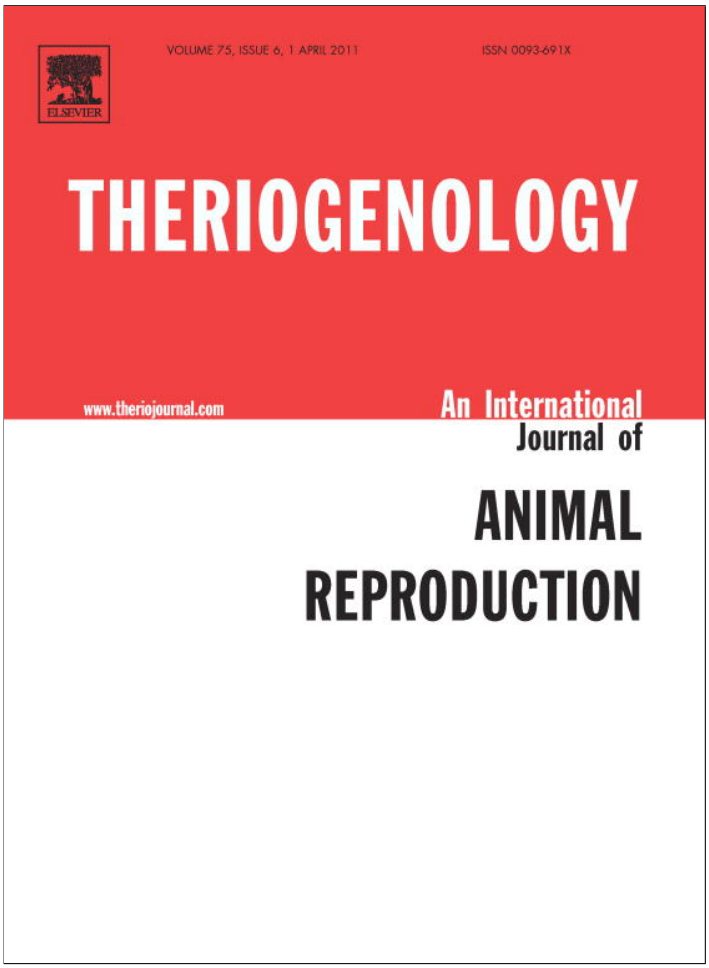

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Theriogenology 75 (2011) 995–1004

Theriogenology

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Effect of different culture media on the temporal gene expression in the bovine developing embryos

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Received 19 July 2010; received in revised form 4 November 2010; accepted 5 November 2010

Abstract

We have previously shown that the *in vitro* embryonic development and the yield of viable calves were increased by using a two-step chemically defined medium for post-fertilization culture of bovine embryos. In this study, we explored the embryonic development and the temporal behavioral interaction of the genes involved in IFNτ gene expression and how they behave in an orchestrated manner to increase the developmental competence of IVF produced embryos by culturing in the chemically defined medium. Behavior of genes included ETS2, CDX2, GATA2, GATA3, OCT4 and NANOG was analyzed in early bovine IVF produced embryos, (from compact morulae to the blastocyst hatching stages), by semi- and relative quantitative PCR and compared between two *in vitro* culture (IVC) systems, two-step chemically defined medium and modified synthetic oviductal fluid (mSOF) containing 8 mg/mL, BSA. Early embryonic development was found to be better in two-step chemically defined culture system than that of mSOF as indicated by the increment of blastocyst yield, 33.1% in two-step culture system vs 18.8% in mSOF medium, and the blastocyst hatching, 52.3% in two-step culture system vs 33.5% in mSOF medium. Relative quantitative gene expression showed harmonic behavior in the two-step culture system rather than the culture in mSOF, IFNτ showed even increase throughout the embryonic development in the two-step culture medium while it decreased with blastocyst hatching in mSOF culture condition. Temporal dominance of OCT4 over all the transcription factors was found in regulation of IFNτ expression (the major factor of expression regulation but in inverse manner). However, ETS2, CDX2, GATA2 and GATA3 are potent IFNτ stimulator in cumulative manner but in case of OCT4 decrement. CDX2 directly related with IFNτ, but still under OCT4 dominance and also regulated by the subservient of OCT4 which is NANOG. In conclusion, this study confirmed our previous results about the usefulness of using the two-step chemically defined culture medium for increasing the developmental competence of IVF produced embryos and elucidated the dominance of OCT4 over the other genes implicated in regulation of IFNτ expression.

