Differences in developmental competence and gene expression profiles between buffalo (*Bubalus bubalis*) preimplantation embryos cultured in three different embryo culture media

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Abstract The objective of this study was to compare effects of in vitro culture systems on embryonic development and expression patterns of developmentally important genes in preimplantation buffalo embryos. After IVM/IVF presumptive zygotes were cultured in one of three systems: undefined TCM-199, mCR2aa medium supplemented with 10 % FBS and defined PVA–myo-inositol–phosphate–EGF medium. No ($P > 0.05$) differences at 2-cell, 4-cell and 8-cell to 16-cell stages were observed among the three cultured media used, however, increased ($P < 0.05$) blastocyst yield, cell number and hatching rate were found in defined medium compared to undefined media. The expression patterns of genes implicated in embryo metabolism (*GLUT-1*), anti-apoptosis (*BCL-2*), imprinting (*IGF-2R*), DNA methylation (*DNMT-3A*) and maternal recognition of pregnancy (*IFNT*) were increased ($P < 0.05$) in hatched blastocysts derived from defined medium compared to undefined media. In conclusion, serum-free, defined medium improved developmental competence of in vitro cultured buffalo

embryos. Whether these differences in morphological development and gene expression have long-term effects on buffalo calves born after embryo transfer remains unknown. However, it is possible that early adaptations of the preimplantation embryo to its environment persist during fetal and post-natal development.

Keywords Buffalo · In-vitro fertilization · Embryo · Culture media · Expression profile

Introduction

Madan et al. (1991) reported the first birth of buffalo (*Bubalus bubalis*) calf from an in vitro fertilized oocyte. This land mark achievement led to production of a large number of buffalo embryos and pregnancies from abattoir-derived ovaries, but the success rate was low (Gasparrini 2002; De Rosa et al. 2005; Madan 2005; Hufana-Duran et al. 2007). In comparison to cattle, buffaloes show a similar maturation rate (94 vs 87 %, respectively), but a significantly lower cleavage rate (84 vs 65 %, respectively) (Gasparrini 2002; Neglia et al. 2003). Also, the blastocyst rate of buffalo is poor (20 %) compared to cattle (35–48 %) (Liang et al. 2007). This limits the applicability of in vitro embryo production (IVEP) technology in this species (Sadeesh 2015). It is therefore important to improve IVEP so it can be widely used in this species.

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Abundant evidence presented over the last decade indicates that developmental potential of embryos *in vitro* is mainly dependent on oocyte quality from which the embryo originates (Sirard et al. 2003; Rodriguez and Farin 2004) and the culture environment to which embryos are exposed (Lonergan et al. 1999; Van Soom et al. 2002; Yuan et al. 2003).

In order to improve *in vitro* culture systems for bovine blastocyst production, culture media have been strengthened with a variety of antioxidants, growth factors and/or macromolecules. Among the latter, bovine serum albumin (BSA) and fetal bovine serum (FBS), which are widely extensively used as protein sources for embryo culture media, are complex and undefined mixtures of proteins, growth factors and peptides. Serum and BSA can have a stimulatory effect on embryo growth (Kane and Headon 1980; Gardner 1994; Pinyopummintr and Bavister 1994). Thompson (2000) clearly demonstrated that BSA plays a substantial nutritive role, especially during morula development. Although BSA has beneficial effect on embryo development, it is difficult to elucidate specific functions of growth factors or other stimulants as BSA is an undefined mixture of compounds. The presence of serum in a standard culture medium alters the pattern of mRNA expression (Wrenzycki et al. 1999; Rizos et al. 2003), affects embryo development (Gutierrez-Adan et al. 2001) and cryotolerance (Rizos et al. 2003), is associated with a reduction in pregnancy rate after transfer to recipients (Lazzari et al. 2002), and may produce longterm effects on post-natal development and behaviour (Fernández-Gonzalez et al. 2007). Chemically defined conditions without serum or serum derived proteins allow more precise observation of effects of growth or other embryo growth factors in a given medium (Giles and Foote 1997; Sirisathien et al. 2003; Oyamada et al. 2004). It was demonstrated that chemically defined medium supported bovine zygote development to blastocysts with varying rates of success (Keskinetepe et al. 1995; Keskinetepe and Brackett 1996; Lim et al. 2007).

In nearly all studies on buffalo IVEP, presumptive zygotes are subject to an *in vitro* culture (IVC) system, involving use of TCM-199, a complex medium co-culture with buffalo oviductal epithelial cells or granulosa cells and supplemented with serum, even though it has been demonstrated that primary oviduct (Mugnie et al. 2009) and granulosa cell (Nottola et al.

