Research article

Non-invasive analysis of bovine embryo metabolites during *in vitro* embryo culture using nuclear magnetic resonance

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Abstract: The ability to identify embryos that have the highest developmental potential from a cohort would significantly increase the chances of achieving pregnancy. Metabolic analysis is a well-established analytical approach in biological systems. Starting from this idea, we chose to use high-resolution nuclear magnetic resonance (^{1}H-NMR) spectroscopy. The aim of this study was to determine if it is possible to select viable embryos after 48 h of culture using metabolic activity as the parameter. We evaluated embryo metabolism after the first 48 h of culture and compared the activity of cleaved embryos that became blastocysts to cleaved embryos that did not develop to blastocysts, and *in vitro* fertilized (IVF) blastocysts and parthenogenetic-activated (PA) blastocysts. Our results show that citrate, pyruvate, myo-inositol and lysine have great impact on predicting embryo development. When we compared IVF and PA blastocysts, we found that acetate and phenylalanine concentrations are excellent parameters for evaluating blastocyst quality. Combining all these results, we were able to create a formula that predicts zygote development after 2 days of culture. In conclusion, we found that it is possible predict the future development of *in vitro* produced bovine embryos after only 2 days of culture using ^{1}H-NMR.

Keywords: embryo; NMR; metabolism; cattle; early development

1. Introduction

Human *in vitro* fertilization (IVF) was first introduced in 1978 to treat some forms of infertility. Presently, in Western societies up to 4% of all children born are conceived using assisted reproduction technologies (ARTs) [1]. Embryo implantation remains one of the rate-limiting factors in IVF. The ability to identify those embryos that have the highest developmental potential from a given cohort would increase the chances of achieving pregnancy while minimizing the number of embryos transferred in each cycle. Morphological assessment is currently the main method used to determine embryo viability during IVF cycles. It includes observation of the developmental pattern of embryos during culture, including the timing and rate of cleavage, cell number and fragmentation. It has the advantage of being a quick, convenient and inexpensive way of assessment, but it has a modest predictive value, and studies have shown that the predictive power of the usual day 2 and 3 morphological parameters has remained low [2,3,4]. This limited predictive value of early embryo morphology has led many researchers to look for additional techniques to assess the reproductive potential of a given embryo. Various non-invasive analytical tools have recently been used for prediction of embryonic potential [5–10]. One such tool has been the development of quantitative techniques for the non-invasive assessment of embryo metabolism, and its value as predictors of embryo viability is the subject of ongoing investigations [11]. Metabolic analysis is a well-established approach in biological systems, defined as a non-targeted quantitative analysis of tissue and biofluids for endogenous metabolites with a low molecular organic mass. Metabolic profiles and their changes over time due to physiological and/or pathophysiological stimuli such as disease, toxicity, nutrition and other effects provide important information to assist understanding of biological regulation and pathophysiological mechanisms [12]. The field is presently moving away from metabolic target analysis (analysis restricted to single or selectively defined metabolites) for diagnostic purposes towards metabolic profiling (analysis looking for selected groups of target compounds and their metabolite intermediates using a single analytical technique) and a more comprehensive analysis of the metabolome [8]. Starting with this idea, we chose to use high-resolution nuclear magnetic resonance (^{1}H-NMR) spectroscopy to analyze the metabolic profile of bovine embryo culture medium. ^{1}HNMR spectroscopy has proven to be one of the most powerful technologies for biological fluid metabolomic analysis. It is capable of analyzing intact tissues and fluids while producing a comprehensive profile of metabolites. An important aspect of NMR spectroscopy is that the fundamental physicochemical mechanism is completely different from other common analytical techniques and provides an additional scientific perspective [13]. NMR spectroscopy is distinctly different from chromatographic procedures (such as HPLC), which in combination with mass spectrometry are currently the most commonly used methods for identification and quantification of molecular species in a sample. Because chromatography requires thorough knowledge of the sample's chemical properties, it is limited to the chemical dynamic range and separation methods [13], whereas NMR is limited only by magnetic field strength and does not require sample separation [8]. Therefore, the aim of this study was to assess if was possible select viable embryos after 48 hours of culture using the metabolic pattern as the parameter. In the first experiment, we evaluated the embryo metabolism from the first 2 days of culture and compared the metabolism of cleaved oocytes that became blastocysts to that of cleaved oocytes that did not develop to that stage. The second experimental goal was to find a method to discriminate the high potential embryos from the low potential embryos. We selected *in vitro* fertilized (IVF) blastocysts

