PLOS ONE

RESEARCH ARTICLE

Blastulation time measured with time-lapse system can predict in vitro viability of bovine blastocysts

Carmen Huayhua[1], **Misael Rodríguez**[1], **Jhorjhi Vega**[1], **Mario Briones**[2], **Lleretny Rodriguez-Alvarez**[2], **Edwin Mellisho**[1]*

1 Centro de Investigación en Tecnología de Embriones (CIETE), Animal Improvement Program, Universidad Nacional Agraria La Molina, Lima, Perú, 2 Department of Animal Science, Faculty of Veterinary Sciences, Universidad de Concepción, Chillán, Concepción, Chile

* emellisho@lamolina.edu.pe

Abstract

The objective of this study was to evaluate the time of blastulation monitored by time-lapse technology to predict in vitro viability of bovine blastocysts. This technology can be a powerful tool for bovine embryos selection with higher implantation capacity and competence. Also, in humans an early blastulation is associated with higher quality and pregnancy rate. Cumulus oocyte complexes (COCs) were matured for 20 to 22 h and then fertilized by co-incubation of COCs and spermatozoa (10,000 sperm per oocyte) for 18 h. Presumptive zygotes were placed individually in microwells, in droplets of commercial culture medium. The Primo Vision TL system (EVO+; Vitrolife) captured digital images of developing embryos every 15 minutes. The time frame from IVF to the start of blastulation (tSB) and to blastocyst development (tB) was recorded. After day 7.5, the blastocysts were in vitro culture for 48 h until day 9.5 after IVF to evaluate post hatching development. In vitro viability was evaluated at day 9.5: those with a diameter greater than 200 μm and a total cell count greater than 180 were classified as viable (value 1), while the rest were classified as non in vitro viable (value 0). The area under the ROC curve (AUC) was estimated to determine the predictive power of *in vitro* viability through blastulation time. In addition, binary logistic regression analysis was used to generate a mathematical model with morphokinetic variables that allow the best prediction of *in vitro* viability. In 13 sessions, the blastocyst production rate was 46.2% (96/208). The cut-off time to discriminate early or late blastulation was 149.8 h. The post-hatching development of the embryos with early blastulation was 63.3% (31/49), being statistically superior (p = 0.001) than the late blastulation group 14.9% (7/47). Likewise, the time of blastulation showed an accuracy of 90.8% (p < 0.001) in predicting *in vitro* viability of bovine blastocysts. In conclusion, the selection of blastocysts based on blastulation time (< 155 h) and blastocyst diameter measured on day 7.5 after IVF (> 180 μm) maximizes the *in vitro* viability.

Introduction

For the world embryo industry, *in vitro* fertilization (IVF) is the most important innovation that allow the trade of genetics of various species [1]. Over 1.5 million *in vitro*-produced (IVP) bovine embryos were recorded in 2021 [2]. Although, low pregnancy rate (33.5%; [3]) and high proportion of chromosomal abnormalities (20 to 25%; [4]), increased pregnancy loss [5], abnormal placental development [6], heavier fetuses [7], higher rates of dystocia, congenital anomalies [8], stillbirths and neonatal mortality [9] have been suggested as a major cause of failure of IVP system in cattle. Therefore, non-invasive criteria are required to achieve an objective and more precise embryo selection [10].

Early embryonic development involves a series of orchestrated events between the first cell cleavages and the differentiation of the first cell lineages [11,12], leading to the differentiation of the inner cell mass into hypoblast and epiblast (blastocyst formation) important events for the implantation of the embryo in the uterus [13]. Also, in humans, blastulation timing is associated with mitochondrial content, chromosome status, and embryonic quality and competence [14–16], synchrony between embryo development and endometrial receptivity [17] and high precision of pregnancy rate (80%; [18]). In cattle, blastulation timing may have a high potential for embryo selection over other embryo morphologic parameters and may be a criterion for the selection of blastocysts to be transferred that can predict the embryo viability or competence.

The competence of the oocyte and/or embryo is of great importance for the in vitro embryo production programs, impacting on the successful establishment of pregnancy after transfer to recipients [19]. The traditional selection of in vitro produced bovine embryos is mainly based on morphological characteristics [20], though this classification is considered subjective and inadequate [21]. Time-lapse technology is a non-invasive method that allows obtaining continuous digital images to monitor embryonic development *in vitro* [22]. Studies in humans indicate that the selection of competent embryos using continuous monitoring technologies is the more accurate method to identify embryos with greater implantation capacity [22–24]. However, it is little used in bovine embryo transfer [25,26]. The objective of this work is to evaluate the value of blastulation timing monitored by time-lapse technology to select embryos with greater post-hatching development.

Materials and methods

The Graduate School of the La Molina National Agrarian University did not request the approval of the ethics committee, since the research only used commercial frozen semen and ovaries collected at a local slaughterhouse. The chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA), and the *in vitro* embryo culture media from Vitrogen (YVF Biotech LTDA EPP, Sao Paulo, Brazil).

Experimental design

Presumptive zygotes were cultured individually in microwell plates (16-microwell, Vitrolife, Gothenburg, Sweden). Embryo development was monitored with images captured by the Primo Vision TL system (EVO+; Vitrolife, Gothenburg, Sweden) for 7 days. The morphokinetic parameters were annotated: t2 (h) division time to 2 cells; t3 (h) division time to 3 cells; t4 (h) division time to 4 cells, t5 (h) division time to 5 cells, t8 (h) division time to 8 cells; t9+ (h) division time to 16 cells, tM (h) time to morula stage; tSB (h) time of starting blastulation, tB (h) time to blastocyst stage and tBX (h) time to expanded blastocyst stage. At day 7.5, morphological characteristics of blastocysts, embryo quality, developmental stage and blastocyst diameter were determined, using criteria described in the IETS manual [27]. In addition, on

day 7.5, embryos were kept in individual culture, to assess their post-hatching development (in vitro viability) based on growth performance and diameter (Fig 1).

