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Culture medium composition affects the gene expression pattern and *in vitro* development potential of bovine somatic cell nuclear transfer (SCNT) embryos

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ABSTRACT

Different culture systems have been studied that support development of somatic cell nuclear transfer (SCNT) embryos up to the blastocyst stage. However, the use of sequential and two-step culture systems has been less studied. The objective of the present study was to examine the developmental potential and quality of bovine SCNT embryos cultured in different two-step culture media based on KSOM, SOF and the macromolecules FBS and BSA (K-K/FBS, K-S/BSA and K-K/BSA, respectively). No differences were observed in the cleavage rate for any of the culture systems. However, there was a significant difference ($P<0.01$) in the rate of blastocyst development, with the K-K/FBS culture system yielding a higher rate of blastocysts (28%) compared to other treatments (18 and 15%, for K-S/BSA and K-K/BSA, respectively). Although quality of embryos, as assessed by the total number of cells, was not different, the apoptosis index was significantly affected in the sequential culture system (K-S/BSA). Gene expression analysis showed alterations of *DNMT1*, *IGF2*, *LIF*, and *PRDX6* genes in embryos cultured in K-S/FBS and of *SOD2* in embryos cultured in K-K/BSA. In conclusion, we demonstrated that culture medium may affect not only the developmental potential of SCNT embryos but also, more importantly, the gene expression pattern and apoptotic index, presenting the possibility to manipulate the culture medium composition to modulate global gene expression and improve the overall efficiency of this technique.

Key words: Cattle, gene expression, KSOM, nuclear transfer embryos, SOF.

INTRODUCTION

Potential applications of animal cloning in agriculture, biotechnology, biomedicine and basic research (Cibelli et al., 1998, Wells et al., 1999, Keefer 2004) have increased interest in this technology for many researchers around the world. Nevertheless, despite the original success of cloning demonstrated by the generation of live offspring in different species (Cibelli et al., 1998, Wakayama et al., 1998, Baguisi et al., 1999, Polejaeva and Campbell 2000, Chesne et al., 2002, Shin et al., 2002, Galli et al., 2003, Zhou et al., 2003, Li et al., 2006), the efficiency of nuclear transfer is still far from optimum (Wilmut et al., 1997, Wakayama et al., 1998, Miyoshi et al., 2001). For instance, nuclear transfer success rates in cattle are around 11% with adult somatic cells (Kubota et al., 2000, Gibbons et al., 2002, Panarace et al., 2007), although in a few instances higher efficiencies have been described when blastomeres were used as nuclear donors (Peura and Trounson 1998). These data demonstrate that low efficiency is still a major drawback for widespread use of cloning technology.

Different factors have been recognized as contributing to the low efficiency of nuclear transfer, including aberrant reprogramming of the donor nucleus (Stice et al., 1998, Jouneau and Renard 2003), lack of cell cycle coordination between the recipient oocyte and the donor nucleus (Campbell et al., 1994, Gibbons et al., 2002), atypical oocyte activation (De La Fuente and King 1998, Alexander et al., 2006, Bhak et al., 2006) and *in vitro* culture conditions for the reconstructed embryos (Zakhartchenko et al., 1999, Mastromonaco et al., 2004). Improvement of culture conditions for *in vitro* fertilization (IVF) and somatic cell nuclear transfer (SCNT) embryos has been pursued for many years and a variety of culture media that support bovine embryo development have been developed, including synthetic oviductal fluid (SOF) (Tervit et al., 1972, Krisher et al., 1999), CR1aa medium (Rosenkrans and First 1994, Sims and First 1994), potassium simplex optimization medium (KSOM) (Liu and Foote 1995, Bhuiyan et al., 2004), Beltsville embryo culture medium (BECM) (Dobrinsky et al., 1996, Lim et al., 1999), G1/G2 medium (Krisher et al., 1999, Wang et al., 2011) and IVD101 (Abe and Hoshi 2003). Additionally, culture conditions based on defined components free of serum, BSA or cell constituents which may introduce uncharacterized factors into the culture and to some extent have been attributed to problems associated with large offspring syndrome (Young et al., 1998), are also being developed (Lim et al., 2007, Jang et al., 2011).

Culture conditions for bovine embryos can also involve the addition and subtraction of particular components of the basal medium in a second step of the culture (Gardner and Lane 1998, Lane et al., 2003) or the use of a different medium, as is the case in sequential and two step culture systems (Bavister 1995, Nedambale et al., 2004, Felmer et al., 2011). The main principle of these culture systems is that *in vivo* embryos move from the oviduct to the uterus; the secretions and gas atmosphere of these compartments differ in composition (Biggers et al., 2005, Hugentobler et al., 2008). Therefore, these culture systems are being developed in an effort to mimic the physiological conditions that embryos would have *in vivo*.

