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Stability of reference genes for normalization of reverse transcription quantitative real-time PCR (RT-qPCR) data in bovine blastocysts produced by IVF, ICSI and SCNT

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Summary

Reverse transcription quantitative real-time polymerase chain reaction (RT-qPCR) is a sensitive and accurate tool for quantitative estimation of gene transcription levels in preimplantation embryos. To control for possible experimental variations, gene expression data must be normalized using internal control genes commonly known as reference genes. However, the stability of reference genes can vary depending on the state of development and/or experimental conditions; hence the assessment of their stability is essential before initiating a gene expression analysis. In the present study, we used RT-qPCR to measure the transcript levels of 10 commonly used reference genes and analyzed their expression stability in bovine blastocysts produced by *in vitro* fertilization (IVF), intracytoplasmic sperm injection (ICSI) and somatic cell nuclear transfer (SCNT). Using the geNorm program, we found the best combination of genes to normalize gene expression data in bovine embryos at the blastocyst stage produced by IVF (*HMBS, SF3A1,* and *HPRT1*), ICSI (*H2A, HMBS,* and *GAPDH*), SCNT (*ACTB, SF3A1,* and *SDHA*) and/or between blastocysts produced by these methods (*GAPDH, HMBS* and *EEF1A2*). We also demonstrated that not only the culture conditions may affect the expression patterns in bovine blastocysts but also the choice of embryo production method may have an important effect.

Keywords: Gene expression, ICSI, IVF, Reference genes, SCNT

Introduction

Preimplantation development is a dynamic process characterized by a series of developmental events that cover the transition time from gametes to a pluripotent embryo (Lonergan *et al.*, 1999). This orchestrated process is regulated by differential expression of many genes, therefore the acquisition of knowledge on the expression patterns of these genes provides new insights into the complex molecular pathways that control early embryonic development in mammals (Steuerwald *et al.*, 1999; Khurana & Niemann, 2000).

Reverse transcription quantitative real-time PCR (RT-qPCR) is a powerful technique for quantitative analysis, capable of combining accuracy, sensitivity, specificity and reproducibility, properties that make it currently the method most widely used to detect and quantify differences in gene expression. However, RT-qPCR assays are prone to errors and experimental variations, making necessary to minimize these variables for a well conducted study (Bustin, 2002; Huggett *et al.*, 2005; Wong & Medrano, 2005; Van

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Table 1 Information on the primers used for RT-qPCR

Gene	Function	Accession no.	Product size (bp)	Primer sequence (5′→3′)	E (%)	r^2
ACTB	Cytoskeletal structural protein	AY141970	87	CCTCACGGAACGTGGTTACA; TCCTTGATGTCACGCACAATTT	95.2	0.989
EEF1A2	Translation elongation factor activity	BC108110.1	196	GCAGCCATTGTGGAGATG; ACTTGCCCGCCTTCTGTG	96.7	0.999
GAPDH	Oxidoreductase in glycolysis and gluconeogenesis	XM583628	119	TTCAACGGCACAGTCAAGG; ACATACTCAGCACCAGCATCA	89.9	1
H2A(H2AFZ)	Nucleosome structure	NM_174809	176	GCCATCCTGGCGTACCTCAC; TGGATGTGTGGAATGACACC-	93.3	0.993
HMBS	Heme biosynthesis and porphyrin metabolism	BC112573.1	80	CTTTGGAGAGGAATGAAGTGG; AATGGTGAAGCCAGGAGGAA	94.4	0.998
HPRT1	Purine nucleotide synthesis	AF176419	154	TGCTGAGGATTTGGAGAAGG; CAACAGGTCGGCAAAGAACT	101	0.999
PPIA	Catalysis of the *cis-trans* isomerization of proline	NM_178320.2	203	CTGGCATCTTGTCCATGGCAAA; CCACAGTCAGCAATGGTGATCTTC	107.9	0.998
SDHA	Electron transporter in the TCA cycle and respiratory chain	NM_174178.2	185	GCAGAACCTGATGCTTTGTG; CGTAGGAGAGCGTGTGCTT	88.3	0.999
SF3A1	Structural component of the splicing system	XM_878187.1	125	GCGGGAGGAAGAAGTAGGAG; TCAGCAAGAGGGACACAAA	95.1	1
YWHAZ	Signal transduction	BM446307	120	GCATCCCACAGACTATTTCC; GCAAAGACAATGACAGACCA	89.3	1

E, PCR efficiency; r^2, correlation coefficient.