In vitro embryo production

Ovaries were obtained from local abattoirs following the standard procedure described by Rodríguez et al. [28]. COCs in groups (10 to 12) were *in vitro* matured (IVM) in a drop (70 μL) of IVM medium (Vitrogen®, Brazil) for 20 to 22 h. Immediately after sperm selection, 10,000 motile spermatozoa per oocyte were used in IVF and co-incubated with COCs at 38˚C in an atmosphere of 6% CO_2 in air, similar to other authors [29,30]. After 18 h of IVF, presumptive zygotes were mechanically denuded by pipetting and then *in vitro* cultured (IVC) in IVC medium (Vitrogen, Brazil) at 38˚C in 6% CO_2 in air. During *in vitro* culture, 16 presumptive zygotes were individually (Fig 2) monitored using the Primo Vision TL® equipment (Vitro-life, Sweden) that takes images every 15 minutes from day 1 to 7.5 post IVF. On day 3 and 5 post IVF, culture media was refresh by changing 50% of the culture medium. The morphokinetics parameters (t2, t3, t4, t5, t8, t9+ tM, tSB, tB, tBX) were recorded during the culture of the embryos until day 7.5 post IVF. The starting point of IVF was considered as time zero (t0).
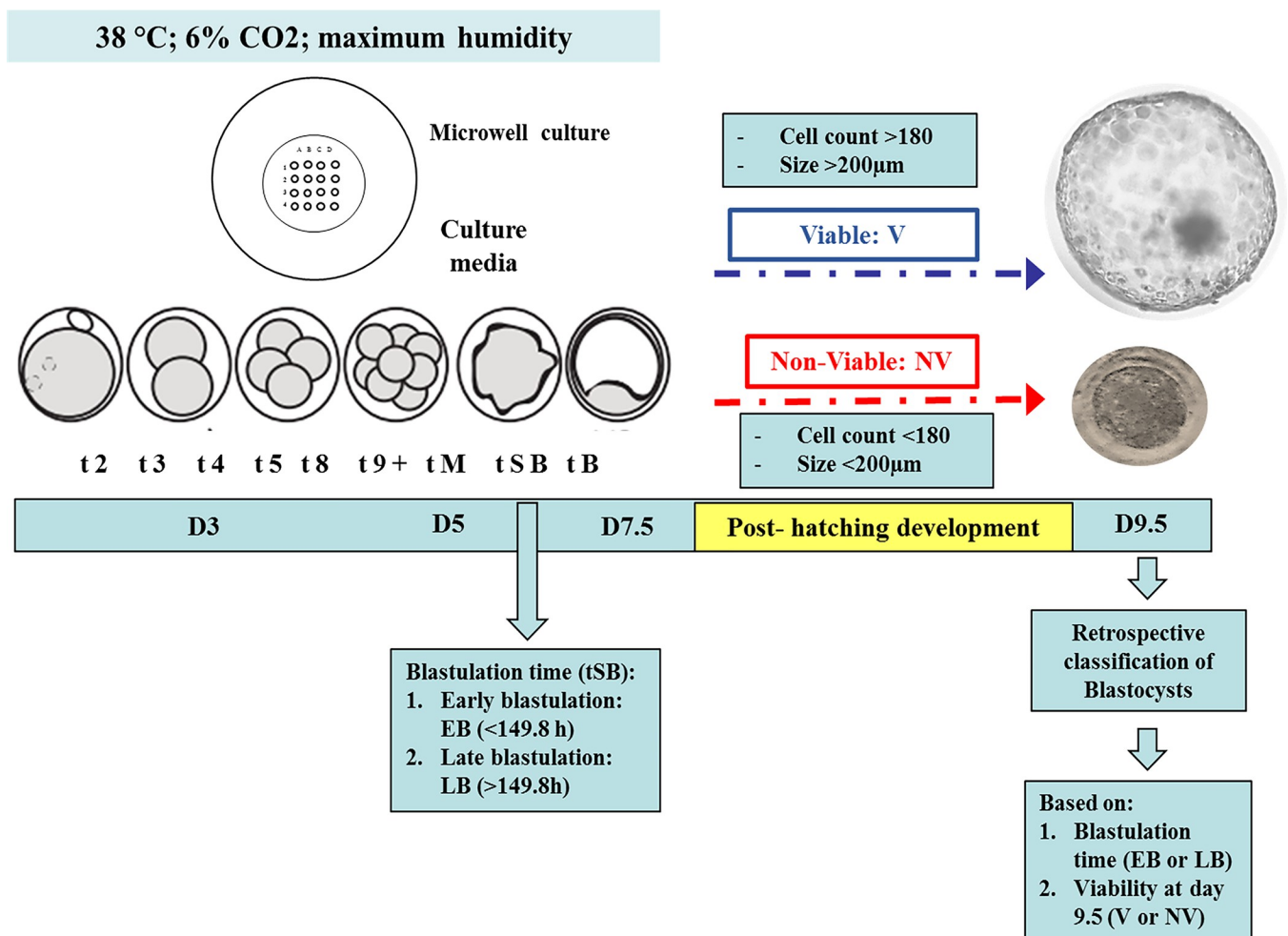
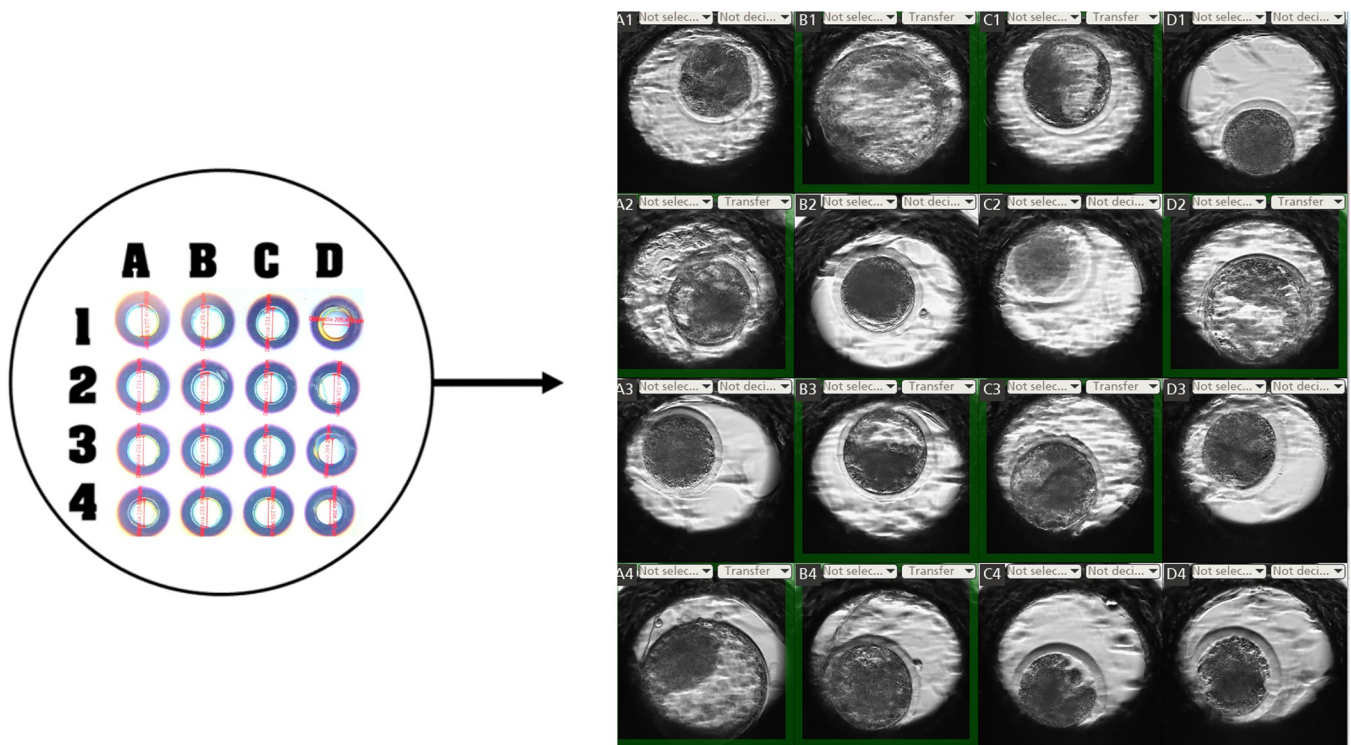**Fig 1. Schematic illustration of experimental design.** Presumptive zygotes were cultured individually and embryonic development monitored with images captured by the Primo Vision TL system for a period of 7.5 days. At day 9.5 post IVF, blastocysts were classified according to their post-hatching development (V: Viable, NV: Non-viable).

Fig 2. Individual culture to monitor embryonic development (Primovision®, Vitrolife, Sweden).

https://doi.org/10.1371/journal.pone.0289751.g002

Variables related to the duration of cell cycles were also determined and designated: duration of second cell cycle t3–t2, duration of third cell cycle t4–t3, duration of cell cycle between t5–t4, duration of cell cycle between t8–t5, duration of cell cycle between t9+–t8, duration of cell cycle between tM–t9+, duration of cell cycle between tSB–tM, duration of cell cycle between tB–tSB, duration of cell cycle between tBX–tB, which combines the concepts of cell cycle and synchrony (S1 Fig).

At the end of individual embryonic development monitoring (day 7.5 post IVF), each video was processed to establish embryonic division times. In addition, the images with calibrated measurements were saved in JPG format to be processed with the ImageJ software to measure the diameters of the embryos at tSB, tB and tBX times. At day 7.5, morphological characteristics of the blastocysts were determined; embryo quality, developmental stage and blastocyst diameter. In vitro viability was evaluated at day 9.5: those with a diameter greater than 200 μm and a total cell count greater than 180 were classified as viable (value 1), while the rest were classified as non in vitro viable (value 0).

As laboratory control, embryos were cultured in groups of 10 to 12 zygotes in 70 μL microdrops under mineral oil. On day 3 and 5 post IVF, a 50% change of the culture medium was performed and on day 3 and 7.5 post IVF the cleavage and blastocysts rate were evaluated, respectively.

For cell count, embryos were individually fixed in 1% paraformaldehyde in saline phosphate buffer for 30 minutes at 4°C. Subsequently, the fixed embryos were incubated individually at 37°C for 15 to 20 min in a dark environment for blastomere nuclei staining in drops of 10 μL of manipulation medium supplemented with 2 μL of DAPI solution (NucBlue™ Fixed Cell ReadyProbes™ Reagent, Thermo Fisher Scientific) and covered with mineral oil. Finally, embryos were mounted on a slide for counting the nuclei using a fluorescence microscope (Axioscope, Carl Zeiss, USA) with a magnification of 100x at a wavelength of 365 nm.

