

Assessing the Quality of Bovine Embryos Produced *In Vitro* Through the Inner Cell Mass and Trophectoderm Ratio

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Embryo quality and implantation potential are the most important factors influencing the rate of successful pregnancies. These two are related to the occurrence of the three morphogenetic process (i.e., compaction, blastulation, and hatching) and the allocation of embryonic cells to the inner cell mass (ICM) and trophectoderm (TE) in response to proper timing of embryonic development. This research was conducted to determine the allocation of ICM and TE of bovine embryos *in vitro* in relation to its developmental stage and age. The account of this event can be used as benchmark for comparison of good quality embryos for transfer. Using a defined medium – modified synthetic oviductal fluid for IVC – 85 bovine embryos derived from the slaughter house were assessed for cell number and ICM and TE ratio using the Hoechst 33342-propidium iodide differential staining method. Embryos collected on days 7, 8, and 9 were stained, viewed, and examined using fluorescence microscope and Nikon Imaging Software - Basic Research. The results revealed that in terms of total cell number (mean \pm SD), the expanded blastocyst on the 7th day (109.29 ± 41.09) and hatched blastocyst on the 8th day (139.5 ± 43.13) yielded the highest total cell number. From these two stages, chi square test determined that the 7th day expanded blastocyst with an ICM:TE count (ratio) of [34.4 ± 15.4]:[73.2 ± 34.9] (0.47) fits to the 1:3 ratio given for a good quality embryo. The results of the present study indicate that the 7th day expanded bovine blastocyst developmental stage and age has the highest potential for pregnancy when transferred owing to its being able to achieve the desired cell number and ICM and TE.

Key words: bovine, embryo, inner cell mass, total cell number, trophectoderm

INTRODUCTION

Improving the *in vitro* production and embryo transfer technologies (IVEP-ET) has been done to continuously meet the goal of genetic improvement in livestock. The IVEP-ET has been recognized to be the most efficient method in producing large number of superior animals (Selokar et al. 2012). In order to achieve this, a good maternal and paternal linkage will allow the development

of quality embryos to be transferred and produce offspring that will satisfy the need for large scale production of milk and meat.

However, embryo quality and implantation potential has become two of the most important factors influencing the decrease in the rate of successful pregnancies done by this technology. Advances in this research require accurate measures to assess the embryo competence for post-transfer development (Hansen 2006). Likewise, recognizing the quality of the embryo before doing the

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transfer is important to save pointless costs and effort of transferring poor quality embryos that eventually will not develop to full term.

In almost all laboratories, the most useful criteria for evaluating the quality is done by visual inspection of their morphology, which produces conflicting results as it is influenced by subjectivity. Whereas the use of TUNNEL assay, metabolic assays, flow cytometer, computer assisted sperm analyzer, spectrophotometer and other equipment are of great help, these are elaborative, expensive, and require high technical skill (Selokar et al. 2012). Meanwhile, research of Thouas and colleagues (2001) acknowledged cell number – the combination of the inner cell mass (ICM) and trophectoderm (TE) – as a valid indicator of embryo quality.

Differences in the morphology of the embryo involve visual changes (e.g., compaction) and blastocyst formation, followed by hatching. At the cellular level, embryonic cells differentiate functionally to TE cells and ICM. ICM cells will give rise to the embryonic tissues as well as other parts of the extra-embryonic tissues, whereas the TE will constitute the other parts of the extra-embryonic tissues and the formation of placenta (Hardy et al. 1989).

Although different studies have been conducted in examining the quality of embryo using ICM-TE cells, they focused only on the age of the embryo or its developmental stage (Iwasaki et al. 1990; Rivera et al. 1996; Van Soom et al. 1997; Macha'ty et al. 1988) but the simultaneous occurrence of the two was not given much attention. Reproductive biotechnologists or embryologists have little choice regarding the quality of embryo to transfer since very few are able to develop to the blastocyst stage. Thus, an assessment of its quality in relation to its developmental stage and age is needed to increase the likelihood of pregnancy after transfer.