and parthenogenetic-activated (PA) blastocysts as possible high and low developmental potential embryos, respectively. PA embryos were used for low developmental potential embryos because it is known that they will not continue to develop past the blastocyst stage. By combining the results from these experiments, we were able to extrapolate some parameters that can give objective information on the development of the blastocyst and its quality. We evaluated 5 sugars (pyruvate, citrate, formate, lactate, myo-inositol, acetate) and 10 amino acids (alanine, leucine, isoleucine, valine, histidine, tyrosine, lysine, methionine and phenylalanine). These parameters gave us a relatively complete perspective of all the metabolic activity. Cattle were used as an animal model for this study because of their similarities to human reproductive processes, including similar fetal developmental time frame and gestation length. In addition, both species are monotocous (single offspring bearing species)[14,15].

2. Material and Methods

2.1. Reagents and media

Unless otherwise stated, all reagents were purchased from Sigma-Aldrich (*USA*).

The IVF medium was Tyrode's modified medium [16] without glucose and bovine serum albumin (BSA), supplemented with 95.6 USP/ml heparin, 30 µM penicillamine, 15 µM hypotaurine, 1 µM epinephrine, and 1% bovine serum (BS). The IVC medium consisted of Synthetic Oviduct Fluid (SOF) medium [17], with 30 µL/mL essential amino acids, 10 µL/mL non-essential amino acids, and 5% BS.

2.2. In vitro embryo production

Matured oocytes were purchased from DeSoto Biosciences (Seymour*, TN, USA*). *In vitro* matured cumulus-oocyte complexes (COCs) were washed in IVF medium and transferred, 20–30 per well, into 300 µL of IVF medium covered with mineral oil. For each replicate, two straws of frozen semen (from a bull previously tested for IVF) were thawed at 37 °C for 40 sec. The sample was processed via a Percoll discontinuous gradient (45–80%) [18]. After processing, pellets were diluted with IVF medium and added to the fertilization wells at the concentration of 1×10^6 sperm/mL. Gametes were co-incubated for 18–20 h at 39 °C, in 5% CO_2 in air, after which presumptive zygotes were vortexed for 2 min to remove cumulus cells in HEPES-TCM (H199)(Lonza 12–117F) with 5% BS, and washed twice in the same medium. Presumptive zygotes were placed in mSOF with 5% BS, where they were incubated in a humidified mixture of 5% CO_2, 6% O_2, and 89% N_2 at 39 °C.

2.3. Chemical activation

Prior to activation, *in vitro* matured COCs were denuded by gentle pipetting in H199 medium. Oocytes were activated by 5 µM ionomycin in H199 for 5 min. Oocytes were washed thoroughly in hSOF, transferred to 20 µl drops (10 oocytes/drop) of SOF medium in the presence of 6-DMAP in order to suppress maturation promoting factor (MPF) kinase activity and were placed in mSOF with 5% BS, where they were incubated in a humidified mixture of 5% CO_2, 6% O_2, and 89% N_2 at 39 °C.

2.4. ¹H-NMR spectroscopy

Samples of media (40 µL) were thawed and added to 660 µL of a stock solution prepared by dissolving 5.0 mg of sodium 3-(trimethylsilyl)-2,2',3,3'-tetradeuteropropionate (TSP) in 50 mL of deuterium oxide. The TSP acted both as a chemical shift reference and as an internal standard for the purposes of quantitation. The resulting diluted samples were transferred to a 5-mm NMR tube. Samples were analyzed on a 750-MHz Agilent VNS750NB spectrometer (Agilent Technologies, Santa Clara, CA) equipped with a 5 mm Varian HCN PFG X,Y Z-probe at 23 °C. Shimming of the sample was performed manually on the residual water signal. ¹H-NMR spectra were recorded with a 90° radio frequency pulse (pulse width = 6.3 µs, transmitter power = 63 dB). Sixty-four scans were acquired in 16 K data points with a spectral width of 7,100 Hz and a relaxation delay of 10 s. The residual water signal at 4.8 ppm was suppressed using the "presat" pulse sequence (saturation power = 9 dB, presaturation delay = 2 s). Spectra were processed with MestReNova© software, version 6.0 (Mestrelab Research, Santiago de Compostela, Spain). Prior to Fourier transformation, the FIDs (Free Induction Decay) were zero-filled to 32 K. All spectra were manually phased and corrected for baseline distortion. Peak integrals were obtained using the MestReNova software integral function and normalized with respect to the number of protons comprising the signal. The concentration of the analytes was calculated by determining the ratio of the normalized integrals of the corresponding ¹H-NMR signals to the TSP signal integral (0 ppm, singlet, Si $(CH_3)_3$). Signal assignments were made on the basis of previously published data [19,20,21].