Statistical analysis

Morphokinetics variables (t2, t3, t4, t5, t8, t9+ tM, tSB, tB, tBX) and cell cycle and synchrony (t3-t2, t4-t3, t5-t4, t8-t5, t9+-t8, tM-t9+, tSB-ttM, tB-tSB, tBX-tB) measurements were classified according to embryo viability and blastulation time and analyzed for normality of distribution using Kolmogorov–Smirnov test and for homogeneity of variance using Levene's Test after which these variables were analyzed with ANOVA.

For exploring the relationship between two quantitative variables and one categorical variable we performed a scatterplot analysis with groups. Principal component analysis (PCA) was used for the multidimensional data set to emphasize variation and highlight strong patterns of morphokinetics and cell cycle variables and synchrony of embryonic development according to their in vitro viability (V = viable and N = non-viable) applying the same approach adopted by Mellisho et al. [26].

The predictive model of embryo *in vitro* viability proposed here was based on binary logistic regression to describe the dichotomous dependent variable of the blastocyst (viable = 1 and non-viable = 0). A set of independent morphokinetic variables were analysed with multiple regression. The logistic regression generated the coefficients, the standard errors and level of significance of the model for calculating the probability to predict the viability of the embryos, where values between 0.5 and 1 indicated blastocyst viability. To verify the predictive power of the algorithm, the following indicators were used: ROC-AUC (receiver operating characteristic analysis with determination of the area under the curve), percentage of correct predictions and omnibus tests. Statistical significance was determined at the $P < 0.05$ level. A rough guide for classifying the accuracy of a predictive model was 0.90–1 = excellent, 0.80–0.90 = good, 0.70–0.80 = fair, 0.60–0.70 = poor, 0.50–0.60 = fail. Statistical analysis was performed with the IBM SPSS Statistics program, version 20 (IBM, Armonk, NY, USA).

Results

In this work, 923 viable COCs (quality 1 and 2) were used in 13 *in vitro* embryo production sessions (Table 1). The blastocyst rate obtained on day 7.5 post IVF and cultured in a time-lapse system (42.8%) was superior (p = 0.0001) to the laboratory control group (30.7%).

Impact of blastulation time on embryonic development in vitro

Out of 208 embryos cultured in microwell culture system (time-lapse system), 174 (83.65%) had a first cleavage and 96 (42.8%) developed to the blastocyst stage. The 96 embryos that formed blastocysts reached mean values of t2, t3, t4, t5, t8, t9+ tM, tSB, tB and tBX at 29.41, 35.30, 43.21, 50.58, 65.74, 80.76, 109.66, 149.79, 164.42 and 171.98 h post IVF, respectively (see S1 Table). Also, they were retrospectively classified as viable (>200 μm and >180 cell count) 38/96 (39.58%) and as non-viable (<200 μm and <180 cell count) 58/96 (60.42%) (Table 2).

In this study, of 96 embryos (96/208) that reached blastulation, 49/96 (51.04%) embryos showed early blastulation (<149.79 h) and 47/96 (48.95%) embryos showed late blastulation

Table 1. Production of bovine embryos *in vitro* in a time-lapse system and culture in drops (laboratory control).

Treatment	Rep.	COCs (quality 1 and 2)	Cleavage rate (D3 post IVF) (x± SD)	Blastocyst rate (D7.5 post IVF) (x± SD)
Laboratory Control (in drops)	13	715	82.1± 5.2 [a]	30.7± 3.1 [a]
Microwell (Individually) Time-lapse system	13	208	83.7± 7.0 [a]	42.8± 9.2 [b]

a,b Different letters in the same column show significant differences (P<0.05). X: Mean, SD: Standard Deviation.

https://doi.org/10.1371/journal.pone.0289751.t001

Table 2. Characteristics of *in vitro* cultured blastocyst according to *in vitro* viability determined by extended culture up to 9.5 days post IVF.

Viability *in vitro*	n	Blastulation time (tSB)		Blastocyst stage (tB)		Blastocyst (7.5 post IVF)
		Time (h)	Diameter (μm)	Time (h)	Diameter (μm)	Diameter (μm)
Viable	38	142.55±7.79 [a]	155.36± 4.45 [a]	159.75±8.76 [a]	168.93±7.77 [a]	209.29±17.19 [a]
Non-viable	58	154.54±12.92 [b]	154.81±4.60 [a]	167.91±9.95 [b]	167.21±7.53 [a]	176.35±15.02 [b]

a,b Different letters in the same column show significant differences (P<0.05).

(>149.79 h). In Table 3, we observed that blastulation time is critical for *in vitro* viability, presenting high and significant viability the embryos with early versus late blastulation (63.27 vs 14.89%, respectively). The average blastulation timing for the 96 embryos resulted in 149.79 h.

In Fig 3A and 3B, the morphokinetic parameters of embryonic development were classified and compared statistically according to *in vitro* viability. The variables tM, tsB and tBx (Fig 3A) and tm-t9+ (Fig 3B) showed statistical differences (P<0.05). Scatter plot showed that embryos with time of starting blastulation (tSB) less than 155 h maximized their in vitro viability to 54.54% (Fig 3C, 3D and 3E), although embryos with time of starting blastulation (tSB) greater than 155 h reduce to 6.67% their viability (see S1 Table). In Fig 3E, we show that a combination between time of starting blastulation (tSB) less than 155 h and embryo diameter measured at day 7.5 post IVF greater than 180 μm ensures maximum viability of *in vitro* produced blastocysts.

In Fig 4A, *in vitro* viability shows a positive and significant Pearson's correlation coefficient with embryo diameter at day 7.5 post IVF (0.75) and a negative and significant correlation with embryo quality at day 7.5 post IVF (-0.56). Also, in Fig 4B and 4C, PCA analysis of multivariable data sets does not allow to emphasize variation or highlight patterns or cluster embryo morphokinetics variables (Fig 4B) and morphokinetics variables and embryo diameter after the start of blastulation (Fig 4C) according to their in vitro viability.

Mathematical model to predict viability *in vitro*

Mathematical model-1 and model-2 included univariable data of the time of starting blastulation (tSB) and blastulation time (BT) had a fair predictor (ROC-AUC 0.7–0.8) of viability. While, the mathematical model-3 included the univariate data of embryo diameter at day 7.5 post IVF (D75) and had an excellent predictor of viability (ROC-AUC 0.92) (Tables 4 and 5). Additionally, the mathematical model-5 included four quantitative parameters of blastocyst (DtSB, DtB, DtBX, D75) after blastulation time and had the highest precision for predicting viability (ROC-AUC 0.93). Mathematical models 3, 4 and 5 showed a high ROC-AUC value >0.90 (Table 5).

The logistic function (logit) for Model-3

$$Y = \ln[p/(1-p)] = -24.393 + 0.125 * D75$$

Table 3. Characteristics of blastocyst cultured *in vitro* according to blastulation time.

Group	N	Blastulation time (h) (x± SD)	Blastocyst rate (7.5 post IVF)		Post hatching development (9.5 post IVF)		
			%	Diameter (μm)	%	Total count cell	Diameter (μm)
Early	49	140.01±6.48[a]	49 (100%)	204.04±19.59[a]	31 (63.27%) [a]	268.03±72.97	335.60±73.51
Late	47	159.99±8.70[b]	47 (100%)	174.11±13.82[b]	7 (14.89%) [b]	260.7±65.94	309.65±85.46