2006) cultures secrete a series of factors that act in a paracrine manner stimulating development to the blastocyst stage. This system is complex and associated blastocyst yield is very low. Sub-optimal laboratory culture conditions for media preparation have been documented by Chauhan et al. (1998) for buffalo *in vitro* fertilized embryos. These authors reported that the quality of water and chemicals used in media preparation may adversely affect *in vitro* embryo development. Nandi et al. (2003) used media such as synthetic oviductal fluid (SOF) and Charles Rosenkrans (CR) medium for *in vitro* culture of buffalo embryos, but no report is available to ascertain which of these media to support the development of buffalo zygotes to morula and blastocysts stages. Elamaram et al. (2012) reported that use of suboptimal culture conditions is one of the primary reasons for low blastocyst rate during IVEP in buffalo. Simple media such as SOF and CR were found capable of supporting development of buffalo zygotes to blastocyst stage even in the absence of co-culture with somatic cells. However, these media not fully mimic *in vivo* conditions on embryo development. In addition a BSA- and FBS-free defined culture medium to enhance blastocyst production has not been used in buffalo as reported in cattle (Lim et al. 2007).

Superior quality embryos classified according to morphological criteria may display altered developmental capacities with only a certain percentage of these embryos capable of establishing pregnancy after transfer into recipients (Jousan et al. 2008). Therefore, qualitative or morphological assessment alone is not sufficient to provide accurate and efficient estimation of embryo quality and embryonic developmental potential (Wrenzycki et al. 2004). Precise control of gene expression during the preimplantation phase of development is particularly important. Thus, analysis of expression patterns of developmentally important genes essential in early development provides a useful tool to assess quantitative description of produced embryos and to optimize *in vitro* embryo production and related assisted reproduction technologies. Several major developmental events occur during this period, including the first cleavage division, the timing of which is important in determining subsequent embryo development; embryonic genome activation and morula compaction which involves establishment of the first intimate cell-to-cell contact in the embryo and blastocyst formation involving differentiation of

two cell types, the trophectoderm and inner cell mass (Bavister 1995; Lonergan et al. 1999). Many of the differences in embryo quality can be attributed to culture environment medium-induced changes in mRNA abundance (Wrenzycki et al. 1996; Corcoran et al. 2006). Studies on the embryonic genome indicate that currently used in vitro embryo development conditions cannot fully mimic in vivo conditions with regard to mRNA expression. During the culture period, the presence of serum (10 %) can induce a number of subtle modifications in blastocyst development, including increased expression of stress responsive genes and decreased expression of embryo quality genes in bovine (Rizos et al. 2003; Tavares et al. 2008). Similarly, no report is available regarding the efficacy of different embryo culture media to support effective expression of developmentally important genes to increase the efficiency of healthy embryo production in buffalo under in vitro culture. Therefore, the present investigation compared the developmental competence and mRNA expression of developmentally important genes in buffalo IVF embryos cultured in undefined TCM-199 and mCR2aa media containing 10 % FBS and serum-free, defined medium containing polyvinyl alcohol (PVA)–myo-inositol–phosphate–epidermal growth factor (EGF). This study of inhibitory/stimulatory effects of different culture conditions on developmental competence and expression of candidate genes involved in embryo development helps elucidate the role of post-fertilization culture medium on in vitro developmental characteristics of the buffalo embryo. Thus, it may be possible to establish a suitable culture system which encourages greater in vitro development of embryos for commercial application of this technology in buffaloes.

Materials and methods

Chemicals and reagents

Chemicals and media were purchased from Sigma Chemical Co. (St. Louis, MO, USA) unless otherwise indicated. Most chemicals used in the present study were of embryo or cell culture tested grade. Fetal bovine serum (FBS) was from Hyclone (Thermo Scientific, Wilmington, DE, USA), FBS was from the same batch throughout the study. Disposable four-well

multi-dishes and six-well tissue culture plates were procured from Nunc (Roskilde, Denmark). Membrane filters (0.2 µm) were sourced from Pall Life Sciences (Pall Corporation, Ann Arbor, MI, USA). The reagents for molecular biology work (mRNA extraction, cDNA synthesis, and gene amplification) were of biotechnology grade and purchased from Invitrogen (Carlsbad, CA, USA) and Fermentas (Waltham, MA, USA) unless otherwise indicated.

Collection of oocytes

Ovaries from reproductive organs of adult, apparently healthy, female buffaloes were collected from an abattoir within 30 min of slaughter and washed three times with warm isotonic saline (37 °C) containing 400 IU/mL penicillin and 500 µg/mL streptomycin, then transported to the laboratory within 4 h. Aspiration of cumulus oocyte complexes (COCs) was performed as described earlier (Sadeesh et al. 2014). Briefly, oocytes from follicles (2–8 mm) were aspirated with an 18 gauge needle attached to a 10 mL syringe (Sigma Chemical Co., #Z248029) containing aspiration medium (TCM-199 with 0.3 % BSA, 0.1 mg/mL glutamine and 50 µg/mL gentamicin). Oocytes were washed four to six times with washing medium which consisted of TCM-199 with 10 % FBS, 0.09 mg/mL sodium pyruvate, 0.1 mg/mL L-glutamine and 50 µg/mL gentamicin. The COCs selected for in vitro maturation (IVM) had compact and unexpanded cumulus mass with equal to or greater than three layers of cumulus cells and homogenous granular ooplasm.