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Although a number of studies have compared the effects of these culture systems in IVF embryos, only few studies so far have evaluated these systems in SCNT embryos (Wang et al., 2011). This is particularly relevant for cloned bovine embryos, since the majority of the studies use only a single culture medium such as mSOF (Choi et al., 2002, Zhang et al., 2007).

In the present study we assessed the effects of three different two-step culture media on the *in vitro* development potential and quality of bovine SCNT embryos. Additionally, we studied the expression pattern of a number of developmentally important genes in pools of embryos cultured in these media, to establish differences in gene expression as result of the culture medium composition.

MATERIAL AND METHODS

Unless stated otherwise, all chemicals were purchased from Sigma Chemical (St Louis, MO, USA).

Derivation of donor cells

A tissue biopsy was obtained from the ear of a 6-year-old cow (Fries Hollands breed) and taken immediately to the laboratory in Dulbeccos´s PBS medium (Gibco Life Technologies Corporation, Grand Island, NY, USA) containing 10% (v:v) penicillin/streptomycin (100,000 U/ml penicillin G, 100,000 μg/ml streptomycin) at 4 ºC. The tissue biopsy was cut into small pieces, and tissue explants were cultured in Dulbecco´s Modified Eagle Medium (DMEM) (Gibco Life Technologies Corporation, Grand Island, NY, USA), supplemented with 10% inactivated fetal bovine serum (FBS) (Hyclone Laboratories, Inc., Logan, UT, USA) and 1% (v:v) penicillin/streptomycin (10000 U/ml penicillin G, 10000 μg/ml streptomycin) at 38 ºC in 35 mm tissue culture plates in a humidified atmosphere of 5% CO_2 in air. After 10 days in culture, explants were removed and cells harvested by trypsinization, counted, and seeded in 75 cm^2 tissue culture flasks. When cells reached confluency they were collected by trypsinization and frozen until use in DMEM medium supplemented with 40% FBS and 10% dimethyl sulfoxide (DMSO).

Collection of ovaries, selection of oocytes and in vitro maturation

Ovaries were collected from a local slaughterhouse (Frigorifico Temuco, Temuco, Chile). Cumulus-oocyte complexes (COCs) were aspirated from 2-7 mm follicles using an 18-gauge needle. Good quality oocytes surrounded by more than 6 compact layers of cumulus cells and displaying uniformly granulated cytoplasm were selected and matured in TCM-199 medium supplemented with 10% inactivated FBS and 6 μg/mL LH (Sioux Biochemical, Inc., Sioux City, IA, USA), 6 μg/mL FSH (Bioniche Life Science Inc., Belleville, Ontario, Canada) and 1 μg/mL estradiol, and then incubated for 17 h at 38.5 ºC in 5% CO_2 and saturation humidity.

Nuclear transfer procedure

After 17 h of maturation, oocytes were stained with Hoechst 33342 and enucleated by aspiration of the MII plate using an inverted microscope (Nikon TS100, Nikon Instruments Inc., New York, NY, USA) and Narishige micromanipulators (Narishige International, Inc., New York, NY, USA). Prior to nuclear transfer, nuclear donor cells (passage number 3-5), were grown to confluency for 5 days in order to induce quiescence. These cells were microsurgically placed into the perivitelline space evacuated during enucleation and cell-cytoplast complexes were fused in sorbitol media with a single DC pulse of 150 volts/mm and 15 seconds delivered by an Electrocell Manipulator 830 (BTX, Harvard Apparatus, Inc, Holliston, MA, USA). Activation was carried out with 5 μM Ionomicin for 5 minutes followed by incubation in KSOM (EmbryoMax, Millipore, Billerica, MA, USA) + 0.4% BSA medium containing 2 mM DMAP for 4 h.

Embryo culture

After activation, 730 NT units (10 biological replicates) were randomly distributed into 50 μl drops of the following culture media: (1) KSOM + 0.4% FAF-BSA for 3 days and then KSOM + 5% FBS to day 7, hereafter referred to as K-K/FBS; (2) KSOM + 0.1% BSA for 3 days and then SOF (custom made, Caisson Laboratories, Inc., UT, USA) + 0.8% BSA to day 7 (K-S/BSA), and (3) KSOM + 0.4% BSA for 3 days and then KSOM + 0.8% BSA to day 7 (K-K/BSA) (Table 1) and cultured at 38.5 °C with a gas mixture of 5% CO_2, 5% O_2 and 90% N_2. These culture systems are based on different combinations of KSOM medium and have been previously evaluated by our group in bovine embryos generated by IVF (Felmer et al., 2011).