Keywords: Cattle; Embryonic development; *In vitro* fertilization; Gene expression; Interferon tau

1. Introduction

In vitro production (IVP) of bovine embryos has become a reliable alternative to conventional superovulation techniques and has been used as a tool to study pre-implantation embryo development [1]. In particular, the 5–6 days post-fertilization period of IVP embryos is the time when various developmentally important events occur, including the first cleavage division that is critical in determining the subsequent development of the embryo [2], activation of the embryonic genome, compaction of the morula and blastulation [3]. This phase in development is also characterized by

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0093-691X/$ – see front matter © 2011 Elsevier Inc. All rights reserved.
doi:10.1016/j.theriogenology.2010.11.006

distinct morphological changes that must include a well-orchestrated expression of genes derived from both maternal and embryonic genomes to allow for compaction, cavitation and blastocoel expansion [4]. Significant losses in IVP occur during the *in vitro* culture (IVC) period suggesting the post-fertilization environment is critical for development [5,6]. The relative abundance of numerous transcripts varies through the early pre-implantation period and is strongly influenced by the culture environment [5–10].

Over the past 25 years, much attention has been given to interferon tau (IFNτ) which is produced by the trophoectoderm during a defined period of peri-attachment of ruminant embryos [11–15]. IFNτ is first detected at the late morula to early blastocyst stages of development. Both mRNA and protein level are relatively low at the blastocyst stage but increase with advancing age of the spherical conceptus [16,17]. IFNτ acts on the uterus to interrupt the luteolytic pathway caused by oxytocin-dependant pulses of prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) so that corpus luteum (CL) function may continue. IFNτ also promotes luteal function by regulating uterine metabolism of various prostaglandins in a concentration dependent manner [18–20]. A majority of failed pregnancies occur within the first 6 weeks after mating in cattle and it is estimated that 10–40% of all failed pregnancies occur during the critical period when IFNτ must interact with the uterus for continuation of pregnancy [21].

The unique pattern of IFNτ expression is regulated by promoter/enhancer regions that are distinct from that of other type I IFN genes [22]. One key component of IFNτ expression is Ets-2 transcription factor. Ezashi et al. [23] first identified it as a regulator of IFNτ transcription. Subsequent findings determined that a consensus Ets-2 binding site is present in all transcriptionally active bovine IFNτ. Moreover, at least three trophoectodermal transcription regulators are also involved with IFNτ expression regulation. Caudal-type homebox 2 (CDX2) stimulates IFNτ promoter activity in the presence of Ets-2 [24,25]. Two additional regulators, GATA transcription factors 2 and 3 (GATA2 and GATA3), were recently found to have role in regulation of IFNτ expression [26]. In addition, the POU homeodomain protein (Oct-4), that is best known as a marker of pluripotency [27], blunts the ETS2 induced IFNτ promoter activity [28] beside the inhibition of other factors during early pregnancy like CDX2 [29]. This study, as a continuation to our previous work, was undertaken to: 1) compare the developmental competence of bovine embryos that were cultured *in vitro* cultured in a two-step chemically defined medium or modified synthetic oviductal fluid medium (mSOF) containing 8 mg/mL, BSA; 2) compare the gene expression patterns in these embryos; and 3) elucidate the interplay and dominance among the major factors controlling the developmental competence markers of bovine embryos specifically IFNτ and its regulator genes (CDX2, ETS2, GATA2, GATA3, NANOG, and OCT4).

2. Material and methods

2.1. Oocyte collection and in vitro maturation (IVM)

Ovaries were collected from a local abattoir into saline at 35 °C and transported to the laboratory within 2 h. Cumulus-oocyte complexes (COCs) from follicles 2–8 mm in diameter were aspirated using an 18 gauge needle attached to a 10 mL disposal syringe. The COCs with evenly-granulated cytoplasm and enclosed by more than three layers of compact cumulus cells were selected, washed three times in HEPES-buffered tissue culture medium-199 (TCM-199; Invitrogen, Carlsbad, CA, USA), supplemented with 10% FBS, 2 mM $NaHCO_3$ (Sigma–Aldrich Corp., St. Louis, MO, USA), and 1% penicillin–streptomycin (v/v). For IVM, COCs were cultured in four-well dishes (30–40 oocytes per well; Falcon, Becton-Dickinson Ltd., Plymouth, UK) for 22 h in 450 μL TCM-199 supplemented with 10% FBS, 0.005 AU/mL FSH (Antrin, Teikoku, Japan), 100 μM Cysteamine (Sigma-Aldrich) and 1 μg/mL 17β-estradiol (Sigma–Aldrich) at 39 °C in a humidified atmosphere of 5% CO_2.