In vitro maturation

The IVM of COCs was performed as described earlier (Sadeesh et al. 2014). Briefly, the COCs were subject to maturation in IVM medium consisting of TCM-199 + sodium pyruvate (0.80 mM) + L-glutamine (2 mM) + 10 % FBS + 5 % follicular fluid (FF) + pregnant mare's serum gonadotropin (PMSG, 20 IU/mL) + human chorionic gonadotropin (hCG, 10 IU/mL) + gentamicin (50 µg/mL). The pH of the medium was adjusted to 7.4 and the medium was filtered through a 0.22 µm membrane immediately before use. The COCs were washed several times with IVM medium and groups of 15–20 COCs were placed independently in 100 µL droplets of IVM medium

covered with sterilized mineral oil in 35 mm petri dishes and cultured for 24 h under 5 % CO₂ at 38.5 °C.

Sperm capacitation

Spermatozoa were capacitated using Brackett and Oliphant medium (Brackett and Oliphant 1975). Frozen-thawed semen from the same bull was used in all experiments. Frozen semen from buffalo bulls stored in 0.25 mL straws were thawed in a water bath at 37 °C for 1 min. Spermatozoa were washed twice at 1430 g for 5 min using semen washing solution of BO medium supplemented with 10 µg/mL heparin, 137 µg/mL sodium pyruvate and 1.942 mg/mL caffeine sodium benzoate. The pellet was re-suspended in around 0.5 mL of the washing BO medium and sperm number was counted using hemocytometer and number of spermatozoa were adjusted to 2×10^6 /mL.

In vitro fertilization (IVF)

For in vitro fertilization, matured oocytes were washed twice in solution of BO medium supplemented with 10 mg/mL of fatty acid free BSA and transferred to 50 µL droplets of capacitation and fertilization BO medium. Spermatozoa in 50 µL of the capacitation and fertilization BO medium was then added to the droplet containing oocytes and covered with sterile mineral oil and placed in CO₂ incubator at 38.5 °C for 18 h for IVF.

In vitro culture (IVC)

Following sperm-oocyte incubation, cumulus cells were washed from oocytes by gentle pipetting. Presumptive zygotes, i.e. (presence of two polar bodies within the perivitellin space), were separated from sperm droplets, divided into three groups and cultured in one of the following three IVC media: (1) TCM-199 + 10 % FBS (2) mCR2aa + 10 % FBS (3) SOFaa + 0.1 mg/mL PVA + 2.77 mM myo-inositol + 1.2 mM phosphate + 10 ng/mL EGF. Zygotes were cultured for up to 8 days post insemination in a humidified CO₂ incubator (5 % CO₂ in air) at 38.5 °C. The culture medium was refreshed every 48 h by replacing half of original medium with similar volume.

Embryo production rate from each group was examined as reported earlier (Sadeesh et al. 2015a).

Randomly collected hatched blastocysts were stained with Hoechst 33342 for 1 h and number of nuclei counted.

Real-time PCR for relative quantification

Morphologically normal embryos at 2-cell, 4-cell, 8-cell to 16-cell, morula and hatched blastocyst stage (n = 10 each) from undefined and defined media in each trial were used for preparation of cDNA using a cDNA kit (Ambion Inc, The RNA Company, Austin, TX, USA) according to the manufacturer's protocol. Due to transcript level variations between early and late cleaved embryos (Roth and Hansen 2002), only early cleaved embryos were collected in each group. Embryos were washed with 200 µL ice cold PBS after which 50 µL of chilled cell lysis buffer was added and the mixture was incubated at 75 °C for 10 min in a thermal cycler. Genomic DNA was degraded by incubating cell lysates in DNase-I at 37 °C for 30 min and remaining activity of DNase-I was inactivated by heating at 75 °C for 5 min. For cDNA synthesis, 10 µL of cell lysates (RNA), 4 µL deoxynucleoside triphosphate (dNTP) mix (2.5 mM each dNTP), 2 µL random decamer were added to a 200 µL PCR tube. The reaction mixture was incubated at 70 °C for 1 min to denature RNA for easier primer binding in a thermal cycler, the tubes were immediately cooled on ice and the following reverse transcriptase reagents were added: 2 µL 10× RT buffer, 1 µL MMLV reverse transcriptase and 1 µL RNase inhibitor. The reaction mixture was incubated in a thermal cycler at 42 °C for 60 min and 95 °C for 10 min to inactivate reverse transcriptase. Synthesized cDNA was stored at −80 °C until used for amplification.

The target genes were selected taking into account their roles in transport and glucose metabolism (*GLUT-1*), oxidative stress (*MnSOD*), apoptosis (*BAX* and *BCL-2*), imprinting (*IGF-2* and *IGF-2R*), DNA methylation (*DNMT-3A*) and maternal recognition of pregnancy (*IFNT*). Real-time PCR (Applied Biosystems 7500 Real-Time PCR system, Foster City, CA, USA) was performed using SYBR Green qPCR supermix (Invitrogen SYBR Green qPCR supermix: Carlsbad, CA, USA) as a double-standard DNA specific fluorescent dye in 25 µL reaction to assess gene expression of *GLUT-1*, *MnSOD*, *BAX*, *BCL-2*, *IGF-2*, *IGF-2R*, *DNMT-3A* and *IFNT*. Validation