Therefore, this research was conducted to determine the allocation of ICM and TE of bovine embryos produced in-vitro in relation to its developmental stage and age as basis of embryo quality. The account of this event can be used as benchmark for comparison of good quality embryos with higher chance of pregnancy upon transfer.

MATERIALS AND METHODS

Experimental Design

Embryos of each developmental stage (early blastocyst, blastocyst, expanded, and hatched), and day of production (7, 8, and 9) were stained for ICM-TE cell counting. The ICM-TE ratio and total cell number (TCN) were noted for each embryo of different stage and age.

In vitro embryo production

The production of bovine embryos *in vitro* was performed following the procedure described by Atabay and colleagues (2007). In brief, bovine ovaries were collected from the slaughter house and transported to the laboratory in 0.9% NaCl solution. The follicles were aspirated, and the cumulus-oocyte complexes were collected, grouped according to morphological appearance, and incubated in the *in vitro* maturation medium (IVM) for 22-24 h at 38° C and 5% CO₂ in air. Fertilization of the oocytes were done using frozen-thawed spermatozoa in Brackett-Oliphant medium (IVF) under the same condition for 6-18 h. After the co-incubation of spermatozoa and oocytes, presumptive zygotes were transferred to the modified synthetic oviductal fluid (SOF) culture medium (IVC). The development of embryos was observed during 7th-9th day from the day of culture for quality evaluation.

ICM-TE differential staining of embryos

In vitro-produced embryos were collected and classified according to its developmental stage and day of production. Differential staining was done following the method of Sripunya and colleagues (2009) with minor modifications. Blastocysts were washed in phosphate buffered saline (PBS) containing 0.2% polyvinyl alcohol (PBS-PVA) and incubated for 30 sec in PBS-PVA containing 0.2% (v/v) Triton X and 0.1 mg propidium iodide (PI). Embryos were then transferred to a well dish containing 25 ug/ml of Hoechst 33342 dissolved in absolute ethanol and incubated for 30 min at 37° C. After staining, the blastocysts were washed in PBS-PVA and mounted on a glass slide. Mounting was done by adding glycerol droplets on the side of the embryo and then flattened with glass slip. The embryo was viewed on a fluorescence microscope (Nikon Inverted Microscope Ti) and focused using UV light. The nuclei of TE cells labeled by Hoechst 33342 will appear color blue, whereas the nuclei of ICM cells labeled by propidium iodide will appear color red (Muenthaisong & Parnpai 2005). The number of TE and ICM cells were counted separately and recording was made based on its age and developmental stage. Images were taken using Nikon Imaging Software - Basic Research.

Analysis of Data

The ICM, TE, and TCN were presented as means ± SD. Descriptive analysis of the differences in the mean TCN of embryos with the same developmental stage and age were done. The obtained results were compared with the published results of Van Soom et al. (1997). Meanwhile, chi-square test for the goodness of fit was used to compare the ICM-TE ratio in an expected 1:3 based on studies of Thouas and colleagues (2001).

RESULTS

Table 1 presents the number of embryos stained according to its developmental stage and age. Majority of the embryos assessed were at the expanded blastocyst stage (44) and at the 7th day (47). The total number of embryos assessed were 85. There were unequal number of embryos assessed per developmental stage and age since the development was based on the innate capacity of the embryo. Generally, embryos developing at the 9th day were already late, thus there were very few in the population.

Table 1. Total number of embryos produced according to its developmental stage and age.

	Early Blastocyst	Blastocyst	Expanded Blastocyst	Hatched Blastocyst
7 th day	3	16	27	1
8 th day	7	7	15	2
9 th day	2	1	2	2

Table 2 shows the mean \pm SD of the TCN of the embryos based on its developmental stage and age in comparison with the previous report of Van Soom and colleagues (1997) on the developmental stage of its embryo regardless of age. In the results, it was only the 7th day expanded blastocyst and the 8th day hatched blastocyst that were similar on the cell count obtained by Van Soom and colleagues (1997). With these results, one can infer that the quality of the embryos based on total cell count were comparable with the quality of produced embryos by other researchers. However, some embryos with developmental stages: early blastocyst and blastocyst, failed to reach the given cell count, thus can already be classified as inferior quality. Using the cell count as the basis, both the 7th day expanded blastocyst and 8th day hatched blastocyst were of good quality.