a,b Different letters in the same column show significant differences (P<0.05). X: Mean, SD: Standard Deviation.
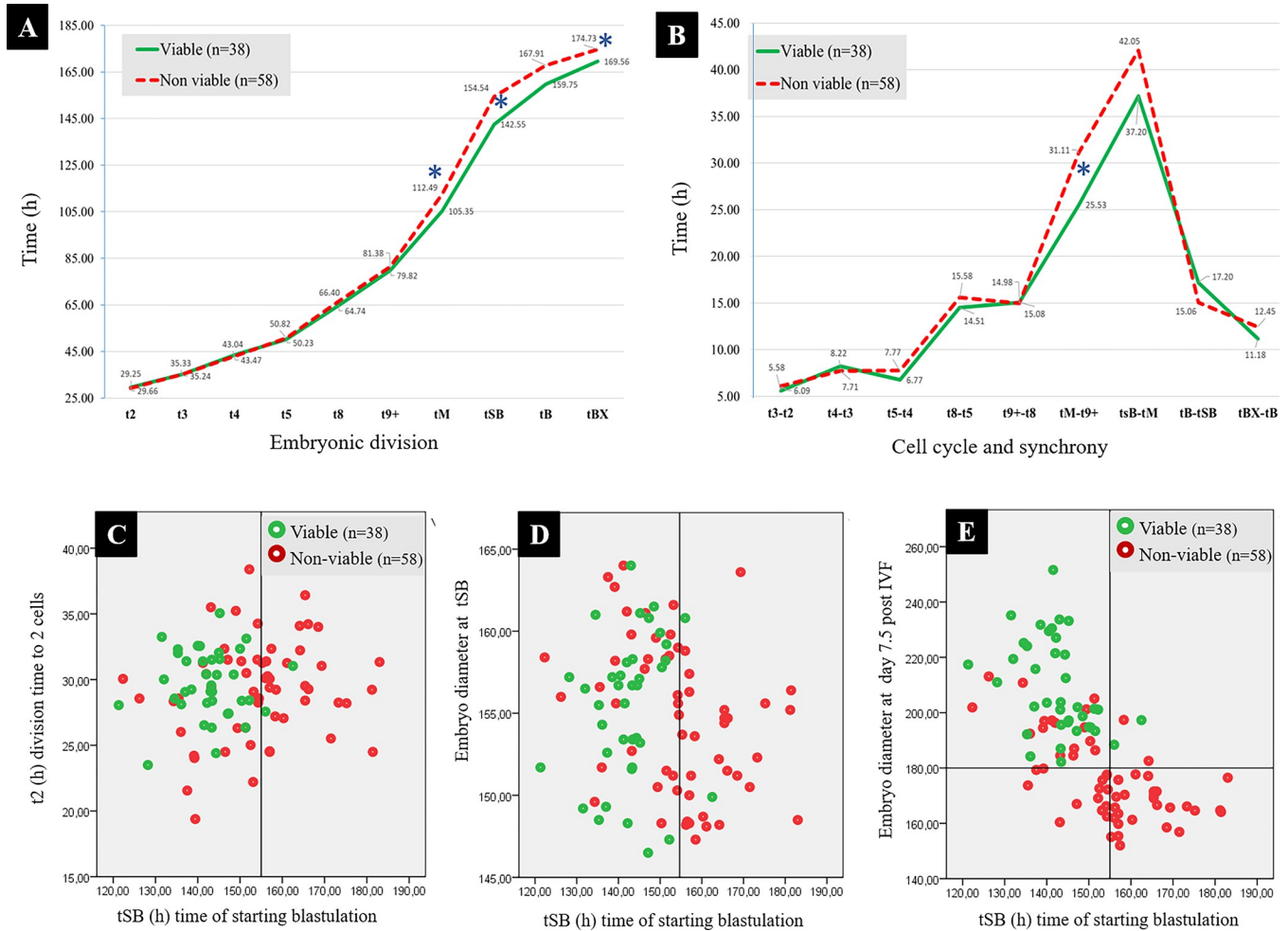
Fig 3. Bovine embryonic development in microwell culture system according to viability (Viable "green line" and non-viable (red line). A) Morphokinetic parameters of embryonic divisions t2, t3, t4, t5, t8, t9+, tM, tSB, tB and tBX; B) Time of cell cycle and synchrony t3-t2, t4-t3, t5-t4, t8-t5, t9+-t8, tM-t9+, tSB-ttM, tB-tSB, tBX-tB. (*) It indicates that in this morphokinetic parameter there is a statistical difference (P<0.05); Scatter plot illustrating two variables from the morphokinetics data, where colour represents *in vitro* viability, C) t2 (h) division time to 2 cells; D) Embryo diameter at tSB; and E) Embryo diameter at day 7.5 post IVF, with tSB (h) time of starting blastulation, respectively.

The logistic function (logit) for Model-2

$$Y = \ln[p/(1{-}p)] = 2.83 - 2.287 * BT$$

The logistic function (logit) for Model-1

$$Y = \ln[p/(1{-}p)] = 15.274 - 0.106 * tSB$$

The estimated probability is:

$$p = \mathrm{Exp}\, Y / (1 + \mathrm{Exp}\, Y)$$

Where:
ln is the natural logarithm, log exp, where exp = 2.71828...
ln[p/(1-p)] is the log odds ratio, or "logit"
p/(1-p) is the "odds ratio"

p is the probability that the event Y occurs, p (Y = 1)

If. p > 0.5, Predicts, embryo viable

p < 0.5, Predicts, embryo non-viable

Example: Estimated value of probability to predict viability for model 3:

D75 (µm))	Y	p	Predicted
173.729	-2.68	0.06	Non viable
179.884	-1.91	0.13	Non viable
163.567	-3.95	0.02	Non viable
166.116	-3.63	0.03	Non viable
196.371	0.15	0.54	viable
158.534	-4.58	0.01	Non viable
164.131	-3.88	0.02	Non viable
194.569	-0.07	0.48	Non viable
201.487	0.79	0.69	Viable
229.554	4.30	0.99	Viable
202.061	0.86	0.70	Viable
211.086	1.99	0.88	Viable
213.196	2.26	0.91	Viable
231.816	4.58	0.99	Viable
225.276	3.77	0.98	Viable
235.291	5.02	0.99	Viable

https://doi.org/10.1371/journal.pone.0289751.t006

Discussion

Time-lapse system of embryonic development is currently one of the most advanced techniques used in *in vitro* embryo production in humans [31], cattle [32] and ovine [33]. In humans and bovines, morphokinetics has generated a database of parameters which has included the development of machine learning technologies to provide further insights to interpret and understand the embryonic development data.
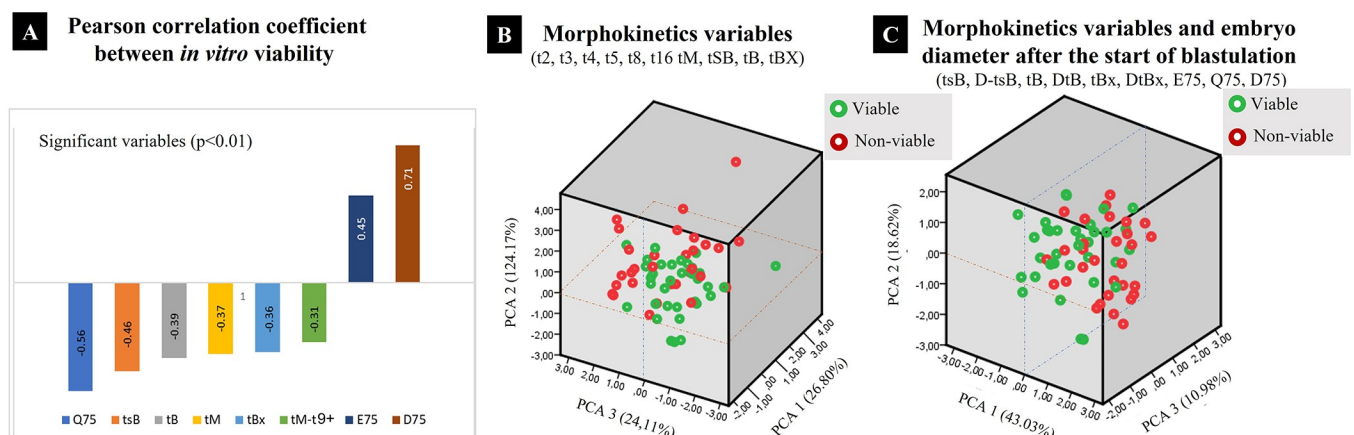
Fig 4. Pearson correlation coefficient and principal component analysis for all variables with *in vitro* viability. A) Variables con Pearson's correlation coefficient significative (p<0.01). PCA analysis of morphokinetics variables (B) and morphokinetics variables and embryo diameter after the start of blastulation (C) according to their in vitro viability (V = viable and N = non-viable).

https://doi.org/10.1371/journal.pone.0289751.g004

Table 4. Non-invasive predictive models of *in vitro* viability using binary logistic regression.