Cell number count and TUNEL assay

Cell number in day 7.5 expanded blastocysts (10 per treatment) was scored by incubating embryos in media (TCM-199) containing 10 μg/mL bisbenzimide in absolute ethanol at room temperature for 10 min. Blastocysts were then treated in 50% (v/v) glycerol, mounted onto a glass slide and visualized under an epifluorescent microscope coupled with a UV-2E/C DAPI filter. For TUNEL assay, blastocysts were permeabilized with 0.2% Triton for 5 minutes and fixed at room temperature (~24 ºC) in 4% paraformaldehyde for 30 min. Embryos were additionally permeabilized for 5 minutes with 0.1% sodium citrate containing 0.1% Triton, washed twice with PBS/BSA and incubated with labelling reagent according to the manufacturer's instructions (Cell Death Detection kit, Roche Biochemical, Indianapolis, IN, USA). Finally, embryos were mounted onto a glass slide on drops of 10 μL of antifade and examined under an epifluorescence microscope coupled with an EGFP filter as above. Positive controls for TUNEL were carried out by treating embryos with 15 U DNAse I for 15 min at 37 ºC before the TUNEL assay, and negative controls by incubating embryos with the fluorescent labelling reagent in the absence of the terminal transferase dUTP enzyme.

RNA extraction, reverse transcription and gene expression analysis

Fifteen good quality expanded blastocysts divided into 3 pools per treatment (5 blastocysts/pool) were lysed in 20 μL of extraction buffer (XB; Arcturus, Carlsbad, CA, USA) by incubation at 42 °C for 30 minutes followed by centrifugation at 3,000 x g for 2 minutes. RNA was kept frozen at -80 °C in the kit´s extraction buffer until all samples were collected for analysis. Total RNA was extracted from each pool of embryos using the PicoPure RNA Isolation Kit (Arcturus, Carlsbad,

TABLE 1

Composition of culture media used for SCNT embryo culture.

Components (SIGMA)	Medium 1		Medium 2		Medium 3	
	KSOM		KSOM	SOF	KSOM	
	Step 1	*Step 2*	*Step 1*	*Step 2*	*Step 1*	*Step 2*
	mM		mM	mM	mM	
$CaCl_2$ $2H_2O$	1.71		1.71	1.78	1.71	
KCl	2.5		2.5	7.16	2.5	
KH_2PO_4	0.35		0.35	1.19	0.35	
$MgSO_4$ 7 H_2O	0.2		0.2	1.51	0.2	
NaCl	95		95	107.7	95	
$NaHCO_3$	25		25	25.07	25	
Na lactate	10		10	5.35	10	
Na pyruvate	0.2		0.2	7.25	0.2	
L-glutamine	1		1	0.2	1	
EDTA	0.01		0.01		0.01	
D- glucose	0.2		0.35		0.2	
Taurine			0.4			
Tri-Sodium citrate				0.34		
Myo- Inositol				2.77		
BSA-FAF (%)	0.4		0.1	8	0.4	8
FBS (%) (Hyclone)	5					
MEM non-essential solution 100x (ml/ml)	10		10	4	10	
BME essential solution 50x (ml/ml)	10		10	12	10	
Penicillin (U/ml)	100		100		100	
Streptomycin (g/l)	0.05		0.05		0.05	

CA, USA) according to the manufacturer´s instructions, and residual genomic DNA was removed by DNAse I digestion using 0.125 units final concentration of RNase-Free DNase Set (Qiagen, Valencia, CA, USA). Final RNA was eluted from the purification column using 11 µL of the kit´s elution buffer.

Reverse transcription was carried out with the RevertAid H Minus First Strand Kit (Thermo Scientific Inc., Pittsburgh, PA, USA), according to the manufacturer´s instructions. Briefly, 10 µL total RNA and 200 ng of random hexamers were added to each 0.5 mL of RNase-free tube. The reaction tubes were incubated in a preheated PCR machine at 70 °C for 5 minutes and transferred to ice. After denaturation, the following reagents were added to each reaction tube: 4 µL of 5X first-strand reaction buffer, 2 µL of 10 mM dNTPs, and 1 µL of Riboblock. After gentle mixing, reaction tubes were incubated at 25 °C for 5 minutes. Then, 1 µL of RevertAid^{TM} M MuLV RT was added and the mixture incubated at 42 °C for 60 minutes in a dry bath. The reaction was terminated by heating at 70 °C for 10 minutes and then chilled on ice. This first-strand cDNA was diluted 5 times and used for real time experiments.