2.2. Sperm preparation, in vitro fertilization (IVF) and in vitro culture of embryos (IVC)

Motile spermatozoa were purified and selected using the Percoll gradient method [30]. Briefly, spermatozoa were selected from the thawed semen straws by centrifugation on a Percoll discontinuous gradient (45–90%) for 15 min at 1500 rpm. The 45% Percoll solution was prepared with 1 mL of 90% Percoll (Nutricell, Campinas, SP, Brazil) and 1 mL of capacitation-TALP (Nutricell). The sperm pellet was washed two times with capacitation-TALP by centrifugation at 1500 rpm for 5 min. The active motile spermatozoa from the pellet used for insemination of matured oocyte (At 24 h of IVM). Oocytes were inseminated (day 0) with 1–2 $\times$ 10^6 spermatozoa/mL for 18 h in 30 μL microdrops of IVF-TALP medium (Nutricell) overlaid with mineral oil at 39 °C in a humidified atmosphere of 5% CO_2. Presumptive zygotes were denuded and cultured in two different media, the two-step defined culture medium

(first 5 days with D1 then transferred to the later stage medium D2) and mSOF and overlaid with mineral oil (Sigma–Aldrich) [5]. All incubations were done at 39 °C in an atmosphere of 5% O_2, 5% CO_2 and 90% N_2. Cleavage rates were recorded on days 2 and embryonic development was monitored according to the stages of the International Embryo Transfer Society (IETS); C4: compact morula; C5: early Blastococysts; C6: middle Blastocysts; C7: expanded Blastocysts; C7H: Blastocysts in hatching and C8: hatched Blastocysts (Fig. 1A). Hatching of blastocysts was recorded on day 9.

2.3. Counting total cells of blastocysts

The blastocysts of stages C7, C7H and C8 were washed in PBS then stained by Hoechst 33342 (Sigma–Aldrich) for 20 min, mounted on glass slides and the number of nuclei in the embryos were counted using a fluorescence microscope (Nikon TE2000, Tokyo, Japan).

2.4. Determination of the relative abundance of genes transcripts in single embryo by semi-quantitative RT-PCR

Single embryo of each stage (C4–C8) were washed in PBS three times then transferred into 5 μL of diethylpyrocarbonate (DEPC) treated water (Invitrogen) and stored at −80 °C or used freshly for total RNA extraction using the RNeasy total extraction kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instruction. Reverse transcription was carried out at 50 °C for 50 min. Individual RT reaction was performed using random hexamer and superscript™ III reverse transcriptase (Invitrogen) in a 20 μL reaction. 1–2 μL