Variable	Model-1 (tSB)	Model-2 (BT)	Model-3 (D75)	Model-4 (E75, Q75, D75)	Model-5 (DtSB, DtB, DtBX, D75)
Constant	15.274	2.830	-24.393	-24.11	36.014
Blastocyst kinetics					
BT (early = 1, late = 2)	–	-2.287	–	–	–
tSB	-0.106	–	–	–	–
Blastocyst morphometry					
D-tSB	–	–	–	–	-0.008
D-tB	–	–	–	–	-0.052
D-tBX	–	–	–	–	-0.364
E75	–	–	–	-0.038	–
Q75	–	–	–	-0.025	0.216
D75	–	–	0.125	0.125	–
Algorithm power					
ROC-AUC (0.9–1 = excellent, 0.8–0.9 = good, 0.7–0.8 = fair predictor)	0.79	0.75	0.92	0.92	0.93
Correct prediction (%)	70.8	74.0	81.3	81.3	82.8
Coef. Omnibus test	0.000	0.000	0.000	0.000	0.000
Nagelkerke R-Square	0.308	0.309	0.655	0.655	0.695
Cox and Snell R squared	0.227	0.228	0.484	0.484	0.521

Note: Time of starting blastulation (tSB), blastulation time (BT; early = 1, late = 2), time to blastocyst stage (tB), time to expanded blastocyst stage (tBX), diameter to time of starting blastulation (DtSB), diameter to time to blastocyst stage (DtB), diameter to time to expanded blastocyst stage (DtBX), embryo stage at day 7.5 post IVF (E75), embryo quality at day 7.5 post IVF (Q75) and embryo diameter at day 7.5 post IVF (D75).

The first division is an indicator of the developmental potential of embryos produced *in vitro* [34] and first division time is related to the state of polyadenylation and transcription of genes that are important for early embryonic development [35]. In this work, the average first cleavage time in bovine embryos was 31.18 h (Min 19.39 h and max: 69.33 h). Likewise, only 7.4% (2/27) of embryos cleaved after 36 h IVF developed into a blastocyst, similar to those reported by Dinnyés et al. [36] that showed very few blastocysts (5%) developed from embryos cleaved after 36 h IVF. Also, in human [37] and bovine [25] studies, time lapse monitoring has revealed that first cleavage after fertilization can be indicative of implantation potential. The first cleavage rate reported in this study was 83.65% (174/208), being similar to those reported by Rizos et al. [38] 84.3%, and Sanches et al. [39] 78.8%.

A conventional culture system (microdrops) allows the development of 30% of the oocytes matured *in vitro* to blastocysts [40–42] similar to the result reported in our laboratory control. However, the use of microwell culture to monitor embryonic development showed blastocyst rates greater than 30% in cattle [43] and humans [44–46] when cultured in a time-lapse system versus the traditional microdrops system. The difference in the higher rate of blastocysts in the

Table 5. Logistic regression parameters of model-3 to predict viability *in vitro*.

Variable	B	95% CI de OR	P value
Constant	-24.393		0.000
D75	0.125	1.077–1.192	0.000

Regression coefficient (B), odds ratio (OR), Confidence interval (95% CI of OR).

time-lapse system could be due to the lower exposure to changes in pH, temperature, osmotic pressure and lighting during the observation and evaluation of embryonic development [47]. In addition, culture in a time-lapse system allows the non-invasive observation of key developmental markers, such as the extrusion of polar bodies, the formation of pronuclei, division times, and the duration of cell cycles that may be indicative of a greater potential of blastocyst development, which could greatly influence embryo selection by offering new opportunities and approaches for embryologists [48].

It is important to understand that a competent oocyte must develop to the blastocyst stage, and then, when transferred to the uterus of a recipient, it must have good interaction with the maternal environment and has the ability to implant. That is why during the last decades, optimizing *in vitro* systems that impact embryonic quality and competence has been a priority issue for laboratories and embryologists [49]. Likewise, non-invasive techniques that include observation or morphological and morphokinetic parameters for the classification of embryonic development are more frequently used in IVF programs [50,51]. In this study, embryos with *in vitro* viability have statistically different characteristics from non-viable embryos, being the key factor the speed of blastulation onset (Tables 2 and 3).

Post-hatching development (*in vitro* viability) in extended culture emerged as a more accurate alternative method to assess the development capacity of the embryo, without the need to be transferred to recipients and maintaining *in vitro* conditions similar to all embryos [52,53]. This stage of development can be used not only to assess the quality of embryos produced by different technologies, but also as a model to study embryonic loss during the period of cell differentiation and embryo elongation [53]. On the other hand, determining the viability of blastocysts under *in vivo* conditions has been carried out by transferring embryos at day 7 and recovering them after day 14 or 16 of development from the cow uterus [54–57]. Although this invasive technique may be more accurate in determining viability, the use of surgical procedures in the recovery of elongated blastocysts is poorly repeatable.

Our results show that post-hatching viability of embryos with early blastulation (63.27%; 31/49) was higher (P<0.05) than in late blastulation (14.87%; 7/47) embryos. Blastulation is an essential event in preimplantation embryonic development during which many molecular and morphological changes occur [58]. In addition, between the compact morula and blastocyst stages, the first differentiation of the cell lineage occurs, forming the inner cell mass (ICM) and the trophectoderm [59,60]. Likewise, early division favors the abundance of transcripts in all stages and increases blastocyst production [61], while early blastulation has been related to a better synchrony of embryonic development [18,62].

On the other hand, the use of time-lapse technology to monitor early development, pronuclear formation and fusion, and time to first division is quite common in humans [63,64]. However, the determination of the impact of the blastulation moment is little studied [65,66]. In humans, Ho et al. [14] and Moustafa et al. [16] indicated that time of blastulation has a high potential for embryo selection over other embryo morphologic grading components. Likewise, Lee et al. [15]. and Moustafa et al, [16]. reported that embryos with chromosomal abnormalities (aneuploid and mosaic) showed delayed blastulation. Nevertheless, in cattle, there are few reports regarding the moment of blastulation and its impact on subsequent in vitro development.

In humans, researchers have analyzed the prediction of embryonic morphokinetic evaluation (based on time-lapse system results) on implantation, results of combined mathematical models showed less precision than those shown in this work with ROC-AUC of 0.7 [67], 0.602 [66], 0.70 [68] and 0.71 [69]. Although, the ROC-AUC value was reduced to 0.561, when it only included the blastocyst morphology parameters [66]. However, models that consider morphokinetic variables are very useful for predicting blastocyst formation (ROC-AUC of

0.849). On the other hand, Alpha Executive and ESHRE Special Interest Group of Embryology (2011) proposed a blastocyst morphological evaluation system based on the combination of developmental stage and quality criteria. Even though, predictive models based on morphological parameters have low precision (ROC-AUC of 0.55) to predict successful pregnancy [70].

In cattle, some work has been done to predict pregnancy or implantation capacity in relation to morphological parameters and the time of the first embryonic division [71–73] although, the results have been variable and contradictory. Holm et al. [71] affirm that the time-lapse system is a superior method to study embryonic kinetics in cattle and to select embryos with a high probability of being competent (63 to 80%). On the other hand, Mellisho et al. [26]. used a mathematical model, with ROC-AUC value of 0.724, combining morphological and morphokinetic variables of the embryo (blastulation time, stage of development, quality, and diameter of the blastocyst at day 7.5) to predict *in vitro* viability. In this study, it was shown that the moment of blastulation affects the development of blastocysts diameter and quality at day 7.5 and *in vitro* viability.

The use of time-lapse technology has allowed the acquisition of morphokinetic parameters for the selection of viable embryos. These parameters include the duration of the first division of 1 to 2 cells, the time between division of 2 to 3 cells, the time between division of 3 to 4 cells, the cycle patterns of uniform divisions with short intervals in stage of 3 and 5 cells, and the time of the abrupt first cell division into 3 or more cells [23,22]. In humans, some studies report improved clinical outcomes when predictive morphokinetic models are used to select embryos for transfer [74], although the results are still controversial with other reports [22,48].

Conclusion

The results from this work support that embryo morphokinetic variables at early stages of development could be used simply and routinely to predict developmental viability of in vitro produced bovine embryos. However, the high cost of time lapse monitoring equipment could limit its use in cattle. We propose two variables that require only two observations at the end of in vitro culture without the need for complex devices. Therefore, selection of blastocysts based on a blastulation time of less than 155 h and a blastocyst diameter measured on day 7.5 after IVF greater than 180 μm maximizes their viability in vitro.