studies were performed using *GAPDH* as the internal reference gene and *GAPDH* expression was consistent across various stages between embryos derived from different IVC media. All reactions were run in triplicate and three biological replicates were carried out. Amplification was carried out in 25 μ L volume reaction mixture containing 12.5 μ L SYBR Green qPCR mastermix, 1 μ L primer (10 pM each forward and reverse primer), 2 μ L cDNA template and 9.5 μ L nuclease free water. Samples not exposed to reverse transcriptase (RT) were used as negative controls. For PCR, samples were activated at 95 °C for 10 min, followed by 40 cycles of denaturing at 95 °C for 45 s, then annealing at the specific primer annealing temperature (Table 1) for 30 s and extension at 72 °C for 30 s. The comparative cycle threshold (CT) method was used for relative quantification of target gene expression levels. Quantification was normalized to the internal control *GAPDH* gene. Within the exponential phase of the amplification curve, each cycle doubled the amplified product. The Δ CT value was determined by subtracting the *GAPDH* CT value for each sample from the target gene CT value. Calculation of $\Delta\Delta$ CT value involved using the

highest sample method Δ CT as an arbitrary constant to subtract from all other Δ CT samples values. Fold changes in relative mRNA expression of target genes were determined using the formula $2^{-\Delta\Delta CT}$ (Livak and Schmittgen 2001).

Experimental designs

The present study comprised three experiments. In experiment 1, in order to compare embryo developmental competence between undefined and defined media, after in vitro fertilization, presumptive zygotes were cultured in three different media (TCM-199 + 10 % FBS; mCR2aa + 10 % FBS; SOFaa + 0.1 mg/mL PVA + 2.77 mM myo-inositol + 1.2 mM phosphate + 10 ng/mL EGF). In experiment 2, after evaluating the effect of PVA, myo-inositol, phosphate and EGF in defined medium, presumptive zygotes were cultured in defined medium without EGF to provide insight as to whether PVA, myo-inositol, phosphate and EGF combinations maximize developmental rates in serum-free, defined medium. In experiment 3, to assess the quality of preimplantation embryos cultured in the defined and undefined media

Table 1 Details of primer sequence

Gene name	Sequence (5'–3')	Fragment size (bp)	Annealing temp (°C)	GenBank accession no.
<i>GAPDH</i>	Forward	CCTGCCAAGTATGATGAGA	54	GU324291
	Reverse	GAAGGTAGAAGAGTGAGTGT		
<i>GLUT-1</i>	Forward	CCGTCTCTTCCTATCCAA	55	HQ434959
	Reverse	GGTCTTCTTGAATAGTGAGTT		
<i>MNSOD</i>	Forward	GGGCCATATCAATCACAG	55	JN687584
	Reverse	GTCCCTGCTCCTTATTGA		
<i>BAX</i>	Forward	ATCCACCAAGAAGCTGAG	54	HE661581
	Reverse	CTGCGATCATCCTCTGTA		
<i>BCL-2</i>	Forward	GGCCCCTGTTTGATTCT	55	U92434
	Reverse	CTTATGGCCCAGATAGGC		
<i>IGF-2</i>	Forward	CTTCAGCCGACCATCCAG	55	FJ032306
	Reverse	GGGGTGGCACAGTAAGTC		
<i>IGF-2R</i>	Forward	GCAGATTTATTTCTTCTCCAC	55	J03527
	Reverse	CACTCAAACCTCGTAGAAGCA		
<i>DNMT-3A</i>	Forward	GACAAGAATGCCACCAAAGC	55	AY271298
	Reverse	ATCCACCAAGACACAATGCG		
<i>INFT</i>	Forward	GACTTTGCTTTCCTCAG	54	JQ343920
	Reverse	GTGTGGAAGAGGTTGAAG		

in terms of gene expression related to preimplantation development, the relative abundance of gene expression (*GLUT-1*, *MnSOD*, *BAX*, *BCL-2*, *IGF-2*, *IGF-2R*, *DNMT-3A* and *IFNT*) was examined.

Statistical analysis

Data were analysed using GraphPad Prism (version 6.05). Experimental results were presented as mean $\pm$ SEM (standard error mean). Data were subject to analysis of variance (ANOVA), and the Tukey test was used to identify if the differences between means were statistically significant ($P < 0.05$).

Results

Development rates in vitro

A total of 577, 512 and 527 presumptive zygotes were incubated in the undefined TCM or mCR2aa media containing FBS and defined SOFaa medium containing PVA, myo-inositol, EGF and phosphate as energy source, respectively, in an eight replicate experiment (supplementary table). There were no morphological differences between the embryos in different media (not shown). Development rates of buffalo IVF embryos generated in the different systems are summarized in Table 2. No differences ($P > 0.05$) in the number of embryos at 2-cell, 4-cell and 8- to 16-cell were observed amongst the experimental groups. In vitro fertilized embryos derived from the defined medium produced higher ($P < 0.05$) numbers of

embryos at the morula stage than from the undefined TCM medium. However, no differences ($P > 0.05$) were observed between defined and undefined mCR2aa media. While the blastocyst yield in the defined medium was similar to that in undefined mCR2aa medium, it was significantly higher than that in undefined TCM-199 medium. An increased ($P < 0.05$) number of blastocysts hatched in defined medium than in undefined media. The total cell number of hatched blastocysts was increased ($P < 0.05$) in defined medium than undefined media. Supplementing the defined medium with EGF stimulated blastocyst yield, hatched blastocyst and cell number compared to its absence in defined medium (Table 3).