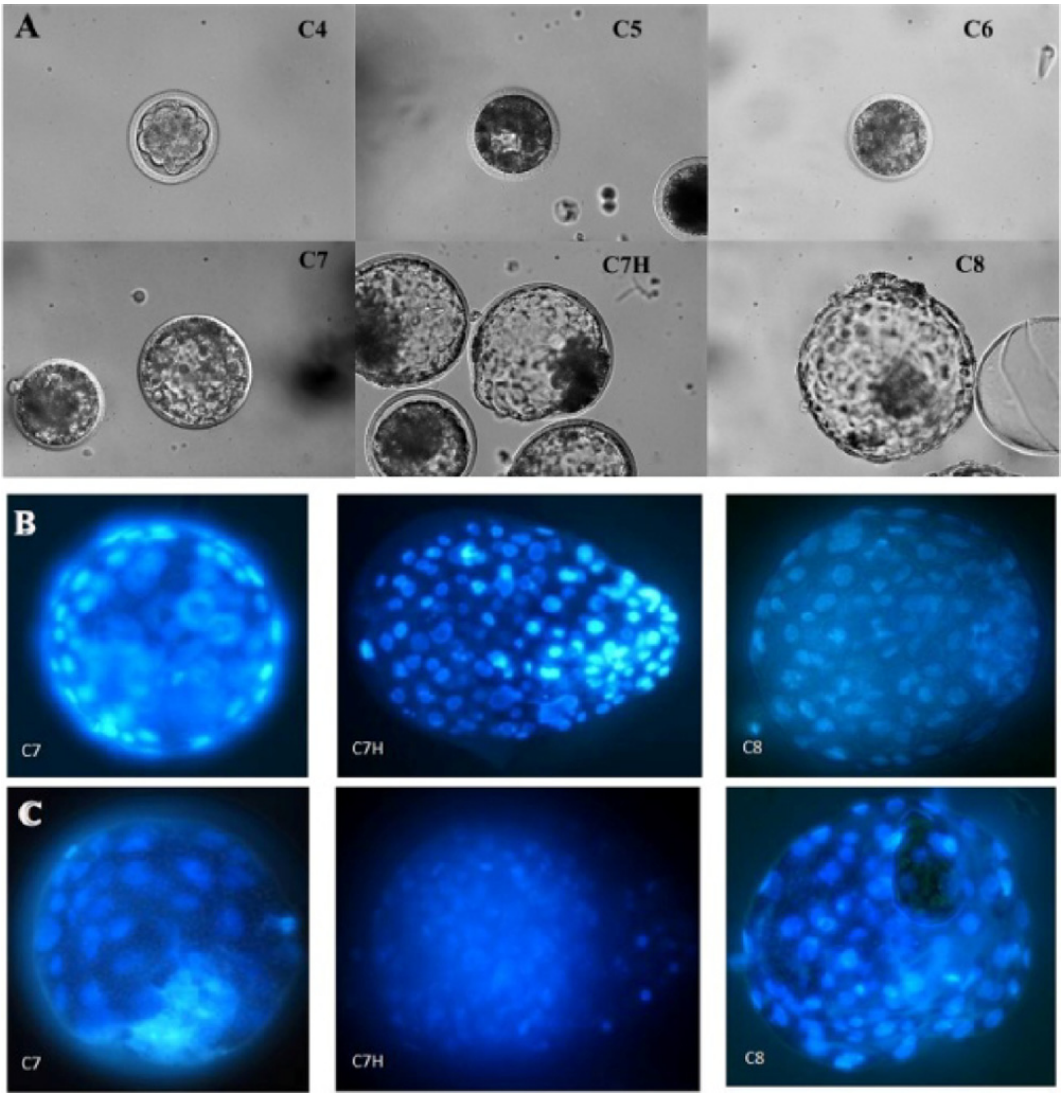

Fig. 1. (A) Representative developmental embryos from compact morulae (C4) to hatched blastocyst (C8), (B) blastocysts in stages C7, C7H and C8 stained by Hoechst 33342 cultured in two-step culture system and (C) in mSOF medium.

Table 1
Primers used for RT-PCR and real-time RT-PCR

Gene	Primer sequences (5′----3′)	Annealing temperature (°C)	Cycle numbers	Fragment size (bp)	GenBank accession number	References
GAPDH*	F:TGCACCACCAACTGCTTGGC R:CACGTTGGGAGTGGGGACGC	60	38	267	NM_001034034.1	[48]
	F:GGCGTGAACCACGAGAAGTA R:CCCTCCACGATGCCAAAGT	60	40	120		
IFNτ*	F:GACGATCTCTGGGTTGTTAC R:GTG ATGTGGCATCTTAGTCA	55	38	565	AF238611.1	[43]
	F:TCCATGAGATGCTCCAGCAGT R:TGTTGGAGCCCAGTGCAGA	60	40	103		
OCT4*	F:GGTTCTCTTTGGAAAGGTGTTC R:ACACTCGGACCACGTCTTTC	60	38	314	AF022987	[41]
	F:CCACCAGCAGGCAAACAC R:GAGAAGGCGAAGTCAGAAGC	60	40	223		
NANOG	F:TTCCCTCCTCCATGGATCTG R:ATTTGCTGGAGACTGAGGTA	58	38	219	NM_001025344	[47]
CDX2	F:GCCACCATGTACGTGAGCTAC R:ACATGGTATCCGCCGTAGTC	59	38	140	DQ126146	[46]
ETS2	F:GTGGGCCTATCCAGCTGTG R:TTCCCTGACGTCTTGTGGAT	58	38	227	DQ126148	[46]
GATA2	F:GAGGACTGTAAGCGTAAAGG R:AAGAACCAAGTCTCCCCAT	60	38	140	XM_583307	[26]
GATA3	F:ATGAAACCGAAACCCGATGG R:TTCACAGCACTAGAGAGACC	60	38	185	NM_001076804	[26]

* The upper and lower line primers of GAPDH, IFNτ and OCT4 were for RT-PCR and real-time PCR, respectively.

cDNA subjected to reverse transcription-polymerase chain reaction (RT-PCR) using Maxime PCR PreMix kit-*i*-starTaq (Intron Biotech., Seoul, Republic of Korea). Primer sequences, annealing temperatures and approximate size of the amplified fragments are listed in Table 1. The PCR amplification carried out for one cycle of denaturation at 95 °C for 5 min and subsequent cycles with denaturation at 95 °C, annealing for 30 sec, extension at 72 °C for 45 sec and final extension at 72 °C for 5 min. and then ten μL of PCR products were fractioned on 1% agarose gel (Intron Biotech.) and stained with RedSafe™ (Intron Biotech.). Expression level for each gene was determined densiometrically by Image J software (1.40 g, NIH, USA). Relative expression levels of each gene at specific stages of embryo development were represented as a ratio to that of GAPDH gene expression.