Table 3 shows the mean ICM, TE and ratio of the embryo based on developmental stage and days of production. In relation with the previous report of Van Soom and colleagues (1997), the mean of ICM \pm SD according to developmental stage regardless of days of production were as follows: 36.8 ± 10.1 (early blastocyst), 35.0 ± 4.9

(normal blastocyst), 42.4 ± 8.6 (expanded blastocyst), and 57.9 ± 19.8 (hatched blastocyst). Comparing the results with these given data, the ICM mean \pm SD of days 7, 8, and 9 expanded blastocysts fit on the given range. The 8th day hatched blastocyst was also comparable to the given range. Using the chi-square goodness of fit test at 0.05 level of significance, from those that qualified with the TCN, only the 7th day expanded blastocyst fits on the given 1:3 ratios for ICM-TE cells.

Table 3. Mean allocation of ICM \pm SD and TE \pm SD according to developmental stage and age of the embryos.

	ICM	TE	Ratio
7th day			
Early Blastocyst	10.00	18.00	0.55
Blastocyst	17.90 ± 12.64	48.50 ± 20.79	0.36
Expanded Blastocyst	34.42 ± 15.43	73.21 ± 34.88	0.47
Hatched Blastocyst	36.00	84.00	0.43
8th day			
Early Blastocyst	15.60 ± 9.76	41.40 ± 25.19	0.38
Blastocyst	35.20 ± 20.75	31.60 ± 20.23	0.89
Expanded Blastocyst	30.14 ± 16.33	58.00 ± 32.37	0.52
Hatched Blastocyst	60.00 ± 49.50	74.50 ± 13.43	0.81
9th day			
Early Blastocyst	11.50 ± 0.71	21.50 ± 14.85	0.53
Blastocyst	9.00	30.00	0.30
Expanded Blastocyst	31.50 ± 20.51	37.50 ± 9.19	0.84
Hatched Blastocyst	24.00	74.00	0.32

DISCUSSION

The most common method performed by embryologists in identifying the quality of an *in vitro* produced embryo is through the morphological grading. This type of grading is subjective and does not fully recognize the embryo based on its intrinsic characteristics. Although there is a standard for the embryo quality according

Table 2. Mean TCN \pm SD based on developmental stage and day of production.

	Early Blastocyst	Blastocyst	Expanded Blastocyst	Hatched Blastocyst
7 th day	26.3 ± 2.89	58.5 ± 18.79	109.3 ± 41.09	120.00
8 th day	52.4 ± 28.80	71.1 ± 24.93	86.7 ± 41.38	139.5 ± 43.13
9 th day	33.0 ± 15.57	39.00	69.0 ± 29.70	82.5 ± 21.92
Van Soom et al. (1997)	105.0 ± 23.4	115.0 ± 5.6	159.0 ± 14.10	171.4 ± 34.10