Analysis of mRNA expression

Relative abundance of developmentally important genes transcripts in buffalo embryos derived from undefined and defined media are shown in Fig. 1.

Gene related to transport and metabolism of glucose

Embryos cultured in vitro under undefined and defined culture media affected mRNA abundance of *GLUT-1*. There was no difference ($P > 0.05$) between defined and undefined mCR2aa media embryos in expression of *GLUT1* at the 4-cell, 8- to 16- cell stages. Relative abundance (RA) of *GLUT-1* transcript in defined medium embryos was greater ($P < 0.05$) than that of undefined media embryos at morula and hatched blastocyst stages. At all developmental stages expression of *GLUT-1* transcripts was increased ($P < 0.05$) in

Table 2 Developmental competence of in vitro produced buffalo embryos cultured in undefined and defined media

	Undefined TCM-199	Undefined mCR2aa	Defined
No. of presumptive zygotes cultured	577	512	527
No. of 2-cell (% $\pm$ SEM)	301 (61.9 $\pm$ 14.2) ^a	247 (63.6 $\pm$ 13.5) ^a	344 (68.2 $\pm$ 9.3) ^a
No. of 4-cell (% $\pm$ SEM)	217 (46.6 $\pm$ 10.9) ^a	184 (47.5 $\pm$ 16.9) ^a	264 (52.0 $\pm$ 7.1) ^a
No. of 8- to 16 -cell embryo (% $\pm$ SEM)	109 (22.5 $\pm$ 4.2) ^a	104 (26.3 $\pm$ 5.9) ^a	169 (32.1 $\pm$ 5.6) ^a
No. of morula (% $\pm$ SEM)	78 (14.6 $\pm$ 3.3) ^a	82 (20.9 $\pm$ 5.0) ^{ab}	129 (25.7 $\pm$ 4.1) ^b
No. of blastocyst (% $\pm$ SEM)	43 (8.2 $\pm$ 3.2) ^a	61 (15.8 $\pm$ 4.1) ^{ab}	102 (20.4 $\pm$ 2.5) ^b
No. of hatched blastocyst (% $\pm$ SEM)	13 (2.2 $\pm$ 1.3) ^a	23 (4.5 $\pm$ 1.4) ^b	74 (14.1 $\pm$ 3.6) ^c
Total cell number (% $\pm$ SEM)	108.4 $\pm$ 8.4 ^a	158.6 $\pm$ 6.8 ^b	186.3 $\pm$ 4.3 ^c

Different superscripts within rows denote differences ($P < 0.05$). Each group is pool of 8 replicates each. Embryos in relation to total oocytes cultured

Table 3 Effects of supplementation with myo-inositol and EGF on the in vitro developmental competence of buffalo embryos cultured in defined medium

	SOFaa + PVA + phosphate + myo-inositol	SOFaa + PVA + phosphate + myo-inositol + EGF
No. of presumptive zygotes cultured	168	174
No. of 2-cell (% $\pm$ SEM)	107 (63.7 $\pm$ 8.4) ^a	120 (68.5 $\pm$ 6.7) ^a
No. of 4-cell (% $\pm$ SEM)	82 (48.8 $\pm$ 6.1) ^a	90 (51.5 $\pm$ 4.1) ^a
No. of 8–16 cell embryo (% $\pm$ SEM)	48 (28.5 $\pm$ 7.6) ^a	61 (34.9 $\pm$ 4.1) ^a
No. of morula (% $\pm$ SEM)	36 (21.4 $\pm$ 5.6) ^a	44 (25.1 $\pm$ 6.9) ^a
No. of blastocyst (% $\pm$ SEM)	16 (9.5 $\pm$ 2.1) ^a	36 (20.5 $\pm$ 5.8) ^b
No. of hatched blastocyst (% $\pm$ SEM)	14 (8.3 $\pm$ 1.2) ^a	25 (14.4 $\pm$ 1.8) ^b
Total cell number (% $\pm$ SEM)	138.4 $\pm$ 5.4 ^a	184.4 $\pm$ 3.7 ^b

Different superscripts within rows denote differences ($P < 0.05$). Each group is pool of 8 replicates each. Embryos in relation to total oocytes cultured

Fig. 1 Relative expression profile of developmentally important gene transcripts in buffalo embryos at a given stage of development obtained from TCM-199 (white bars), mCR2aa (dark grey bars) and defined (light grey bars) culture media.

a *GLUT-1*, **b** *MnSOD*, **c** *BAX*, **d** *BCL-2*, **e** *IGF-2*, **f** *IGF-2R*, **g** *DNMT-3A*, **h** *INFT*. The values are expressed as mean $\pm$ SEM. Within a developmental stage, bars without a common superscript differ significantly ($P < 0.05$)

the defined medium compared to undefined TCM media embryos. However, expression of *GLUT-1* in defined medium embryos was down-regulated until the 8- to 16- cell stage and up-regulated towards later developmental stages when compared to undefined media embryos.