2.5. Relative quantification (RQ) of transcripts by real-time RT-PCR (qPCR)

Real-Time RT-PCR was done according to the Takara Bio Inc. guidelines. Total 22 μL PCR reaction was made by adding 2 μL cDNA, 1 μL forward primer, 1 μL reverse primer, 8 μL SYBR Premix Ex Taq, 0.4 μL ROX Reference (Takara Bio Inc. Shiga, Japan) and 9.6 μL of Nuclease-free water (Ambion Inc., Austin, TX). The reaction was done by using 7300 Real Time PCR System (Applied Biosystems, Forest City, CA) according to the company instructions. The thermal profile for real-time RT-PCR was 95 °C for 10 min, followed by 40 cycles of 95 °C for 10 sec, 60 °C for 20 sec, and 72 °C for 40 sec. All primers are described in Table 1.

2.6. Experimental design

In this study, we compared embryonic developmental competence between two-step chemically defined medium (D1-D2) and mSOF, by incubating presumptive zygotes produced via in vitro fertilization in the two media. Cleavage rates, morulae compaction, blastocyst formation and blastocyst hatching were observed in both groups with regard to the stage codes by IETS. Total cell numbers of three developing blastocysts of codes C7, C7H and C8 from each group were counted after staining the nuclei with Hoechst 33342 (Sigma-Aldrich). The relative abundance of gene expression (IFNτ, ETS2, CDX2, GATA2, GATA3, OCT4 and NANOG) was evaluated and represented as a ratio to that of GAPDH gene expression. Three embryos from each stage were subjected to RT-PCR and qPCR with repeating each sample three times. In real-time RT-PCR, the relative quantification of each gene expression (IFNτ, OCT4 and CDX2) was compared to the

calibrator which is the relative quantity of the target gene of C4 stage with its GAPDH expression in both experimental groups.

2.7. Statistical analysis

In each experimental group, presumptive zygotes were randomly distributed. All data were subjected to one-way ANOVA followed by Tukey test to determine differences among the two experimental groups using GraphPad Version 4.0 [31]. Statistical significance was determined when a P-value was less than 0.05.

3. Results

3.1.Embryo development and fate of blastocysts after IVC in the two-step chemically defined media and mSOF

A total of 184 and 170 presumptive zygotes were incubated in the two-step culture system and mSOF, respectively (Table 2). There was no difference in cleavage rate (78.3% vs 74.7%) and morulae compaction (50.5% vs 45.3%) between the two groups (Table 2). However, the blastocyst yield was significantly increased in D1-D2 medium than that of mSOF (33.7% vs 18.8%) and significantly more blastocysts hatched in D1–D2 medium (52.3% vs 33.5%).

3.2. Total cell numbers of blastocysts after IVC in the two-steps chemically defined media and mSOF

After blastocyst formation, expansion (C7) and while hatching (C7H); there was no significant difference between the two groups (Table 2 and Fig. 1B and C), while the total cell number of blastocysts was higher in D1–D2 medium after hatching (C8) than that of mSOF (140 ± 8.25 vs 122 ± 6.42; $P < 0.05$).

3.3. Semi-quantitative analysis and relative quantification of transcripts among the different stages of embryonic development

In order to analyze the semi-quantitative differential expression of the eight designated genes, the level of GAPDH expression from single embryo compared to the expression of the other genes by pixel comparison using imaging analysis program. The gene expression in embryos cultured in two-step culture medium as shown in figure 2A were; IFNτ and CDX2 showed an even increase in expression then CDX2 showed slight decrease in expression at the stage of blastocyst hatching while OCT4 and NANOG showed decreased expression with the advancement of embryo growth. GATA2 and GATA3 expression were low in embryos until the mid-blastocyst stage but became high after blastocyst expansion. However, the gene expression of

Table 2
Embryonic development, total blastocyst cell number and fate of blastocyst after IVC of bovine IVF produced embryos in chemically defined two-step medium (D1-D2) and mSOF*

Item		Two-step culture system			*mSOF*		
Zygote		184			170		
**Cleaved (%)		144 (78.3)			127 (74.7)		
**Morulae (%)		93 (50.5)			77 (45.3)		
BL* (%)		62 (33.7)[a]			32 (18.8)[b]		
**BL hatching (%)		52.3[a]			33.5[b]		
BL total cell number							
C7		97 ± 9.21			93 ± 8.88		
C7H		119 ± 11.34			113 ± 6.69		
C8		140 ± 8.25[a]			122 ± 6.42[b]		
	Embryo stage	IFNτ	OCT4	CDX2	IFNτ	OCT4	CDX2
RQ*** of gene expression	C4	1	1	1	1	1	1
	C5	1.16	2.05	0.95	1	0.94	0.99
	C6	2.14	1.86	1.15	2.43	1.45	1.01
	C7	2.89	1.57	1.3	2.45	0.95	1.03
	C7H	3.18	0.65	1.33	2.5	1.37	1.08
	C8	3.59	0.38	0.97	1.28	1.83	1.07

[a,b] Values for different superscripts in the same row are significantly different ($P < 0.05$).
* mSOF, modified synthetic oviductal fluid.
** Cleaved, Morulae, BL and BL hatching were recorded on Day 2, 5, 7 and 9, respectively.
*** BL, Blastocyst; ***RQ, realtive quantitification by real-time RT-PCR.