Quantification of a panel of 19 developmentally-important genes (primer sequences published in Felmer et al. (2011) was carried out by RT-qPCR using Brilliant II SYBR Green QPCR Master Mix (Agilent Technologies, Palo Alto, CA, USA) in a thermocycler MX3000P (Agilent Technologies, Palo Alto, CA, USA). All PCR reactions were performed in duplicate wells in a final volume of 20 µL containing 4 µL of diluted cDNA, 10 µL of Master mix, 4 µL of primer mix (300 nM final), and 2 µL of PCR-grade water. The PCR program consisted of an initial incubation at 95 °C for 5 minutes to activate the Taq DNA polymerase, followed by 40 cycles of template denaturation at 95 °C for 20 seconds, a primer annealing step at 58 °C for 20 seconds (same annealing for all primers), and an extension step at 72 °C for 20 seconds. A control for removal of genomic DNA after DNAse treatment (-RT) was performed with primers for *ACTB* and negative control tubes without cDNA template were included in each assay. At the end of the PCR reaction, melting curve analyses were performed for all genes, and the specificity as well as integrity of the PCR products was confirmed by the presence of a single peak (data not shown). PCR efficiencies (E) were estimated using relative standard curves derived from a pooled cDNA mixture from *in vitro* produced embryos (a 10-fold dilution series with five measuring points). These values were determined by the slopes of the curves according to the equation $E= 10^{(-1/slope)}$ established by Pfaffl (2001).

The comparative *Ct* method was used to quantify of mRNA expression levels using the amplification efficiency of each

gene as a correction factor (Livak and Schmittgen 2001). For normalization we used the geometric average of the *BAX*, *GAPDH* and *YWHAZ* reference genes, after analysis with the geNorm Visual Basic Application program for Microsoft Excel as described by Vandesompele et al. (2002), confirming their stability under our laboratory conditions (data not shown). To measure the differences in expression between the different culture media, we used the pairwise fixed reallocation randomization test in the Relative Expression Software Tool (REST; V2.0.7, Copyright 2008, Corbett Research Pty. Limited, Munich, Germany) (Pfaffl et al., 2002).

Statistical analysis

Data analysis for embryo development and cell count was carried out by descriptive statistics based on the mean and standard error calculated for each of the variables, using Statgraphics Plus 5.1 software (StatPoint Technologies Inc., Warrenton, VA, USA). One-way ANOVA was used to test for statistically significant differences among treatments for cleavage, blastocyst rate and cell counting after arcsine transformation of the proportional data. Post hoc analysis to identify differences between groups was carried out using the Scheffé test. An error probability of P<0.05 was considered significant.

RESULTS

Effect of culture medium on the in vitro developmental competence of bovine SCNT embryos.

The result of nine replicates with a total of 730 NT embryos randomly distributed among the three different culture media showed no differences in the cleavage rate at 72 h of culture (75, 77 and 74% for K-K/FBS, K-S/BSA and K-K/BSA, respectively) (Table 2). However, significant differences in the rate of blastocysts were observed on day 7 of culture (p<0.001). A greater proportion of embryos reached the blastocyst stage when they were cultured in K-K/FBS (28%) medium compared to K-S/BSA (18%) and K-K/BSA (15%) (Table 2 and Fig. 1). Hatching rates were higher in K-K/FBS (71%) and K-S/BSA (52%) compared to K-K/BSA (42%) (Table 2).

Effect of culture medium on the total number of cells and frequency of apoptotic nuclei in bovine SCNT embryos.

Total number of cells and apoptosis index were assessed to determine any effect of the culture medium on embryo quality. As observed in Table 3, the total number of cells was not different between culture media (128.0±4.4, 119.7±4.6 and 124.6±3.0 for K-K/FBS, K-S/BSA, and K-K/BSA, respectively)

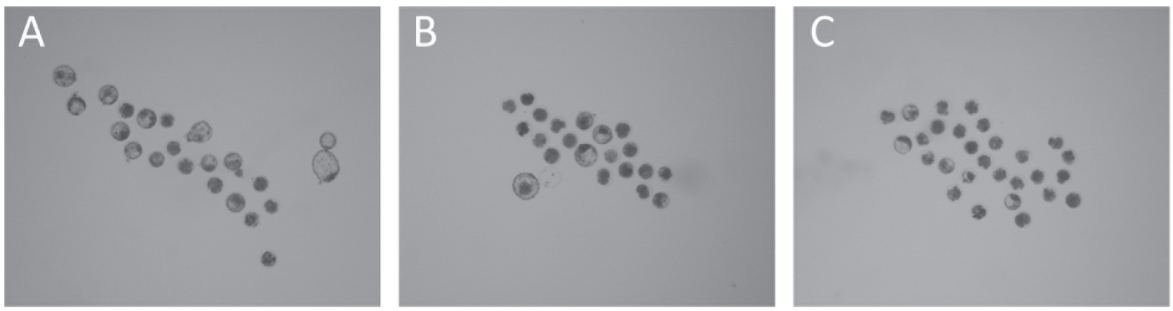

Figure 1. *In vitro*-produced bovine SCNT embryos from K-K/FBS (A), K-S/BSA (B), and K-K/BSA (C) culture systems on day 7, magnification: 40 x.