Guilder *et al.*, 2008). These differences can be controlled by normalizing data using reference genes, which should be expressed consistently in the samples regardless of tissue, experimental condition and/or treatment (Thellin *et al.*, 1999; Dheda *et al.*, 2004; Ohl *et al.*, 2005; Bar *et al.*, 2009). However, the stability of reference genes can also vary depending on the state of development and experimental conditions, hence its experimental validation is essential for each model, as inappropriate use of these reference genes can lead to erroneous normalization of RT-qPCR data and therefore to a misinterpretation of the biological significance of the generated results (Haller *et al.*, 2004; Zhang *et al.*, 2005; McCurley & Callard, 2008).

In the present study, we used RT-qPCR to measure the transcript levels of 10 genes commonly used as reference genes mostly in studies of gene expression in bovine embryos produced *in vitro* (Goossens *et al.*, 2005; Perez *et al.*, 2008; Vireque *et al.*, 2009; Walker *et al.*, 2009a). We analyzed the relative gene expression stability of these genes in bovine blastocysts produced by *in vitro* fertilization (IVF), intracytoplasmic sperm injection (ICSI) and somatic cell nuclear transfer (SCNT), in order to identify the most stable genes and their optimum number to normalize RT-qPCR data for gene expression studies in bovine blastocysts produced by these methods.

Materials and methods

Biological material

Gene expression analysis was carried out on day 7 expanded bovine blastocysts produced in our laboratory by IVF, ICSI and SCNT, according to the methods already described (Felmer & Arias, 2011; Felmer *et al.*, 2011; Arias *et al.*, 2012). Embryos generated by these methods were cultured under the same culture medium and culture conditions. Briefly, embryo culture was carried out in 50 μl drops (25 embryos per drop) under mineral oil at 38.5°C and 5% CO_2, 5% O_2, and 90% N_2, in a humidified atmosphere. Culture medium consisted of KSOM (EmbryoMax, Millipore Corp, Billerica, MA, USA) 0.4% FAF-BSA for 3 days and then KSOM 5% FBS to day 7.

Selection of reference genes and primer design

Ten candidate genes previously used as reference genes for normalization of gene expression data were

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selected for evaluation (Table 1). Primer sequences for *ACTB* and *HPRT1* were taken from Goossens *et al.* (2005), *EEF1A2, HMBS* and *SF3A1* from Perez *et al.* (2008) and *H2A* from Vireque *et al.* (2009). Primer sequences for remaining genes (*SDHA, YWHAZ, GAPDH,* and *PPIA)* were re-designed based on RNA or DNA bovine sequences found in the GenBank database using the FAST PCR software tool, as sequences described by Goossens *et al.* (2005) and Walker *et al.* (2009a) did not allow us to set up the same annealing temperature for all genes under study.

RNA extraction and cDNA synthesis

Three pools of embryos for each treatment (n = 5 blastocysts/pool) were lysed in 20 μl of extraction buffer (XB; Arcturus, Carlsbad, CA, USA) by incubation at 42°C for 30 min followed by centrifugation at 3000 *g* for 2 min. 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, CA, USA) according to the manufacturer's instructions; 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. Due to the low cell number used for RNA extraction, RNA quantity could not be measured by a NanoDrop 2000C (ThermoScientific) spectrophotometer.