Supporting information

S1 Fig. The morphokinetics parameters in embryo culture using time lapse system. (TIF)

S1 Table. Morphokinetic development of bovine embryos. (XLSX)

Author Contributions

Conceptualization: Lleretny Rodriguez-Alvarez, Edwin Mellisho.

Data curation: Edwin Mellisho.

Formal analysis: Misael Rodríguez, Mario Briones, Edwin Mellisho.

Funding acquisition: Edwin Mellisho.

Investigation: Misael Rodríguez, Jhorjhi Vega, Edwin Mellisho.

Methodology: Carmen Huayhua, Misael Rodríguez, Jhorjhi Vega, Edwin Mellisho.

Project administration: Edwin Mellisho.

Resources: Lleretny Rodriguez-Alvarez, Edwin Mellisho.

Software: Mario Briones, Edwin Mellisho.

Supervision: Edwin Mellisho.

Validation: Lleretny Rodriguez-Alvarez, Edwin Mellisho.

Visualization: Carmen Huayhua, Edwin Mellisho.

Writing – original draft: Carmen Huayhua, Jhorjhi Vega, Edwin Mellisho.

Writing – review & editing: Carmen Huayhua, Misael Rodríguez, Jhorjhi Vega, Mario Briones, Lleretny Rodriguez-Alvarez, Edwin Mellisho.

References

1. Blondin P. Logistics of large scale commercial IVF embryo production. Reproduction, fertility, and development. 2016; 29:32–36. https://doi.org/10.1071/RD16317 PMID: 28278791

2. IETS. Embryo Technology Newsletter. International Embryo Technology Society. 2022; 40(4):45p.

3. Pontes JH, Nonato-Junior I, Sanches BV, Ereno-Junior JC, Uvo S, Barreiros TR, et al. Comparison of embryo yield and pregnancy rate between in vivo and in vitro methods in the same Nelore (Bos indicus) donor cows. Theriogenology. 2009; 71: 690–697. https://doi.org/10.1016/j.theriogenology.2008.09.031 PMID: 18995895

4. Demyda-Peyrás S, Dorado J, Hidalgo M, Anter J, De Luca L, Genero E, et al. Effects of oocyte quality, incubation time and maturation environment on the number of chromosomal abnormalities in IVF-derived early bovine embryos. Reproduction, fertility, and development. 2013; 25: 1077–1084. https://doi.org/10.1071/RD12140 PMID: 23182337

5. Agca Y, Monson RL, Northey DL, Peschel DE, Schaefer DM, Rutledge JJ. Normal calves from transfer of biopsied, sexed and vitrified IVP bovine embryos. Theriogenology. 1998; 50(1):129–145. https://doi.org/10.1016/s0093-691x(98)00120-4 PMID: 10734481

6. Miles JR, Farin CE, Rodriguez KF, Alexander JE, Farin PW. Angiogenesis and morphometry of bovine placentas in late gestation from embryos produced in vivo or in vitro. Biol Reprod. 2004; 71(6):1919–1926. https://doi.org/10.1095/biolreprod.104.031427 PMID: 15286036

7. Bertolini M, Moyer AL, Mason JB, Batchelder CA, Hoffert KA, Bertolini LR, et al. Evidence of increased substrate availability to in vitro-derived bovine foetuses and association with accelerated conceptus growth. Reproduction. 2004; 128(3):341–54. https://doi.org/10.1530/rep.1.00188 PMID: 15333785

8. van Wagtendonk-de Leeuw AM, Aerts BJ, den Daas JH. Abnormal offspring following in vitro production of bovine preimplantation embryos: a field study. Theriogenology. 1998; 49(5):883–94. https://doi.org/10.1016/s0093-691x(98)00038-7 PMID: 10732097

9. Numabe T, Oikawa T, Kikuchi T, Horiuchi T. Birth weight and birth rate of heavy calves conceived by transfer of in vitro or in vivo produced bovine embryos. Anim Reprod Sci. 2000; 64(1–2):13–20. https://doi.org/10.1016/s0378-4320(00)00190-1 PMID: 11078963.

10. Farin PW, Britt JH, Shaw DW, Slenning BD. Agreement among evaluators of bovine embryos produced in vivo or in vitro. Theriogenology. 1995; 44:339–349. https://doi.org/10.1016/0093-691x(95)00189-f PMID: 16727734

11. Fogarty NME, McCarthy A, Snijders KE, Powell BE, Kubikova N, Blakeley P, et al. Genome editing reveals a role for OCT4 in human embryogenesis. Nature. 2017; 550: 67–73. https://doi.org/10.1038/nature24033 PMID: 28953884

12. Alberio R. Regulation of cell fate decisions in early mammalian embryos. Annual review of animal biosciences. 2020; 8:377–393. https://doi.org/10.1146/annurev-animal-021419-083841 PMID: 31730400

13. Ortega MS, Kelleher AM, O'Neil E, Benne J, Cecil R, Spencer TE. NANOG is required to form the epiblast and maintain pluripotency in the bovine embryo. Molecular reproduction and development. 2020; 87:152–160. https://doi.org/10.1002/mrd.23304 PMID: 31803983

14. Ho JR, Arrach N, Rhodes-Long K, Salem W, McGinnis LK, Chung K, et al. Blastulation timing is associated with differential mitochondrial content in euploid embryos. J Assist Reprod Genet. 2018 Apr; 35 (4):711–720. https://doi.org/10.1007/s10815-018-1113-9 PMID: 29353449

15. Lee CI, Chen CH, Huang CC, Cheng EH, Chen HH, Ho ST, et al. Embryo morphokinetics is potentially associated with clinical outcomes of single-embryo transfers in preimplantation genetic testing for

aneuploidy cycles. Reprod Biomed Online. 2019 Oct; 39(4):569–579. https://doi.org/10.1016/j.rbmo.2019.05.020 PMID: 31395516

16. Moustafa SM, Rosen EM, Boylan C, Mersereau JE. Time to blastulation is superior to individual components of embryo grading for live-birth prediction. F&S reports 2020; 1: 243–248. https://doi.org/10.1016/j.xfre.2020.09.016 PMID: 34223251

17. Richter KS, Shipley SK, McVearry I, Tucker MJ, Widra EA. Cryopreserved embryo transfers suggest that endometrial receptivity may contribute to reduced success rates of later developing embryos. Fertility and sterility. 2006; 86:862–866. https://doi.org/10.1016/j.fertnstert.2006.02.114 PMID: 16935284

18. Shapiro BS, Daneshmand ST, Garner FC, Aguirre M, Thomas S. Large blastocyst diameter, early blastulation, and low preovulatory serum progesterone are dominant predictors of clinical pregnancy in fresh autologous cycles. Fertility & Sterility. 2008; 302–309. https://doi.org/10.1016/j.fertnstert.2007.06.062 PMID: 17905239

19. Hoelker M, Held E, Salilew-Wondim D, Schellander K, Tesfaye D. Molecular signatures of bovine embryo developmental competence. Reprod Fertil Dev. 2013; 26(1):22–36. https://doi.org/10.1071/RD13255 PMID: 24305174

20. Sugimura S, Akai T, Imai K. Selection of viable in vitro-fertilized bovine embryos using time-lapse monitoring in microwell culture dishes. The Journal of reproduction and development. 2017; 63:353–357. https://doi.org/10.1262/jrd.2017-041 PMID: 28552887

21. Farin PW, Slenning BD, Britt JH. Estimates of pregnancy outcomes based on selection of bovine embryos produced in vivo or in vitro. Theriogenology. 1999; 52:659–670. https://doi.org/10.1016/S0093-691X(99)00160-0 PMID: 10734364

22. Wong CC, Loewke KE, Bossert NL, Behr B, De Jonge CJ, Baer TM, et al. Non-invasive imaging of human embryos before embryonic genome activation predicts development to the blastocyst stage. Nat Biotechnol. 2010 Oct; 28(10):1115–21. https://doi.org/10.1038/nbt.1686 PMID: 20890283

23. Meseguer M, Herrero J, Tejera A, Hilligsoe KM, Ramsing NB, Remohi J. The use of morphokinetics as a predictor of embryo implantation. Human Reproduction. 2011; 26:2658–2671. https://doi.org/10.1093/humrep/der256 PMID: 21828117

24. Lundin K, Park H. Time-lapse technology for embryo culture and selection. Upsala journal of medical sciences. 2020 125:77–84. https://doi.org/10.1080/03009734.2020.1728444 PMID: 32096675

25. Sugimura S, Akai T, Hashiyada Y, Somfai T, Inaba Y, Hirayama M, et al. Promising system for selecting healthy in vitro-fertilized embryos in cattle. PLoS One. 2012; 7(5):e36627. https://doi.org/10.1371/journal.pone.0036627 PMID: 22590579

26. Mellisho EA, Briones MA, Velásquez AE, Cabezas J, Castro FO, Rodríguez-Álvarez L. Extracellular vesicles secreted during blastulation show viability of bovine embryos. Reproduction. 2019; 158:477–492. https://doi.org/10.1530/REP-19-0233 PMID: 31600718

27. Stringfellow DA, Givens MD. Manual of the International Embryo Transfer Society (IETS), 4th ed. Champaign, IL: IETS. 2010.