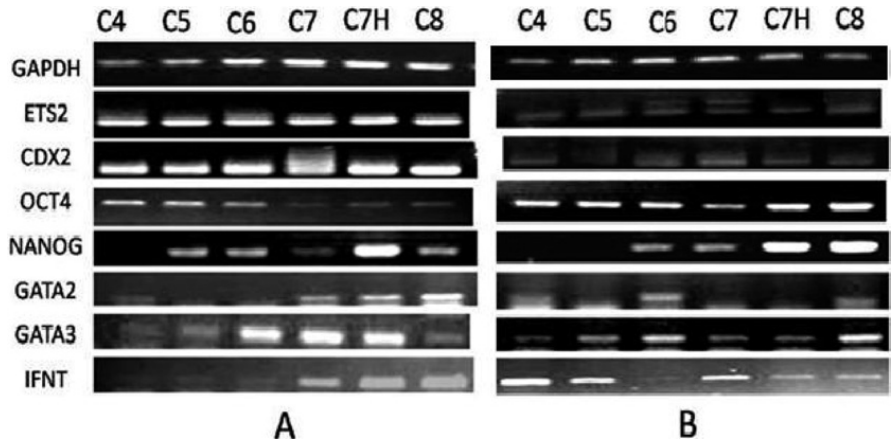

Fig. 2. Representative gel photographs of the mRNA expression of GAPDH, ETS2, CDX2, OCT4, NANOG, GATA2, GATA3, and IFNτ in embryos cultured in (A) two-step culture system and (B) in mSOF medium.

mSOF embryos (Fig. 2B) were; IFNτ and CDX2 showed increased expression until the blastocyst expansion and mid-blastocyst respectively then they showed decrease in expression after these stages while OCT4 behaved in inverse manner to IFNτ. NANOG showed an even increase with the advancement of blastocyst growth. Both GATA2 and GATA3 showed intermittent expression with tendency to increase after blastocyst hatching. ETS2 expression level showed no difference among the stages of embryonic development between the two groups. Relative quantification (RQ) of the IFNτ, OCT4 and CDX2 was confirmed by qPCR analysis and the results were found to be similar to that of RT-PCR (Table 2).

3.4. Temporal dominance of the genes at each stage of embryo development in relation to IFNτ

We summarize the relative gene expression after comparing the pixel of each gene with its expression along the stage of embryo development (Fig. 3 and Table 3). In the two-step culture system, the prominent gene in both stages C4 and C5 was OCT4 then GATA3 was added versus OCT4 in C6. At C7 CDX2 added to the prominent genes. At C7H CDX2 became a stable then at C8 GATA2 and added to the prominent genes. IFNτ showed even increase in expression with the blastocyst advancement. However, in case of mSOF medium; the prominent gene in both stages C4 and C5 was OCT4 like the two-step culture medium, GATA3 and CDX2 added versus OCT4 in C6 and then they became the prominent genes at C7. At hatching blastocyst OCT4 resumed its expression with NANOG then at C8 GATA2 and GATA3 began to express again. IFNτ showed increased expression until C7 when it showed a decrease in expression with blastocyst hatching (C7H).

4. Discussion

In our previous study, IVP embryos derived from chemically defined medium have showed comparable development *in vitro* and higher efficiency of calving rate compared to undefined culture medium, mSOF [5]. This chemically defined medium can allow us to produce safer embryos without unknown pathogens in FBS or BSA. Here, it is confirmed again that the importance of using the chemically defined two-step culture system for IVC of bovine IVP embryos because of the elevated blastocyst yield, cell number and hatching rate. Furthermore, this system of IVC demonstrated the harmonic and orchestrated interplay among the genes expressed at each embryo developmental stage responsible for the embryonic-maternal interaction, especially the IFNτ signal. According to Hernandez-Ledezma et al [32], they suggested that the best way to study IFNτ expression as an indicator of embryo quality is the temporal expression than the absolute expression at a particular stage because the latter is known to vary widely.