TABLE 2

Effect of different culture media on the in vitro development of bovine SCNT embryos

Two steps *in vitro* culture system		Oocytes	Cleavage	Total Blastocysts	Hatching Blastocysts
Step one (72 h)	Step two (96 h)	*n*	*n* (%)	*n* (%)	*n* (%)*
KSOM 0.4% BSA	KSOM 5% FBS	245	183 (75)	68 (28)[a]	48 (71)[a]
KSOM 0.1% BSA	SOF 0.8% BSA	247	191 (77)	44 (18)[b]	23 (52)[a,b]
KSOM 0.4% BSA	KSOM 0.8% BSA	238	177 (74)	36 (15)[b]	15 (42)[b]

Cleavage and blastocyst rates were registered at 72 and 168 h, respectively (9 replicates). *Percentage is based on the total number of blastocysts. Data followed by different letters in the same column are statistically different (P<0.01).

TABLE 3

Effect of different culture media on the quality and apoptosis index of bovine SCNT embryos

Culture treatment		Number of embryos	Cell number (mean ± SEM) Total cells	Apoptotic blastomeres	Apoptotis index (%)
KSOM 0.4% BSA	KSOM 5% FBS	10	128.0 ± 4.4[a]	4.5 ± 0.3[a]	3.52 ± 0.22[a]
KSOM 0.1% BSA	SOF 0.8% BSA	10	119.7 ± 4.6[a]	6.3 ± 0.2[b]	5.35 ± 0.30[b]
KSOM 0.4% BSA	KSOM 0.8% BSA	10	124.6 ± 3.0[a]	4.8 ± 0.2[a]	3.88 ± 0.22[a]

Cell count and apoptosis index was carried out on day 7.5 expanded blastocysts. Data followed by different letters in the same column are statistically different ($p<0.001$).

(Table 3). However, a higher proportion ($P<0.01$) of apoptotic to total cell ratio was observed in embryos cultured in K-S/BSA (5.35± 0.30) compared to embryos cultured in K-K/FBS (3.52±0.22) and K-K/BSA (3.88±0.22) (Table 3).

Effect of culture medium on the gene expression profile of bovine SCNT embryos.

Gene expression analysis carried out on day-7 expanded blastocysts showed an increase in the expression level of *LIF* ($p<0.05$) and a decrease in the expression levels of *DNMT1* ($p<0.05$), *IGF2* ($p<0.05$), and *PRDX6* ($p<0.05$) in embryos cultured in K-S/BSA compared to embryos cultured in K-K/FBS. On the contrary, in embryos cultured in K-K/BSA only one gene was affected (*SOD2,* $p<0.05$) compared to embryos cultured in K-K/FBS (Table 4).

DISCUSSION

A number of different culture media have been extensively evaluated in an effort to improve the quality of embryos produced *in vitro* and to mimic the physiological conditions that embryos would have *in vivo*. Most of this work has been carried out in embryos produced by IVF and only a few studies have focused on the evaluation of different culture media for embryos produced by SCNT, presumably due to the early assumption that both embryo production methods would behave similarly under the same culture conditions and because of the extra technical requirements involved in the generation of SCNT embryos. However, previous studies in mice demonstrated that SCNT embryos had better developmental potential when cultured in somatic cell culture medium (MEMα) rather than in IVF-optimized KSOM medium (Gao et al., 2004). Similarly, in cattle Mastromonaco et al. (2004) observed that replacement of serum with BSA in the maturation medium had no effect on the developmental potential of embryos generated by IVF. However, a lower blastocyst rate was observed in SCNT embryos, demonstrating different culture media requirements for embryos produced by IVF and SCNT.

