins as growth factors [epidermal growth factor (EGF), keratinocyte growth factor, hepatocyte growth factor (HGF), basic fibroblast growth factor (bFGF), transforming growth factor-α (TGFα), TGFβ1, TGFβ2, TGFβ3] and two growth receptors (keratinocyte growth factor receptor and epatocyte growth factor receptor).

The mesenchymal stem cells by bone marrow or fat tissue express mRNA for interleukin (IL)-6, IL-11, leukemia inhibitory factor (LIF), macrophage colony-stimulating factor (M-CSF), and stem cell factor (SCF) (Majumdar, 2000); other studies evidenced expression of vascular endothelial growth factor (VEGF), HGF, bFGF, granulocyte–macrophage colony stimulating factor (GM-CSF) and TGF-β (www.vet-stem.com/technologies/regenerativecells.php).

A growth factor that differentiates the AE-SCs from the BM-MSCs is EGF, that *in vivo* is produced by endometrial cells and for which the embryo possesses receptors. We cannot affirm that this factor is responsible for the different results of the *in vitro* culture of the bovine embryo, but certainly it is one of the greatest candidates to play a positive role of the monolayer of AE-SCs in embryonic development as it happens *in vivo* for the endometrium. Moreover, it can be suggested that these cells secrete biologically active substances, including signalling molecules and growth factors of epithelial nature, different to those of the BM cells of mesenchymal origin.

It is also important to underline that for embryonic development it is not necessary that these factors must be species-specific, considering the different affiliation of species of the equine amniotic epithelial stem cells in comparison with the bovine embryos, the object of this study.

In conclusion, this approach revealed that the complexity of the environment provided by this cellular feeder makes the AE-SCs particularly interesting for the *in vitro* co-culture of embryos and represents the initial step towards standardization of a protocol for the culture of equine embryos.

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