28. Rodríguez L, Navarrete FI, Tovar H, Cox JF, Castro FO. High developmental potential in vitro and in vivo of cattle embryos cloned without micromanipulators. Journal of Assisted Reproduction & Genetics. 2008; 13–16. https://doi.org/10.1007/s10815-007-9194-x PMID: 18205035

29. de Los Santos MJ, Gámiz P, de Los Santos JM, Romero JL, Prados N, Alonso C, et al. The Metabolomic Profile of Spent Culture Media from Day-3 Human Embryos Cultured under Low Oxygen Tension. PLoS One. 2015 Nov 12; 10(11):e0142724. https://doi.org/10.1371/journal.pone.0142724 PMID: 26562014

30. Nastri CO, Nóbrega BN, Teixeira DM, Amorim J, Diniz LMM, Barbosa MWP, et al. Low versus atmospheric oxygen tension for embryo culture in assisted reproduction: a systematic review and meta-analysis. Fertil Steril. 2016 Jul; 106(1):95–104.e17. https://doi.org/10.1016/j.fertnstert.2016.02.037 PMID: 27012651

31. Kalleas D, McEvoy K, Horne G, Roberts SA, Brison DR. Live birth rate following undisturbed embryo culture at low oxygen in a time-lapse incubator compared to a high-quality benchtop incubator. Hum Fertil. 2022; 25(1):147–153. https://doi.org/10.1080/14647273.2020.1729423 PMID: 32098536

32. Lechniak D, Sell-Kubiak E, Warzych E. The metabolic profile of bovine blastocysts is affected by in vitro culture system and the pattern of first zygotic cleavage. Theriogenology. 2022; 188:43–51. https://doi.org/10.1016/j.theriogenology.2022.05.021 PMID: 35661988

33. Fryc K, Nowak A, Kij B, Kochan J, Bartlewski PM, Murawski M. Timing of cleavage divisions determined with time-lapse imaging is linked to blastocyst formation rates and quality of in vitro-produced ovine embryos. Theriogenology. 2021; 159:147–152. https://doi.org/10.1016/j.theriogenology.2020.10.031 PMID: 33157452

34. Lechniak D, Pers-Kamczyc E, Pawlak P. Timing of the first zygotic cleavage as a marker of developmental potential of mammalian embryos. Reprod Biol. 2008 Mar; 8(1):23–42. https://doi.org/10.1016/s1642-431x(12)60002-3 PMID: 18432305

35. Lonergan P, Gutiérrez-Adán A, Pintado B, Fair T, Ward F, Fuente JD, et al. Relationship between time of first cleavage and the expression of IGF-I growth factor, its receptor, and two housekeeping genes in bovine two-cell embryos and blastocysts produced in vitro. Mol Reprod Dev. 2000 Oct; 57(2):146–52. https://doi.org/10.1002/1098-2795(200010)57:2<146::AID-MRD5>3.0.CO;2-2 PMID: 10984414

36. Dinnyés A, Lonergan P, Fair T, Boland MP, Yang X. Timing of the first cleavage post-insemination affects cryosurvival of in vitro-produced bovine blastocysts. Mol Reprod Dev. 1999 Jul; 53(3):318–24. https://doi.org/10.1002/(SICI)1098-2795(199907)53:3<318::AID-MRD7>3.0.CO;2-O PMID: 10369392

37. Mandawala AA, Harvey SC, Roy TK, Fowler KE. Time-lapse embryo imaging and morphokinetic profiling: Towards a general characterisation of embryogenesis. Anim Reprod Sci. 2016 Nov; 174:2–10. https://doi.org/10.1016/j.anireprosci.2016.09.015 PMID: 27720247

38. Rizos D, Gutiérrez-Adán A, Pérez-Garnelo S, De La Fuente J, Boland MP, Lonergan P. Bovine embryo culture in the presence or absence of serum: implications for blastocyst development, cryotolerance, and messenger RNA expression. Biology of reproduction. 2003; 68:236–243. https://doi.org/10.1095/biolreprod.102.007799 PMID: 12493719

39. Sanches BV, Marinho LS, Filho BD, Pontes JH, Basso AC, Meirinhos ML, et al. Cryosurvival and pregnancy rates after exposure of IVF-derived Bos indicus embryos to forskolin before vitrification. Theriogenology. 2013; 80:372–377. https://doi.org/10.1016/j.theriogenology.2013.04.026 PMID: 23746692

40. Farin PW, Crosier AE, Farin CE. Influence of in vitro systems on embryo survival and fetal development in cattle. Theriogenology. 2001; 55:151–170. https://doi.org/10.1016/s0093-691x(00)00452-0 PMID: 11198080

41. Mucci N, Aller J, Kaiser GG, Hozbor F, Cabodevila J, Alberio RH. Effect of estrous cow serum during bovine embryo culture on blastocyst development and cryotolerance after slow freezing or vitrification. Theriogenology. 2006; 65:1551–1562. https://doi.org/10.1016/j.theriogenology.2005.08.020 PMID: 16229883

42. Presicce GA, Xu J, Gong G, Moreno JF, Chaubal S, Xue F, et al. Oocyte source and hormonal stimulation for in vitro fertilization using sexed spermatozoa in cattle. Vet Med Int. 2010 Sep 5; 2011:145626. https://doi.org/10.4061/2011/145626 PMID: 20885928

43. Sugimura S, Akai T, Hashiyada Y, Aikawa Y, Ohtake M, Matsuda H, et al. Effect of embryo density on in vitro development and gene expression in bovine in vitro-fertilized embryos cultured in a microwell system. The Journal of reproduction and development. 2013; 59:115–122. https://doi.org/10.1262/jrd.2012-113 PMID: 23154384

44. Wu L, Han W, Wang J, Zhang X, Liu W, Xiong S, et al. Embryo culture using a time-lapse monitoring system improves live birth rates compared with a conventional culture system: a prospective cohort study. Human fertility. 2018; 21:255–262. https://doi.org/10.1080/14647273.2017.1335886 PMID: 28597711

45. Kermack AJ, Fesenko I, Christensen DR, Parry KL, Lowen P, Wellstead SJ, et al. Incubator type affects human blastocyst formation and embryo metabolism: a randomized controlled trial. Hum Reprod. 2022 Nov 24; 37(12):2757–2767. https://doi.org/10.1093/humrep/deac233 PMID: 36287638

46. Zhang XD, Zhang Q, Han W, Liu WW, Shen XL, Yao GD et al. Comparison of embryo implantation potential between time-lapse incubators and standard incubators: a randomized controlled study. Reprod Biomed Online. 2022 Nov; 45(5):858–866. https://doi.org/10.1016/j.rbmo.2022.06.017 PMID: 36210273

47. Zhang JQ, Li XL, Peng Y, Guo X, Heng BC, Tong GQ. Reduction in exposure of human embryos outside the incubator enhances embryo quality and blastulation rate. Reproductive biomedicine online. 2010; 20:510–515. https://doi.org/10.1016/j.rbmo.2009.12.027 PMID: 20129824

48. Kirkegaard K, Agerholm IE, Ingerslev HJ. Time-lapse monitoring as a tool for clinical embryo assessment. Hum Reprod. 2012 May; 27(5):1277–85. https://doi.org/10.1093/humrep/des079 PMID: 22419744