Reverse transcription assay was carried out using the RevertAid™ H Minus First Strand Kit (Fermentas Inc., MD, USA), according to the manufacturer's instructions. Briefly, the following reagents were added to each 0.5 ml of RNase-free tube: 10 μl total RNA and 200 ng of random hexamers. The reaction tubes were incubated in a preheated PCR machine at 70°C for 5 min and transferred to ice. After denaturation, the following reagents were added to each reaction tube: 4 μl of 5× 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 min. Then, 1 μl of RevertAid™ MuLV RT was added and the mixture incubated at 42°C for 60 min in a dry bath. The reaction was terminated by heating at 70°C for 10 min and chilled on ice. This first-strand cDNA was diluted five times and used for real-time experiments.

Quantitative real-time RT-PCR

Polymerase chain reactions (PCR) were performed using Brilliant II SYBR® Green QPCR Master Mix (Stratagene) in a thermocycler MX3000P (Agilent Technologies, 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. PCR program consisted of an initial incubation step at 95°C for 5 min to activate *Taq* DNA polymerase, followed by 40 cycles of template denaturation step at 95°C for 20 s, a primer annealing step at 58°C for 20 s (same annealing for all primers), and an extension step at 72°C for 20 s. 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 a relative standard curve 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 $E = 10^{(-1/\mathrm{slope})}$ established by Pfaffl (2001; Table 1).

Gene expression stability analysis

RT-qPCR data (Ct values) were transformed into relative quantification data using the formula $Q = (E)^{\Delta Ct}$ described by Livak & Schmittgen (2001) and then exported into an Excel datasheet (Microsoft® Excel 2003). To determine the most stable reference genes, the geNorm Visual Basic Application Program v3.4 described by Vandesompele *et al.* (2002) was used.

Results and Discussion

Given the high sensitivity of RT-qPCR to detect small changes in transcript abundance in bovine blastocysts produced *in vitro*, it is necessary to normalize the data by endogenous control genes commonly known as reference or housekeeping genes. Normalization by these genes is essential to control initial differences in embryo cell number, variations in RNA extraction yield, RNA abundance, reverse transcription efficiency, and the presence of inhibitors, thus enabling comparison of mRNA levels across different samples (Bustin *et al.*, 2009). However, many studies have made use of these genes without a proper validation of their stability (Vandesompele *et al.*, 2002). Furthermore, much evidence suggests that stability of reference genes can vary depending on the state of development and experimental conditions (Bustin, 2000; Warrington *et al.*, 2000); therefore its usefulness should be validated experimentally in each model (Zhang *et al.*, 2005; McCurley & Callard, 2008).

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Table 2 Ranking of candidate reference genes according to their expression stability values (*M*) in each of the *in vitro* embryo production methods

IVF	M	ICSI	M	SCNT	M	Combined	M
HMBS/ SF3A1	0.271	*H2A/HMBS*	0.156	*ACTB/SF3A1*	0.010	*GAPDH/HMBS*	0.441
HPRT1	0.334	*GAPDH*	0.261	*SDHA*	0.043	*EEF1A2*	0.698
GAPDH	0.425	*PPIA*	0.407	*H2A*	0.165	*SF3A1*	0.756
ACTB	0.595	*EEF1A2*	0.492	*PPIA*	0.232	*HPRT1*	0.826
H2A	0.679	*SDHA*	0.593	*EEF1A2*	0.335	*H2A*	0.881
PPIA	0.781	*HPRT1*	0.713	*HPRT1*	0.426	*YWHAZ*	0.932
SDHA	0.830	*YWHAZ*	0.805	*YWHAZ*	0.455	*PPIA*	1.011
EEF1A2	0.909	*ACTB*	0.888	*HMBS*	0.533	*SDHA*	1.081
YWHAZ	1.036	*SF3A1*	0.978	*GAPDH*	0.752	*ACTB*	1.446

Candidate genes are listed according to their expression stability with the highest on top and the lowest at the bottom.