Genes related to oxidative stress response

The RA of *MnSOD* gene steadily increased from 2-cell to 8- to 16- cell stages in undefined mCR2aa medium, whereas, in the defined medium the expression pattern followed a similar trend except it exhibited down-regulation at 2-cell, morula and hatched blastocyst stages. However, expression levels of *MnSOD* in the defined medium were low at all developmental stages when compared to undefined TCM and undefined mCR2aa media except at 4- and 8- to 16-cell stages embryos. No differences ($P > 0.05$) in RA of *MnSOD* were observed between defined and undefined media embryos at 4- and 8- to 16-cell stages, whereas, at morula and hatched blastocyst stages difference in transcript abundance between undefined mCR2aa and defined media embryos were significant. Transcription levels of *MnSOD* were higher ($P < 0.05$) from morula to hatched blastocyst stages in the undefined TCM medium compared to mCR2aa and defined media.

Pro-apoptosis and anti-apoptosis related genes

Greater ($P < 0.05$) mRNA abundance of *BAX* was observed for embryos cultured in the undefined TCM medium compared to undefined mCR2aa and defined media at 2-cell, morula and hatched blastocyst stages. The transcription levels of *BAX* were significant from morula to hatched blastocyst stages between undefined mCR2aa and defined media. However, relative level of *BCL-2* mRNA in defined and undefined media increased with embryonic development. It was found that mRNA expression of *BCL-2* increased ($P < 0.05$) in the defined medium compared to undefined media at 2-cell, 8- to 16-cell, morula and hatched blastocyst stages.

Gene related to imprinting

From the 4-cell stage, *IGF-2* expression level increased up to 8- to 16-cell stage and after that a

continuous decrease was observed in defined medium embryos. In embryos derived from the undefined media expression was greater ($P < 0.05$) compared to embryos derived from defined medium in all developmental stages, except at the 8- to 16-cell stage. Significant differences were found in *IGF-2R* mRNA transcript expression at all embryonic stages, except at 8- to 16-cell and morula stages of IVF embryos between undefined and defined media. *IGF-2R* mRNA transcript expression was consistently lower in undefined media embryos than in defined medium embryos.

Genes related to DNA Methylation

Although a similar pattern of *DNMT-3A* expression was observed in undefined and defined media for all embryonic stages, the defined medium resulted in greater ($P < 0.05$) *DNMT-3A* mRNA abundance compared with undefined media, particularly at the hatched blastocyst stage.

Gene related to maternal recognition of pregnancy

Relative abundance of *IFNT* was greater ($P < 0.05$) in hatched blastocysts derived from the defined medium as compared to undefined TCM and mCR2aa media.

The RT-PCR analysis of developmentally important gene amplifications in pre-implantation embryos is shown in supplementary data.

Discussion

Embryo culture media are chemically defined, but they are typically supplemented with additives like FBS, which are undefined. Thus, the resulting supplemented media are undefined. The undefined in vitro culture media supplemented with BSA and FBS contain a potential source of viral or other infectious pathogenic agents that may infect embryos (Avery et al. 1993; Springfellow and Wrathall 1995). The use of defined medium was introduced, to improve repeatability of the procedure and allow for metabolic studies, in addition to improving health and hygiene. In this study, we showed that by modifying and supplementing defined medium buffalo embryo developmental competency was improved as compared to undefined media.

The present study revealed IVC of presumptive buffalo zygotes in defined medium gave higher embryo developmental competence than those cultured in undefined media. These results are in agreement with those of Lim et al. (2007) who reported higher yield of transferable embryos in defined medium in bovine. Here, the importance of using serum-free defined culture system for IVC of buffalo embryos is confirmed by elevated blastocyst yield, hatched blastocyst and cell number.

In our study, defined medium contained PVA, myo-inositol, phosphate and EGF. Supplementation of myo-inositol, an isomer of a C6 sugar alcohol that belongs to the Vitamin B complex group, alone in defined medium had an effect on hatched blastocyst yield, but development was still lower than in defined medium containing both myo-inositol and EGF. Thus, the combined effects of myo-inositol and EGF might be a key of this defined medium in supporting growth of hatched blastocyst. Our results are consistent with other studies that show significant effect of EGF and myo-inositol on embryo developmental competence in bovine (Mtango et al. 2003; Lim et al. 2007). Another study reported that without phosphate, glucose alone had no detrimental effect on early embryonic development (Kim et al. 1993). In contrast, Barnett and Bavister (1996) and Biggers and McGinnis (2001) showed that in culture media containing glucose, phosphate inhibited embryo development to blastocyst stage. On the other hand, according to our study, defined medium containing the both glucose and phosphate has exhibited better embryo development compared to glucose alone in undefined media.