49. Kushnir VA, Barad DH, Albertini DF, Darmon SK, Gleicher N. Systematic review of worldwide trends in assisted reproductive technology 2004–2013. Reprod Biol Endocrinol. 2017 Jan 10; 15(1):6. https://doi.org/10.1186/s12958-016-0225-2 PMID: 28069012

50. Zollner U, Zollner KP, Hartl G, Dietl J, Steck T. The use of a detailed zygote score after IVF/ICSI to obtain good quality blastocysts: the German experience. Human reproduction. 2002; 17:1327–1333. https://doi.org/10.1093/humrep/17.5.1327 PMID: 11980760

51. Gianaroli L, Magli MC, Ferraretti AP, Fortini D, Grieco N. Pronuclear morphology and chromosomal abnormalities as scoring criteria for embryo selection. Fertility and sterility. 2003; 80:341–349. https://doi.org/10.1016/s0015-0282(03)00596-x PMID: 12909497

52. Vejlsted M, Du Y, Vajta G, Maddox-Hyttel P. Post-hatching development of the porcine and bovine embryo—defining criteria for expected development in vivo and in vitro. Theriogenology. 2006; 65:153–165. https://doi.org/10.1016/j.theriogenology.2005.09.021 PMID: 16257443

53. Machado GM, Ferreira AR, Pivato I, Fidelis A, Spricigo JF, Paulini F, et al. Post-hatching development of in vitro bovine embryos from day 7 to 14 in vivo versus in vitro. Molecular reproduction and development. 2013; 80:936–947. https://doi.org/10.1002/mrd.22230 PMID: 24022836

54. Bertolini M., Beam SW, Shim H, Bertolini LR, Moyer AL, Famula TR, Anderson GB. Growth, development, and gene expression by in vivo- and in vitro-produced day 7 and 16 bovine embryos. Molecular reproduction and development. 2002; 63: 318–328. https://doi.org/10.1002/mrd.90015 PMID: 12237947

55. Clemente M, de La Fuente J, Fair T, Al Naib A, Gutierrez-Adan A, Roche JF, et al. Progesterone and conceptus elongation in cattle: a direct effect on the embryo or an indirect effect via the endometrium?. Reproduction. 2009; 138, 507–517. https://doi.org/10.1530/REP-09-0152 PMID: 19556439

56. Rodríguez-Alvarez L, Sharbati J, Sharbati S, Cox JF, Einspanier R, Castro FO. Differential gene expression in bovine elongated (Day 17) embryos produced by somatic cell nucleus transfer and in vitro fertilization. Theriogenology. 2010 Jul 1; 74(1):45–59. https://doi.org/10.1016/j.theriogenology.2009.12.018 PMID: 20197198

57. Clemente M, Lopez-Vidriero I, O'Gaora P, Mehta JP, Forde N, Gutierrez-Adan A, et al. Transcriptome changes at the initiation of elongation in the bovine conceptus. Biology of reproduction. 2011; 85:285–295. https://doi.org/10.1095/biolreprod.111.091587 PMID: 21508349

58. Watson AJ, Barcroft LC. Regulation of blastocyst formation. Frontiers in bioscience: a journal and virtual library. 2001; 6: D708–D730. https://doi.org/10.2741/watson PMID: 11333210

59. Rossant J, Tam PP. Blastocyst lineage formation, early embryonic asymmetries and axis patterning in the mouse. Development. 2009: 136:701–713. https://doi.org/10.1242/dev.017178 PMID: 19201946

60. Maître JL. Mechanics of blastocyst morphogenesis. Biology of the cell. 2017; 109:323–338. https://doi.org/10.1111/boc.201700029 PMID: 28681376

61. Gutiérrez-Adán A, Rizos D, Fair T, Moreira PN, Pintado B, de la Fuente J, et al. Effect of speed of development on mRNA expression pattern in early bovine embryos cultured in vivo or in vitro. Mol Reprod Dev. 2004 Aug; 68(4):441–8. https://doi.org/10.1002/mrd.20113 PMID: 15236328.

62. Hoelker M, Schmoll F, Schneider H, Rings F, Gilles M, Tesfaye D, et al. Bovine blastocyst diameter as a morphological tool to predict embryo cell counts, embryo sex, hatching ability and developmental characteristics after transfer to recipients. Reprod Fertil Dev. 2006; 18(5):551–7. https://doi.org/10.1071/rd05149 PMID: 16836962

63. Lemmen JG, Agerholm I, Ziebe S. Kinetic markers of human embryo quality using time-lapse recordings of IVF/ICSI-fertilized oocytes. Reprod Biomed Online. 2008 Sep; 17(3):385–91. https://doi.org/10.1016/s1472-6483(10)60222-2 PMID: 18765009

64. Aguilar J, Motato Y, Escribá MJ, Ojeda M, Muñoz E, Meseguer M. The human first cell cycle: impact on implantation. Reproductive biomedicine online. 2014; 28: 475–484. https://doi.org/10.1016/j.rbmo.2013.11.014 PMID: 24581982

65. Muthukumar K, Kamath M S, Mangalaraj AM, Aleyamma T, Chandy A, George K. Comparison of clinical outcomes following vitrified warmed day 5/6 blastocyst transfers using solid surface methodology with fresh blastocyst transfers. Journal of human reproductive sciences. 2013; 6:59–64. https://doi.org/10.4103/0974-1208.112384 PMID: 23869154

66. Motato Y, de los Santos MJ, Escriba MJ, Ruiz BA, Remohi J, Meseguer M. Morphokinetic analysis and embryonic prediction for blastocyst formation through an integrated time-lapse system. Fertility and Sterility. 2016; 105:376–384.e9. https://doi.org/10.1016/j.fertnstert.2015.11.001 PMID: 26598211

67. Swain JE. Could time-lapse embryo imaging reduce the need for biopsy and PGS? Journal of Assisted Reproduction & Genetics. 2013; 1081–1090. https://doi.org/10.1007/s10815-013-0048-4 PMID: 23842747

68. Milewski R, Kuć P, Kuczyńska A, Stankiewicz B, Łukaszuk K, Kuczyński W. A predictive model for blastocyst formation based on morphokinetic parameters in time-lapse monitoring of embryo development. Journal of Assisted Reproduction and Genetics. 2015; 32:571–579. https://doi.org/10.1007/s10815-015-0440-3-015-0440-369 PMID: 25690157

69. Milewski R, Kuczyńska A, Stankiewicz B, Kuczyński W. How much information about embryo implantation potential is included in morphokinetic data? A prediction model based on artificial neural networks and principal component analysis. Advances in Medical Sciences. 2017; 62:202–206. https://doi.org/10.1016/j.advms.2017.02.001 PMID: 28384614

70. Seli E, Bruce C, Botros L, Henson M, Roos P, Judge K, et al. Receiver operating characteristic (ROC) analysis of day 5 morphology grading and metabolomic Viability Score on predicting implantation

outcome. Journal of assisted reproduction and genetics. 2011; 28:137–144. https://doi.org/10.1007/s10815-010-9501-9 PMID: 21063765

71. Holm P, Shukri NN, Vajta G, Booth P, Bendixen C, Callesen H. Developmental kinetics of the first cell cycles of bovine in vitro produced embryos in relation to their in vitro viability and sex. Theriogenology. 1998; 50:1285–1299. https://doi.org/10.1016/s0093-691x(98)00227-1 PMID: 10734442

72. Lequarre AS, Marchandise J, Moreau B, Massip A, Donnay I. Cell cycle duration at the time of maternal zygotic transition for in vitro produced bovine embryos: effect of oxygen tension and transcription inhibition. Biol Reprod. 2003 Nov; 69(5):1707–13. https://doi.org/10.1095/biolreprod.103.017178 PMID: 12890737

73. Kaith S, Saini M, Raja AK, Sahare AA, Jyotsana B, Madheshiya P, et al. Early cleavage of handmade cloned buffalo (Bubalus bubalis) embryos is an indicator of their developmental competence and quality. Reprod Domest Anim. 2015 Apr; 50(2):214–220. https://doi.org/10.1111/rda.12472 PMID: 25604613

74. Alpha Scientists in Reproductive Medicine and ESHRE Special Interest Group of Embryology. The Istanbul consensus workshop on embryo assessment: proceedings of an expert meeting. Human reproduction. 2011; 26:1270–1283. https://doi.org/10.1093/humrep/der037