In the present study, in order to analyze the suitability of candidate reference genes for bovine blastocysts produced by different *in vitro* embryo production methods, the expression stability of 10 previously used reference genes was assessed by the geNorm algorithm (Vandesompele *et al.*, 2002). This program calculates the gene stability measure (*M*) by determining the average pairwise variation of a gene with all other control genes (Vandesompele *et al.*, 2002). In this way, genes with a low *M* value have a low variation, which represents a more stable expression (*M* values <1.5). Using this program we ranked the 10 candidate reference genes in bovine blastocysts produced by the different methods according to their expression stability (Table 2 and Fig. 1). Thus, a gradual stepwise exclusion of the least stable genes allowed us to identify *HMBS* and *SF3A1* as the most stable genes for embryos produced by IVF (*M* values 0.271), *H2A* and *HMBS* for embryos produced by ICSI (*M* values 0.156), and *ACTB* and *SF3A1* for embryos produced by SCNT (*M* values 0.010) (Table 2 and Fig. 1). The same analysis considering the gene expression data of all combined embryo production methods (IVF, ICSI, and SCNT) positioned *GAPDH* and *HMBS* as the most stable reference genes with an *M* value of 0.441, whereas *ACTB* showed the least stability in this analysis (*M* value 1.446; Table 2).

It is necessary to note from these data that each embryo production method showed a different ranking for these genes, with a slightly higher difference for embryos produced by SCNT (Table 2), which could be explained by inefficiencies in the process of nuclear transfer attributable to incomplete or abnormal epigenetic reprogramming (Somers *et al.*, 2006; Beyhan *et al.*, 2007; Suzuki *et al.*, 2008). These differences in the stability of the genes confirm that not only *in vitro* culture conditions may affect the gene expression patterns during embryogenesis in mammals (Niemann & Wrenzycki, 2000; Lazzari *et al.*, 2002; Rizos *et al.*, 2002; Rinaudo & Schultz, 2004; Felmer *et al.*, 2011), but also the methodological procedure used to generate these embryos, which agrees with previous studies between IVF and SCNT embryos (Zhou *et al.*, 2008; Ross *et al.*, 2010). A large variation in *M* value range between the three different embryo production groups was also observed; particularly the *M* values of SCNT embryos are lower (0.01 for the most stable gene to 0.752 for the least stable gene), a finding that suggested that these embryos have little variation in their expression patterns compared to IVF and ICSI embryos, respectively. This effect could be attributed to the differences in gene expression observed in male and female embryos as it would be the case for IVF and ICSI embryos, respectively (Bermejo-Alvarez *et al.*, 2008; Walker *et al.*, 2009b), an effect that is not observed in SCNT embryos as all embryos are of the same gender. It is also interesting to note that despite all candidate reference genes showed a high expression stability, as evidenced by their low *M* values (<1.4), somehow supporting the previous selection of these genes as reference genes (Goossens *et al.*, 2005; Perez *et al.*, 2008; Vireque *et al.*, 2009), differences in gene expression stability were still observed between these genes and the different embryo production methods, confirming that careful selection of the best candidate genes is strongly recommended for each experimental condition. This point is better exemplified by a gene expression analysis carried out with all combined data using the REST program (http://www.gene-quantification.de/rest-2009.html), where selecting *GAPDH* (the most stable) or *ACTB* (the least stable) as reference genes gave different gene expression results (data not shown).

Considering that variations in gene expression stability may always exist for any reference gene subjected to a determined experimental condition (Bustin, 2000; Warrington *et al.*, 2000; Remans *et al.*, 2008), normalization of gene expression data against a single reference gene can bias the generated results