It has been shown that the use of assisted reproductive technologies such as IVF and somatic cell nuclear transfer (SCNT), can affect embryo quality in terms of gene expression and epigenetic markers in oocytes and embryos as compared with in vivo produced embryos (Niemann et al. 2002; Wrenzycki et al. 2007). In this study we analyzed expression of a panel of developmentally important genes in IVF embryos of different developmental stages cultured in undefined and defined media. Our results support the hypothesis that in vitro conditions have an effect on patterns of embryonic gene expression in buffalo, as reported in other species including bovine and mouse (Fernández-Gonzalez et al. 2004; Schwarzer et al. 2012; Arias et al. 2013). Furthermore, the study by Schwarzer et al. (2012), found that 13 different

embryo culture protocols resulted in distinct cellular and molecular phenotypes in mouse embryos. Certain culture medium components can interfere with the epigenetic regulation of genes. It is likely that gene expression analysis at preimplantation stages from different IVC media with different developmental potential may offer better insight regarding the reprogramming process. It is possible that gene expression analysis at different stages of preimplantation development in the present study correspond to gene expression analysis before and after maternal to zygotic transition (MZT) stage. The genes under study were selected because they participate in key cellular processes during preimplantation development and have been reported to become transcriptionally active during preimplantation stages (Li et al. 2006; Amarnath et al. 2007; Wrenzycki et al. 2007; Sadeesh et al. 2015a, b). The genes evaluated include genes involved in embryo metabolism (*GLUT-1*), stress response (*MnSOD*), pro-apoptosis (*BAX*), anti-apoptosis (*BCL-2*), imprinting (*IGF-2*, *IGF-2R*), DNA methylation (*DNMT-3A*) and quality (*IFNT*).

Cellular glucose incorporation into embryonic cells is mediated by *GLUT-1* and its expression has been detected during the entire preimplantation period (Lequarre et al. 1997; Bertolini et al. 2002). Studies in mouse show that for in vivo embryos *GLUT-1* decreased 50 % in in vitro blastocysts (Morita et al. 1992). This suggests that viable embryos have higher *GLUT-1* expression. In the present study, defined medium embryos exhibited greater *GLUT-1* expression levels at morula and hatched blastocyst stages than in undefined media counter parts, with variations depending on IVC conditions, in accordance with the observations of Niemann and Wrenzycki (2000) and Lazzari et al. (2002). Decrease in the relative expression of *GLUT-1* in undefined media hatched blastocysts supports the notion that undefined media embryos are inferior to defined medium embryos in their terms of ability to develop. Decreased glucose transporter expression triggers *BAX*-dependent apoptosis in murine blastocysts (Chi et al. 2000). Therefore, it is possible that the low level of *GLUT-1* expression observed in the present study in undefined media embryos is related to higher levels of apoptotic incidence in these embryos. Pantaleon et al. (2001) and Sadeesh et al. (2015a) demonstrated the importance of glucose transporters for early embryo development where down-regulation results in decreased

cell numbers. Based on our results, we inferred that buffalo embryos could detect and respond to defined and undefined IVC conditions by altering expression levels of *GLUT-1*, as previously described in bovine (Lim et al. 2007).

Reactive oxygen species (ROS) are indicated as one of the main factors responsible for low production and poor quality embryos produced in vitro (Takahashi et al. 2000). It has been observed that physiological levels of ROS are necessary for normal regulation of cell growth and development (Hancock et al. 2001). The *MnSOD* is an important enzyme involved in protection against ROS. Lequarre et al. (2001), confirmed culture environment-dependent expression of the *MnSOD* transcript. In the present study, irrespective of whether culture took place in defined or undefined medium, abundance of *MnSOD* mRNA tended to be non-significant from the 4-cell to 8- to 16-cell stage and expression of *MnSOD* was greater ($P < 0.05$) in embryos from undefined media at 2-cell, morula and hatched blastocyst stages compared to defined medium. In cattle, greater expression of *MnSOD* compared to IVF embryos has been noted, despite the acknowledged poor in vivo development potential of SCNT embryos (Amarnath et al. 2007). On the other hand, contradictory results have been observed more recently by Kim et al. (2012) who described no differences in expression levels of *MnSOD* between bovine SCNT and IVF embryos. Recently, in buffalo, higher expression of *MnSOD* has been detected in IVF-derived 8- to 16-cell, morula and blastocyst stage embryos compared with SCNT embryos (Sadeesh et al. 2015a). This divergence among experiments may be due to different protocols used to produce and culture embryos or different methods to assess gene expression profile. The lower developmental potential observed in undefined TCM-199 medium in this study leads us to suggest that embryos generated by this culture medium are of low quality, this fact is corroborated by higher expression of apoptosis genes in embryos cultured from undefined TCM-199 medium.

In several studies, mRNA expression levels of *BAX* have been used to evaluate embryo quality developed in vitro (Amarnath et al. 2007; Mundim et al. 2009; Shiqiang et al. 2010; Kim et al. 2012; Sadeesh et al. 2015b). Data from the current study indicate that *BAX* expression was greater ($P < 0.05$) in embryos produced from undefined media-containing serum than

those produced in its absence, suggesting that over expression of the *BAX* gene, in the cellular apoptotic pathway takes place (Yang and Rajamahendran 2002; Opiela et al. 2008). This non-regulation of *BAX* mRNA expression could cause low quality of embryos in undefined media. Similarly, Byrne et al. (1999) reported that the incidence of apoptosis increases in bovine embryos produced in the presence of serum. *BCL-2* is considered an anti-apoptotic gene and its expression level should be low when apoptotic incidence is high. Another significant finding from this study was that the level of *BCL-2* mRNA in defined medium increased ($P < 0.05$) at 2-cell, 8- to 16- cell, morula and hatched blastocyst stages than for embryos from undefined media. Yang and Rajamahendran (2002) demonstrated that good quality embryos have a greater concentration of *BCL-2* protein than *BAX* protein, whereas there is more *BAX* than *BCL-2* in lower quality embryos. Correspondingly, in this study the apoptotic incidence of embryos derived from defined medium at 2-cell, morula and hatched blastocyst stages were significantly lower than undefined media embryos.