In the present study, presence of serum at later stages of development in the K-K/FBS culture system increased the development rate to the blastocyst stage but replacement of serum by BSA either in K-K/BSA or K-S/BSA culture systems did not have the same stimulatory effect on SCNT embryos. This result is consistent with a number of previous studies in IVF embryos that have demonstrated that presence of serum during the late culture period increases the rate of morulas and blastocysts, regardless of the culture medium composition (Lim et al., 1994, Dobrinsky et al., 1996, Yoshioka et al., 1997, Lonergan et al., 1999). However, in other studies in IVF embryos cultured in modified SOFaa, presence of serum or BSA had no effect on the development of embryos cultured up to day 9 (Mastromonaco et al., 2004). A similar result was observed recently by our group in bovine embryos generated by IVF and cultured in KSOM medium either in the presence of serum (K-K/FBS) or in its absence (K-K/BSA) (Felmer et al., 2011). However, in the present study the lack of serum in SCNT embryos dramatically decreased the developmental potential of embryos in K-K/BSA and K-S/BSA culture systems, confirming the different preferences of IVF and SCNT embryos for varying culture conditions. Furthermore, lack of serum also affected the kinetics of embryos, with embryos cultured in K-K/BSA showing the lowest number of expanded and hatching blastocysts on day 7.

Addition of serum to the culture medium has been previously observed to accelerate the developmental kinetics of embryos, increasing the numbers of blastocysts at early stages (day 6) and hatching and hatched blastocysts on days 8 and 9, respectively (Wang et al., 1997, Gomez and Diez 2000, Holm et al., 2002). This accelerated development has been suggested to induce a number of unfavorable consequences to the embryo, including impaired morula compaction, premature blastulation and an altered gene expression pattern (Holm et al., 2002, Rizos et al., 2003, Wrenzycki and Niemann 2003). However, the late inclusion of serum in the culture system appears to have less negative effects. In fact, previous studies in embryos have demonstrated a biphasic effect of serum inhibiting the early stages of embryo development and improving the development to morulas and blastocysts regardless of the culture composition (Yoshioka et al., 1997, Thompson et al., 1998, Lonergan et al., 1999). Here, we observed that addition of serum to a two-step culture system (K-K/FBS), not only improved the development rate and produced a greater number of blastocysts and hatching blastocysts, but also improved the quality of these embryos, without greatly affecting the gene expression patterns compared to culture systems without serum.

TABLE 4

Analysis of gene expression in day 7 expanded SCNT blastocysts cultured in different media

CULTURE SYSTEMS COMPARED						
	K-K/FBS - K-S/BSA			**K-K/FBS** - K-K/BSA		
Gene	Expression	95% C.I.	P(H1)	Expression	95% C.I.	P(H1)
BAX	2.108	Reference gene		1.168	Reference gene	
CAT	1.006	0.507 - 1.742	0.974	1.298	0.891 - 1.650	0.107
DNMT1	**0.290**	**0.246 - 0.351**	**0.017 (down)**	0.854	0.556 - 1.160	0.550
FGF4	5.119	2.260 - 10.112	0.073	1.422	0.841 - 2.443	0.162
GAPDH	0.374	Reference gene		0.962	Reference gene	
GLUT1	1.833	1.485 - 2.432	0.063	1.242	0.974 - 1.701	0.206
GPX1	4.854	2.770 - 8.611	0.051	1.590	0.675 - 3.286	0.209
GSS	0.983	0.490 - 2.103	0.823	1.360	0.805 - 2.540	0.393
HDAC1	0.628	0.298 - 1.042	0.221	1.183	0.845 - 1.493	0.099
HDAC2	1.492	0.995 - 2.918	0.170	0.988	0.837 - 1.204	0.828
HDAC3	0.726	0.431 - 1.510	0.211	0.849	0.517 - 1.365	0.471
IFNT	0.484	0.121 - 1.294	0.518	1.511	0.335 - 5.264	0.418
IGF2	**0.217**	**0.088 - 0.352**	**0.028 (down)**	1.106	0.725 - 1.529	0.580
LIF	**7.036**	**2.744 - 14.064**	**0.029 (up)**	2.046	0.698 - 5.496	0.118
PRDX6	**0.339**	**0.184 - 0.659**	**0.017 (down)**	1.152	0.658 - 1.664	0.498
SDHA	1.437	0.738 - 2.477	0.269	1.317	1.008 - 1.814	0.099
SOD1	0.577	0.296 - 1.307	0.138	1.074	0.598 - 2.015	0.642
SOD2	3.777	1.688 - 9.726	0.074	**1.730**	**1.104 - 2.880**	**0.040 (up)**
YWHAZ	1.267	Reference gene		0.890	Reference gene	

Gene expression data analysis was carried out by comparing all culture systems relative to K-K/FBS. Reference genes (***BAX***, ***GAPDH*** and ***YWHAZ***) were selected based on pair-wise analysis of their expression stability by the GeNorm program. Expression: Expression ratios obtained by using randomization and boot strapping techniques included in the REST program; 95%CI: range of confidence interval (95%) for the expression ratios; P(H1): probability of the alternate hypothesis that the difference between the sample and control groups is due only to chance. Shown in bold are the genes that were found up or down-regulated in the culture systems evaluated relative to K-K/FBS.