Gene expression between the two groups showed marked variation in their behaviors (Fig. 2 and 3) and this variation is surely due to the culture environment [5,7–10,33–35]. IFNτ expression was even and progressively increased with the advancement of embryonic development in two-step chemically defined system while it showed a decrease in expression by the blastocyst hatching in mSOF medium. This finding is consistent with our result in blastocyst quality and reflects the developmental competence and its ability to

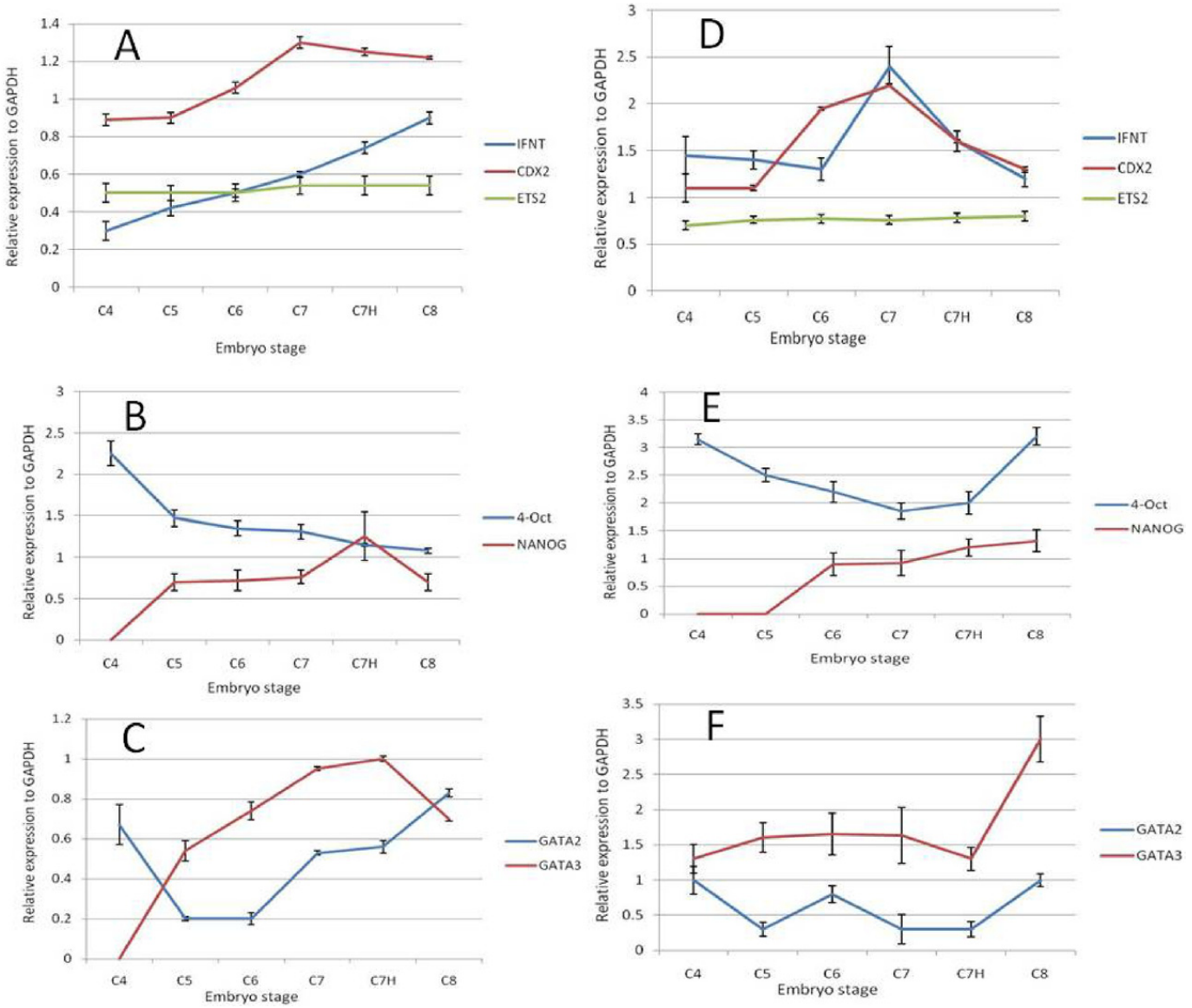

Fig. 3. Densiometric pixel comparison of relative gene expression between two-step culture medium (A, B, C) and mSOF medium (D, E, F).