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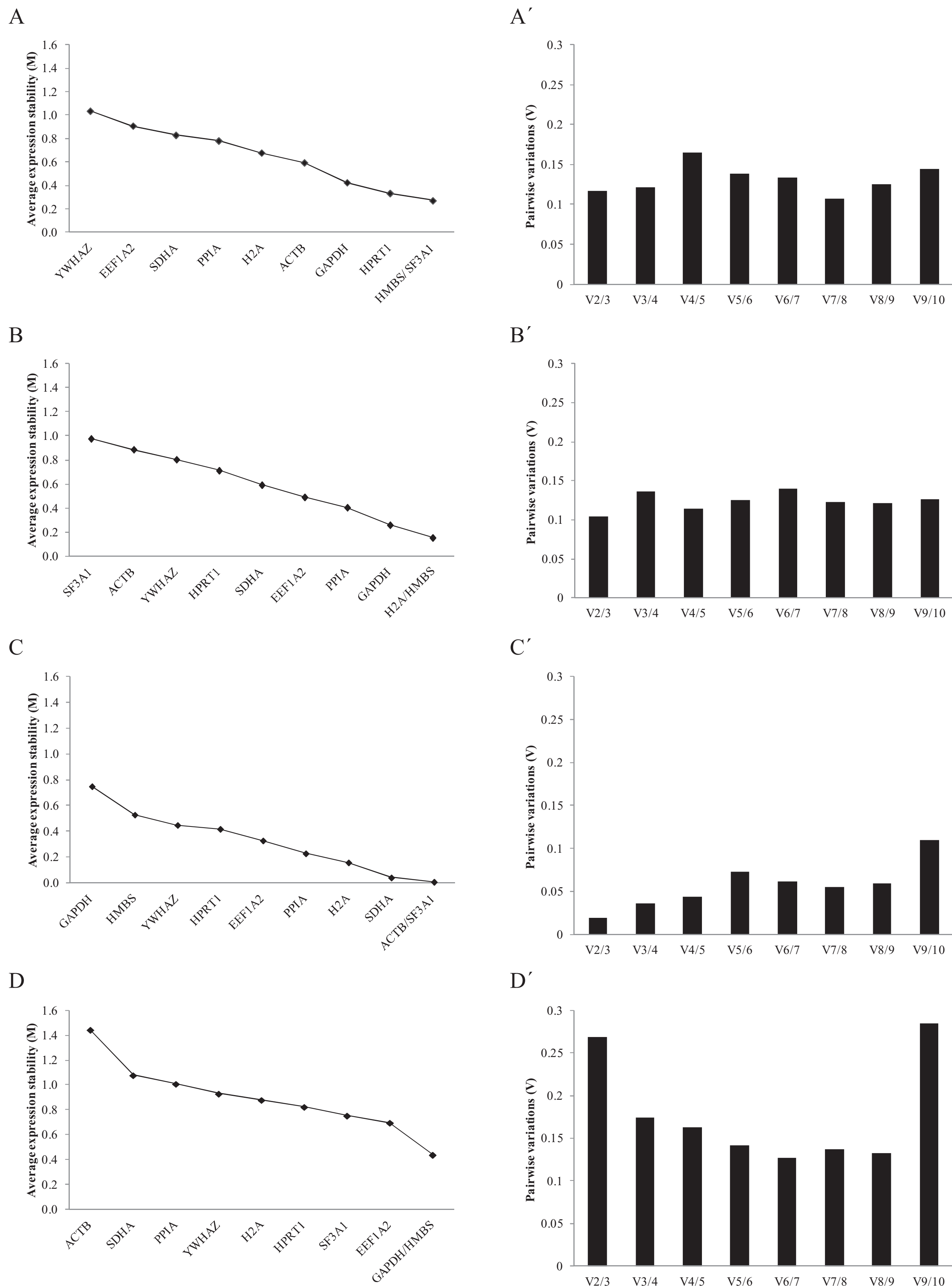

Figure 1 Gene expression stability of candidate reference genes analyzed by the geNorm program. Left panel: Average expression stability values (*M*) of candidate reference genes plotted from the least stable (left) to the most stable (right). (*A*) *In vitro* fertilization (IVF). (*B*) Intracytoplasmic sperm injection (ICSI). (*C*) Somatic cell nuclear transfer (SCNT). (D) All assays combined. (*A′*–*D′*). Right panel: Pairwise variation analysis (V_n/V_{n+1}) between the normalization factors NF_n and NF_{n+1} to determine the optimal number of reference genes for normalization: (*A′*) IVF; (*B′*) ICSI; (*C′*) SCNT; and (*D′*) combined assay.