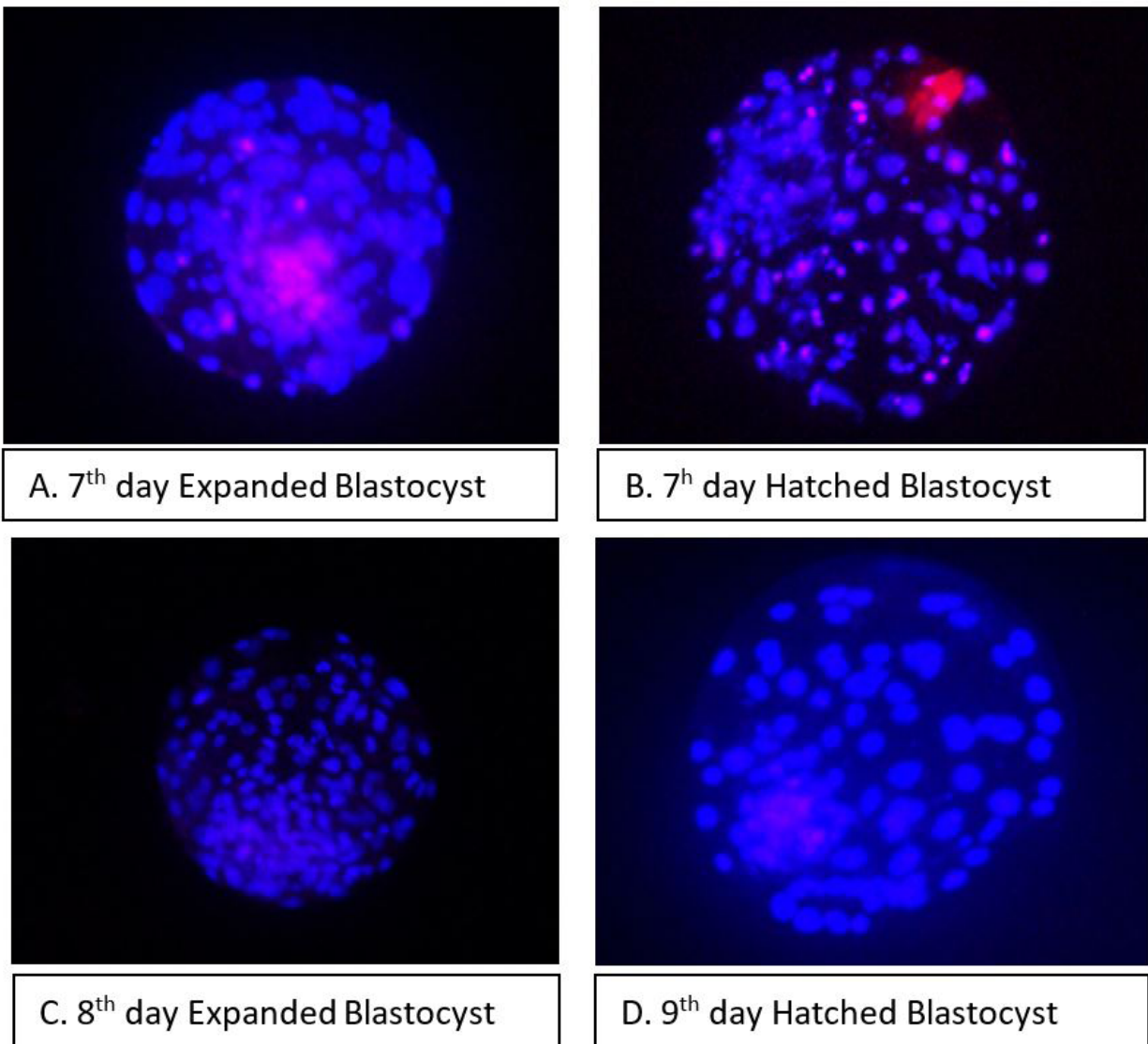


Figure 1. Differential staining of in-vitro produced bovine blastocyst after 7 (a & b), 8 (c) and 9 (d) of in-vitro culture.

to the developmental stage given by Seidel and Seidel (1991) in the FAO for embryo transfer in cattle, that is still based on its visual characteristics. Moreover, efforts being done on the recent years on determining the quality of *in vitro* produced embryo focused only on comparing the efficiency of different culture media. With this, the paper initiated the determination of the quality of embryo based on its developmental stage and age using its internal compositions.

According to the research of Papaioannou and Ebert (1986), cell numbers can be a valid indicator of the viability of pre implantation embryos. Also indicated is that the quality of embryo determined is set to be a more accurate predictor of pregnancy success. Whereas, other researches emphasized that cell lineages (ICM and TE numbers) play a fundamental role in the embryo survival and fetal viability. Accordingly,

they refer that an imbalance of these two and the total cell count may compromise the potential of subsequent pre implantation development of the embryo.