establish pregnancy after embryo transfer (ET) [5,16,17,36]. From the studied genes, ETS2, this was firstly recorded as an essential gene for IFNτ promoter activation [23]. We found that ETS2 expression accompanied IFNτ expression but without a difference in its relative expression either among the stages of embryonic development or between the two experimental group confirming that ETS2 is a basic component of IFNτ expression regardless its relative expression. GATA2 and GATA3 transcriptional factors were described as regulators involved in IFNτ expression [26]. We found that GATA2 and GATA3 varied even with the embryonic stages of the same culture but they seem to be increased toward the blastocyst hatching in both experimental groups. This suggests the supportive effect of GATA2 and GATA3 for increase IFNτ transcription in cumulative and overlapping pattern [37–39]. CDX2 expression was found to be directly related to IFNτ expression along the embryo development until the hatching stage in two-step chemically defined culture system where it tends to be slightly decreased suggesting that CDX2 is a potent regulator of IFNτ expression [25,40–42]. OCT4 and NANOG were found to be inversely affected the IFNτ expression in both experimental groups. In the two-step chemically defined culture system, OCT4 expression becomes minimal with the hatched blastocyst stage that is a good sign of blastocyst quality [43] but in case of mSOF, OCT4 resumed its high expression with blastocyst hatching. It appears from these results that many genes are involved with IFNτ expression, some of them are directly related (CDX2 and GATA2 combined with

Table 3
Relative pixel folds comparison of the analyzed RT-PCR gel photographs of embryos in vitro cultured in the two culture systems

Gene	Two-steps culture system						mSOF medium					
	C4	C5	C6	C7	C7H	C8	C4	C5	C6	C7	C7H	C8
ETS2	+	+	+	+	+	+	+	+	+	+	+	+
CDX2	+	+	+	+++	++	++	+	+	++	+++	+	+
OCT4	+++	++	++	++	+	+	+++	++	++	+	++	+++
NANOG	−	+	+	+	++	+	−	−	+	+	++	+++
GATA2	+	−	−	+	+	++	++	−	++	−	−	++
GATA3	++	+	++	++	+++	++	+	++	++	++	+	+++
Prominent gene/s	OCT4	OCT4	OCT4 GATA3	OCT4 CDX2 GATA3	CDX2 GATA3 NANOG	CDX2 GATA2 GATA3	OCT4	OCT4	OCT4 CDX2 GATA3	CDX2 GATA3	OCT4 NANOG	OCT4 NANOG GATA3
IFNτ	+	+	++	++	+++	++++	+	+	+	+++	++	+

GATA3) and other genes are inversely related (OCT4 and NANOG) to IFNτ expression. From these results, we can say that the interplay of gene expression in the two-step chemically defined culture system showed consistent and orchestrated interaction resulting in an increase of IFNτ expression and subsequent IFNτ secretion which is essential indicator of blastocyst developmental competence. To clarify which gene is the dominant over others, blastocysts hatching stage (C7H) answering this question because we found that CDX2 is directly related to IFNτ but begins to be slightly decrease with this stage, but IFNτ is still dramatically increased. So, the decrease in OCT4 leads the way for the other genes to increase IFNτ like CDX2, GATA2 and GATA3. Niwa et al [29] showed that OCT4 is a potent negative regulator of CDX2 and Chen et al [42] showed that OCT4 directs its subservient, NANOG, to suppress CDX2 expression. This result is also confirmed in mSOF culture in where the maximal level of OCT4 and NANOG the level of CDX2 was the minimal and even GATA2 or GATA3 were unable to increase the IFNτ after blastocyst hatching. Our study suggests the presence of glucose and other additives (PVA, myoinositol and phosphate) in two-step culture medium as our previous published work causes harmonic gene expression especially for the IFNτ, OCT4 and CDX2. Addition of some nutrients in the 2nd stage of culture stables the gene expression of these genes with blastocysts development. Especially CDX2, which showed marked decrease in mSOF medium while it still stable in two-step culture sysyetm. The stable expression of CDX2, as a marker of trophoblast, might be due to the presence of glucose in the 2nd stage of two-step culture medium as glucose is essential for trophoblast [44,45], while in mSOF, these genes were disturbed due to lack of such nutrients.

In conclusion, we showed the importance and the efficiency of using two-step chemically defined media as a good tool for bovine embryos *in vitro* culture system because of: 1) Increased blastocyst yield; 2) Increased blastocyst cell number; 3) Increased blastocyst hatching rate; and 4) Harmonic and orchestrated gene interplay reflected by progressive increase in IFNτ expression which is a good sign for blastocyst developmental competence. Finally, we described the dominance of OCT4 over all the other transcripts in regulation of IFNτ expression and subsequent IFNτ secretion.

Acknowledgments

We thank Dr. Dirk K. Vanderwall (New Bolton Center, University of Pennsylvania) for his assistance in reviewing and editing this manuscript. This study was supported by grants from Research Institute for Veterinary Science, ARPC (#109023-05-1-CG000), NRF (#M10625030005-09N250300510) and (#M10625030005-09N250300510). The authors are grateful for the graduate fellowship provided by Islamic Development Bank, Jeddah, Saudi Arabia and Korean MEST through the BK21 program for veterinary science.

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