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and further compromise its validity (Dheda *et al.*, 2004; Jemiolo & Trappe, 2004). Accordingly, Vandesompele *et al.* (2002) demonstrated that using a single reference gene leads to a moderate error and validated the geometric mean of multiple reference genes as an accurate normalization factor. Therefore, in addition, we calculated the optimal number of reference genes needed for an accurate normalization of gene expression data in bovine blastocysts generated by these *in vitro* embryo production methods. This optimum was determined using the normalization factor (NF_n), which is based on the geometric mean of the expression values of the *n* best reference gene, calculated by the stepwise inclusion of an additional less stable reference gene (Vandesompele *et al.*, 2002). An arbitrary cut-off value of 0.15 indicates acceptable stability of the control gene combination, a value below which the inclusion of an additional reference gene is not required; therefore, in our case, the inclusion of a third gene had no significant effect on the NF value (Fig. 1). However, Vandesompele *et al.* (2002) recommended the minimal use of three reference genes and, therefore, we considered three genes to be sufficient for accurate normalization when analyzing gene expression data in bovine blastocysts produced either by IVF (*HMBS*, *SF3A1*, and *HPRT1*), ICSI (*H2A*, *HMBS*, and *GAPDH*), and SCNT (*ACTB*, *SF3A1*, and *SDHA*) (Fig. 1*A*′, *B*′ and *C*′, respectively). Conversely, the comparison of gene expression data between blastocysts produced by these *in vitro* embryo production methods would require the inclusion of a fifth reference gene (Fig. 1*D*′). The use of five reference genes leads to a waste of resources, particularly in this case when most of the genes showed a relatively stable expression (*M* values <1.5). Therefore, we suggest the use of the geometric average of the three most stable genes, in this case *GAPDH*, *HMBS*, and *EEFIA*2, as the best combination for normalization of gene expression data in bovine blastocysts.

Previous studies on the stability of reference genes determined that *GAPDH*, *YWHAZ*, and *SDHA* were the best endogenous control genes in preimplantation embryo samples and that *ACTB* was the least stable reference gene (Goossens *et al.*, 2005). Our results, which compared all combined gene expression data for bovine blastocysts produced by the different embryo production methods, are in agreement with this study as *GAPDH* and *ACTB* showed the highest and the lowest stability values, respectively. Although some differences could be observed in the ranking for *YWHAZ*, and *SDHA* genes, this finding could be attributed either to the different set of genes used in both studies, the different set of primers (see Materials and methods section), or the different developmental stage of embryos and culture conditions. In a separate study that compared IVF and SCNT embryos, *ACTB* was also found to change significantly at the blastocyst stage, a situation that could be the result of abnormal nuclear reprogramming in SCNT embryos (Ross *et al.*, 2010). A similar result was also observed by Bower *et al.* (2007), who reported unstable expression of *ACTB* in a microarray experiment that compared the transcriptome of SCNT to IVF blastocysts. These data are of particular relevance for *ACTB*, as this gene has been used previously as a single reference gene in different studies of gene expression in embryos; this approach highlights the caution that must be exercised for an appropriate selection of internal control genes. Our data also show that although some reference genes maintained a certain level of similarity in the gene expression stability in each of the *in vitro* embryo production methods, differences were still observed. In addition, the fact that expression of reference genes may also vary under other experimental conditions must be taken into consideration, a situation that is particularly relevant when nuclear transfer embryos are in evaluation.

In conclusion, the results of the present study showed that experimental validation of reference genes is essential for each experimental model and that not only the culture conditions may affect the expression patterns of bovine blastocysts produced *in vitro*, but also the embryo production method may have an important effect. We determined the best combination and the optimal number of reference genes for gene expression studies in bovine blastocysts produced by the different *in vitro* embryo production methods.

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Conflict of interest

The authors declare that they have no conflicts of interest.

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