Due to suboptimal culture conditions and the supplementation of culture media with different macromolecules and growth factors, embryos produced *in vitro* show lower number of cells and a higher apoptotic index compared to embryos produced *in vivo* (Kamjoo et al., 2002, Koo et al., 2002). The quality of embryos in the present study as assessed by the total number of cells was not different in any of the culture media evaluated and was similar in quality to previously described embryos produced *in vivo* or by IVF (Koo et al., 2002). However, differences were observed in the apoptotic index, with sequential culture medium K-S/BSA showing the highest number of apoptotic cells. This result suggests that two-step culture media K-K/FBS and K-K/BSA better support culture conditions for bovine SCNT embryos than the sequential culture medium (K-S/BSA). In this latter case, the transition from one medium to another with different composition might be more detrimental for the quality of the embryos, increasing the number of apoptotic nuclei. It has been previously suggested that a higher apoptosis rate might contribute towards embryo mortality or anomalies in the fetus that could trigger early abortions (Brill et al., 1999). Culture conditions minimizing this effect should therefore be preferred in order to generate better quality embryos. Interestingly, although we observed a similar effect in a previous study comparing these culture media in embryos generated by IVF (Felmer et al., 2011), different results have been described recently by Xiong et al. (2012), comparing the most common single-step culture medium, mSOFaa, and the sequential culture medium G1.5/G2.5. These authors observed a higher apoptotic index in SCNT embryos derived from a single step culture medium (SOFaa) than in a sequential culture medium (G1.5/G2.5). In embryos generated by IVF, however, a much higher apoptotic index was observed when embryos were cultured in G1.5/G2.5 relative to SOFaa (Xiong et al., 2012).

Previous comparative studies in IVF embryos have evaluated the effects of culture media on the *in vitro* development potential and quality of embryos as assessed by the total number of cells and, to a lesser extent, their distribution in the different compartments of the embryo. Considering that these endpoints may not necessarily reflect the actual competence of a culture system, we sought to assess the effect of these culture media on the gene expression pattern of 19 developmentally important genes in order to establish if two-step culture media have an impact on the

expression of these genes. Our results confirmed a differential expression pattern of embryos cultured in these culture media. We observed an increase in the expression level of *LIF* and a decrease of *DNMT1*, *IGF2*, and *PRDX6* in embryos cultured in sequential culture medium K-S/BSA compared to embryos cultured in K-K/FBS. Contrarily, in embryos cultured in the two-step culture medium K-K/BSA only one gene was found altered (*SOD2*) compared to K-K/FBS.

Leukemia inhibitory factor (LIF) plays a crucial role during early differentiation and implantation of embryos. In cattle, great expression of this gene and its receptor were previously observed in IVF versus *in vivo*-produced blastocysts, suggesting that this condition could lead to impaired development of the inner cell mass and trophectoderm compartments of embryos (Eckert and Niemann 1998). A similar result was also observed by Rizos et al. (2002), who detected expression of *LIF* only in IVF blastocysts, regardless of the *in vitro* culture medium used compared to embryos produced *in vivo*. These data suggest that the overexpression of *LIF* observed in our study in SCNT embryos cultured in the K-S/BSA medium could compromise the early differentiation of blastocysts cultured in this medium and subsequent post-implantation development, because down-regulation of this gene is required to allow appropriate differentiation of embryo cell compartments (van Soom et al., 1997).

Insulin-like growth factor 2 (*IGF2*), is an imprinted gene encoding a growth factor involved in fetal and placental development and therefore essential for normal development (Perecin et al., 2009). This gene appears to be biallelically transcribed up to the morula stage but the maternal allele is silenced at the blastocyst stage (Ohno et al., 2001). Previous studies in bovine embryos demonstrated that blastocysts produced by IVF have lower expression of *IGF2* compared to those produced *in vivo*, reflecting a reduction in the viability of embryos produced *in vitro* (Bertolini et al., 2002). In SCNT embryos, Perecin et al. (2009) also observed that embryos at later stages of development (33-36 days) had lower expression of *IGF2* compared to *in vivo* and IVF-produced embryos. It was suggested in this case that abnormal expression of imprinted genes is one of the causes of the low efficiency in the cloning process (Perecin et al., 2009). However, some contradictory results have also been observed with this gene. For instance, Han et al. (2003) described that bovine SCNT blastocysts have increased expression of *IGF2* compared to IVF blastocysts. Furthermore, these authors demonstrated that *IGF2* expression was greater in the SCNT embryos cultured in serum containing medium, suggesting that the presence of exogenous proteins in the culture medium affected critical development points (Pandey et al., 2010). In agreement with some previous findings, the low expression of *IGF2* observed in our bovine SCNT embryos cultured in K-S/BSA medium could be responsible for the low pre-implantation development observed presently. Culture conditions of embryos, particularly the presence of serum in the culture medium, could also affect the expression of this gene, as observed previously by Pandey et al. (2010). In our study however, we did not observe the same effect because no differences were found in embryos cultured in the presence of serum (K-K/FBS) or in its absence (K-K/BSA), suggesting that other factors could be responsible for this effect (e.g. culture medium composition).