In doing embryo transfer, the morphology is always the determining factor whether the blastocyst will be transferred or not. Its fate has also been identified by the way it is seen on the microscope. Hence, an established morphological criterion in relation to its developmental stage and age and in consideration of the ICM-TE allocation is essential to ensure that the embryos to be transferred are of good quality. Moreover, studies already revealed that the three parameters – blastocoele expansion, ICM, and TE – have shown that transferring top-scoring blastocysts (i.e., high grades for all three parameters) resulted in the highest implantation rates (Ahlstrm et al. 2011).

Most of the embryos produced in this study were at the 7th day expanded blastocyst stage. This implies that majority of the embryos produced were early cleaving and early developing because in bovine embryo production, there are certainly more numbers of embryos produced in day 7 as compared to days 8 and 9 (Grisart et al. 1994). In relation to the total cell count of embryos according to developmental stages which was published by Van Soom and colleagues (1997), the produced blastocysts and expanded blastocysts in this study fit the given range, which was 115.0 ± 5.6 and 159.0 ± 14.1 , respectively. In accordance with the day of production of embryo, Thouas and colleagues (2001) reported that day 7 and 8 blastocysts have a total cell count of 151.3 ± 5.48 and 217.8 ± 8.75 , correspondingly. For the 9th day, 105 ± 6.7 total cell count was reported by dela Fuente and King (1997). Relating to this study, only the 7th & 8th day blastocyst and expanded blastocyst fit on the given range. The results were comparable with the observation that embryos developing quickly to the blastocyst stage had a higher total cell count than embryos developing later (Iwasaki et al. 1990), but is decreasing as they age. This is due to the fact that in *in vitro*, blastocyst formation starts earlier, both with respect to embryonic age and to cell number as compared with *in vivo* (Van Soom and colleagues 1997). This also has something to do with the compaction and establishment of functional tight junctions earlier as supplemented by the serum in the culture medium (Holm & Callesen 1998, Mori et al. 2002). From the FAO for embryo production and transfer in cattle, at day 7 (the standard) it should be early blastocyst stage, while at day 8 it has already reached its expanded stage. Embryos developing later than this are already showing considerable degeneration.

After determining the developmental stage and age of embryo that fit on the given range for the total cell count, they were further examined for the allocation of ICM and TE cells. The intense blue color represents the chromatin in nuclei of TE cells, while the red color represents the ICM nuclei. Studies of Macha'ty and colleagues (1998) and Iwasaki and co-workers (1990) contradict the color representation as ICM and TE cells appeared blue and red, respectively. This could be due to the concentration of staining dyes used in this study which were much higher than the previous.

It has been discussed that the possibility of transferring *in vitro*-developed embryos with a lower number of ICM nuclei would have resulted in a lower rate of development and pregnancy (Macha'ty et al. 1998). Other research states that if the number of cells in the ICM of a blastocyst is approximately 30% or more, there is a high risk of fetal loss or developmental injury. Furthermore, the reduction in number of TE cells during early embryogenesis may result

in a reduced placenta size (Rivera et al. 1996). According to Narula and colleagues (1996), the average TCN, TE, and ICM were 103 ± 21.2 , 80 ± 16.1 , and 24 ± 6.1 , respectively. Both cell lineages are vital and essential for embryonic and fetal survival. In other bovine researches, the ratios of ICM-TE were around 1:3.42 and 1:3.36 on the 7th and 8th day, respectively (Thouas et al. 2001). In this paper, a 1:2.12 ratio was obtained from the 7th day expanded blastocyst, which is statistically acceptable for the standard ratio (1:3) for the good quality embryos. According to Seidel and Seidel (1991), excellent embryos are those that are morphologically perfect for its age, while good embryos are with trivial imperfections (e.g., oval zona pellucida), a few, small excluded cells, or slightly asymmetrical shape. These classification of embryos have 58-63% pregnancy rate after ET as compared to the poor quality embryos with 12-31% only.

Therefore, from this study, one may denote that the 7th day expanded bovine blastocysts can have the highest potential for pregnancy when transferred owing to its developmental stage and age and on intrinsic characteristics - TCN, ICM, and TE allocation. Finally, the present findings reinforce the fact that differential staining of ICM-TE cells is a very useful approach in determining the quality of embryo produced from IVF.

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