The growth promoting gene (*IGF-2*) and receptor gene (*IGF-2R*) are among the best studied imprinted genes involved in fetal growth regulation and are essential for normal development. The *IGF-2* ligand is imprinted when inherited maternally and *IGF-2R* is imprinted when inherited paternally (Latham et al. 1994). The expression profiles of *IGF-2* and *IGF-2R* in IVF embryos in different embryo culture media were compared in this study. *IGF-2* gene expression was highest in undefined media-containing serum as compared with serum-free defined medium, suggesting that timing and magnitude of these gene alterations are significantly affected by the presence of exogenous protein in the culture medium in a stage-specific manner, predominantly at critical developmental points. The aberrant behaviour of *IGF-2* mRNA in IVF embryo culture groups may also be due to inappropriate genomic imprinting. Yet, there is little information available with regard to imprinting of this gene in buffalo IVF embryos. *IGF-2R* mRNA levels in 2-cell, 4-cell, and hatched blastocysts cultured in serum free-defined medium were higher ($P < 0.05$) than for undefined media-containing serum. Culture conditions of embryos, particularly the presence of serum in culture medium, could affect expression of this gene, as observed previously by Fernández-

Gonzalez et al. (2009) in mouse and Pandey et al. (2010) in buffalo SCNT. Giraldo et al. (2008) reported that abnormal pattern of transcript may be due to epigenetic modifications due to culture conditions, which directly affect chromatin remodelling and is correlated to imprinted genes. Differences were found in our study for embryos cultured in the presence and absence of serum, may suggest that other factors such as culture medium composition could be responsible for alterations of gene expression.

Earlier studies have revealed species-dependent expression patterns of DNA methyltransferase (*DNMT*) genes in mammalian oocytes and preimplantation embryos (Vassena et al. 2005). The data from the current study indicate that expression of *DNMT-3A* in hatched blastocysts derived from undefined media was reduced ($P < 0.05$) as compared to defined medium. This suggests that differential *DNMT-3A* mRNA levels in undefined media embryos have serious implications for preimplantation embryonic development and result in reduced blastocyst formation in undefined media derived embryos. Whether the consistently low efficiency of undefined media embryos are related to inability of nucleus to undergo normal changes in methylation as indicated by lack of *de novo* methylation triggered by low *DNMT-3A* expression remains unclear in buffalo. However, further studies need to be conducted regarding global levels of DNA methylation and histone acetylation in undefined and defined media embryos using in vivo derived embryos as a control before meaningful conclusions can be drawn in this species.

Studies have detected *IFNT* mRNA in blastocysts but not in morula (Hernandez-Ledezma et al. 1992, 1993; Wrenzycki et al. 1999), suggestive that the beginning of *IFNT* production is closely correlated to blastocoel formation. In our study, hatched blastocysts produced in the defined medium lacking serum produced increased ($P < 0.05$) *IFNT* supporting the notion that mRNA levels for this transcript are higher in good quality embryos. This finding is in agreement with Wrenzycki et al. (1999) and Lim et al. (2007) who, reported increased levels of *IFNT* mRNA in blastocysts produced in the presence of PVA compared to when serum was present.

The novel finding of this study was that embryos generated in the serum-free, defined medium performed better with regards to in vitro development and

gene expression patterns than those generated in an undefined medium-containing serum. The basic culture system was shown to exert strong effects on mRNA expression patterns, with more genes being differentially expressed between undefined and defined medium derived embryos. The expression pattern of genes implicated in embryo metabolism (*GLUT-1*), anti-apoptosis (*BCL-2*), imprinting (*IGF-2R*), DNA methylation (*DNMT-3A*) and quality (*IFNT*) was increased ($P < 0.05$) in embryos derived from serum-free, defined medium compared to undefined media-containing serum. Additionally, variations in RA of target transcripts between undefined and defined media embryos support the suggestion that in buffalo, the method of embryo culture has considerable influence on transcriptional levels of resulting embryos at different stages of development, as reported earlier in bovine and mouse. However, it cannot be ruled out that other gene transcripts could respond in a different way.

In conclusion, a serum-free, defined medium was identified as a good tool for buffalo in vitro embryo culture systems; a, significant over expression of developmentally important genes was found in serum-free, defined medium derived embryos as compared with undefined media-containing serum. Whether these changes in embryo development and gene expression have long-term effects on buffalo calves born through IVF as a result of using serum-free, defined medium derived embryos are unknown. However, it is possible that early adaptations of the preimplantation embryo to its environment persist during fetal and post-natal development.

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Authors' contributions SEM conceived of the study, carried out the experiments and drafted the manuscript. NLS, AKB and PSY assisted with the sample analysis. All authors read and approved the final manuscript.

Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest.

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