Low expression of *IGF2* could also affect post-implantation potential or normal growth of the offspring. Previous studies in the mouse model have demonstrated that low expression of this gene is associated with growth retardation (DeChiara et al., 1990, Eggenschwiler et al., 1997) and therefore this gene could be considered a good marker for embryo quality.

DNA methyltransferase 1 (DNMT1) is responsible for maintenance of methylation patterns of hemimethylated CpG dinucleotides after DNA replication (Bestor 1992). DNMT1 knockout mice show abnormal imprinting, reduced levels of DNA methylation and embryo lethality, confirming its essential role during early development (Li et al., 1992, Li et al., 1993, Robertson et al., 1999). In our study, the low expression of *DNMT1* in SCNT embryos cultured in K-S/BSA medium could also explain the lower development rate observed in these embryos. In a different study, expression level of *DNMT1* was found to be significantly reduced in SCNT embryos compared to embryos generated by IVF. However, low levels of *DNMT1* were also found in *in vivo*-derived embryos, suggesting that the method of embryo production has a greater effect on the expression of this gene than the protocols used for the generation of SCNT embryos (Wrenzycki et al., 2001). On the other hand, Amarnath et al. (2007) found no differences in the expression pattern of *DNMT1* between IVF and SCNT embryos using cumulus cells and skin fibroblasts as nuclear donors (Amarnath et al., 2007). In this case, the differences could be attributed to the experimental procedure employed and selection of reference genes for normalization of gene expression data, which were different in the two studies.

Peroxiredoxin 6 **(**PRDX6) is a bifunctional enzyme with peroxidase and phospholipase A2 activity (Fisher 2011). In cattle, *PRDX6* transcripts have been identified in varying amounts in oocytes and embryos produced *in vitro* (Leyens et al., 2004). Studies in genetically engineered mice and cells indicate that inhibition of this gene increases susceptibility to oxidative damage and leads to cell death (Pak et al., 2002), while its overexpression reduces the levels of H_2O_2 (Phelan et al., 2003) and provides protection against oxidative stress and apoptosis (Manevich et al., 2002). Based on these data, the low expression of *PRDX6* in our SCNT embryos cultured in K-S/BSA could also be negatively compromising the development potential of these embryos.

Reactive oxygen species (ROS) are indicated as being one of the main factors responsible for the low production and poor quality of embryos produced *in vitro* (Takahashi et al., 2000). It has also been observed that physiological levels of ROS are necessary for normal regulation of cell growth and development (Hancock et al., 2001). SOD2 (Manganese superoxide dismutase, located in the mitochondria) is an important enzyme involved in the protection against ROS. Different studies in bovine IVF embryos have associated high levels of expression of *SOD2* with better quality of embryos (Rizos et al., 2002, Lonergan et al., 2003). Recently in cattle, Amarnath et al (2007) suggested that this observation might not apply to SCNT embryos because they showed greater expression of *SOD2* compared to IVF embryos despite the acknowledged poor *in vivo* development potential of SCNT embryos (Amarnath et al., 2007). However, contradictory results have been also observed more recently by Kim et al. (2012), who described no differences in the expression levels of *SOD2* between bovine SCNT and IVF embryos, although a higher expression level of *SOD2* was described in SCNT embryos activated by a repeated ionomycin treatment (Kim et

al., 2012). In our study, an increased expression level of *SOD2* was found in SCNT embryos cultured in K-K/BSA medium. In this case, despite the lower developmental potential observed with this medium we suggest that embryos generated by this culture medium are of good quality, which is corroborated by the morphological, cellular and apoptosis assessments that showed similar quality to those embryos cultured in the presence of serum (K-K/FBS).

In conclusion, the results of the present study show that the use of KSOM medium supplemented with 5% FBS after 3 days of culture can improve the blastocyst formation rate of SCNT embryos and reduce the incidence of apoptosis compared to other two-step culture media supplemented with BSA. Furthermore, the differential expression pattern observed in SCNT embryos cultured in different culture media confirm the feasibility of generating higher quality embryos through changes in culture medium composition, a factor that could be manipulated by the use of chemically-defined media.

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