2.5. Statistical analysis

The embryo qualities were predicted using a logistic model adjusting for 15 potential predictors: formate, citrate, pyruvate, lactate, myo-inositol, acetate, lysine, methionine, leucine, alanine, valine, isoleucine, phenylalanine, histidine, and tyrosine. The outcomes were defined as: cleaved embryos and blastocysts; IVF blastocysts and PA blastocysts. To characterize the variation of the media content with a few variables, reduce the redundancy among variables, and detect linear relationship, a principal component analysis (PCA) was implemented using SAS (SAS Institute, Cary, NC). The first two principal components were plotted. Stepwise selection was performed to create the multivariable logistic regression model of analysis. Only variables with a P-value < 0.05 were retained in the final model and considered significant. The results were presented as estimated effect, standard error of the estimated effect, P-value, and marginal means associated with the effect. Determination of the degree by which variables measured during early cleavage could be predictive of blastocyst development was analyzed by logistic regression

To eliminate the medium effect and reduce the variation in a data set we chose to analyze the metabolites as fraction of the medium before the zygote over the medium after the zygote. Therefore all results below are reported as fractions.

3. Experimental Design

After gamete co-incubation (day 1) (Figure 1), the presumptive zygotes were placed in individual drops of 50 µL of SOF, where they were incubated in a humidified mixture of 5% CO_2, 6% O_2, and 89% N_2 at 39 °C. After 48 h of culture (day 3) (Figure 2), the zygotes (from 2 to 8

blastomeres) were placed into WOW [Well of Well] [22] culture, and the media drops were collected. The percentages of cleaved embryos and embryos reaching the blastocyst stage were determined at day 7 of the culture (day 0 = IVF day). The embryos were scored for quality on the basis of morphological criteria, and only Grade 1 and 2 blastocysts (Bl) were considered in the evaluation of the final embryo rate. We divided the media drops into two groups: 1) drops where the cleaved embryos grew and 2) drops where blastocysts grew. For each group, 20 drops were analyzed for a total of 40 samples. A total of 400 oocytes were fertilized in four replicates.
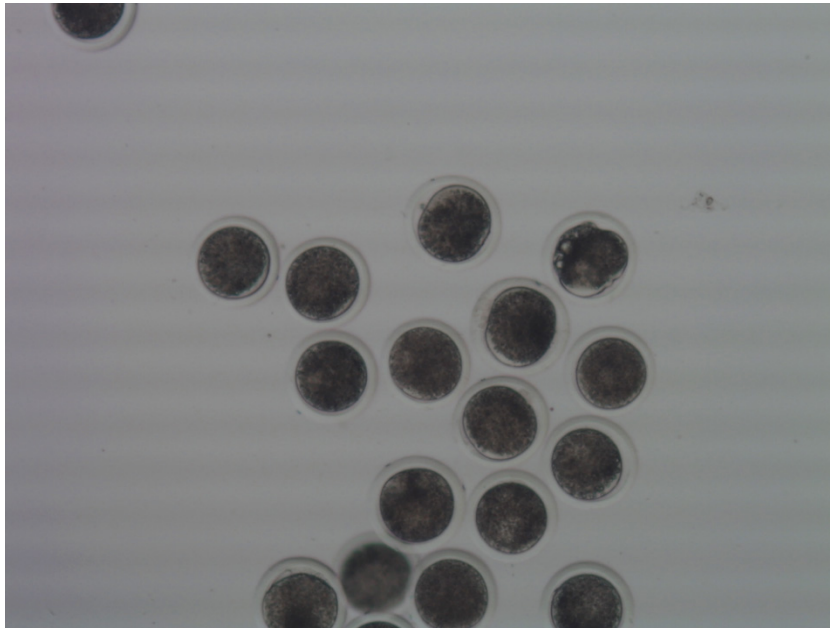
**Figure 1.** Example of presumptive zygotes after co-incubation.
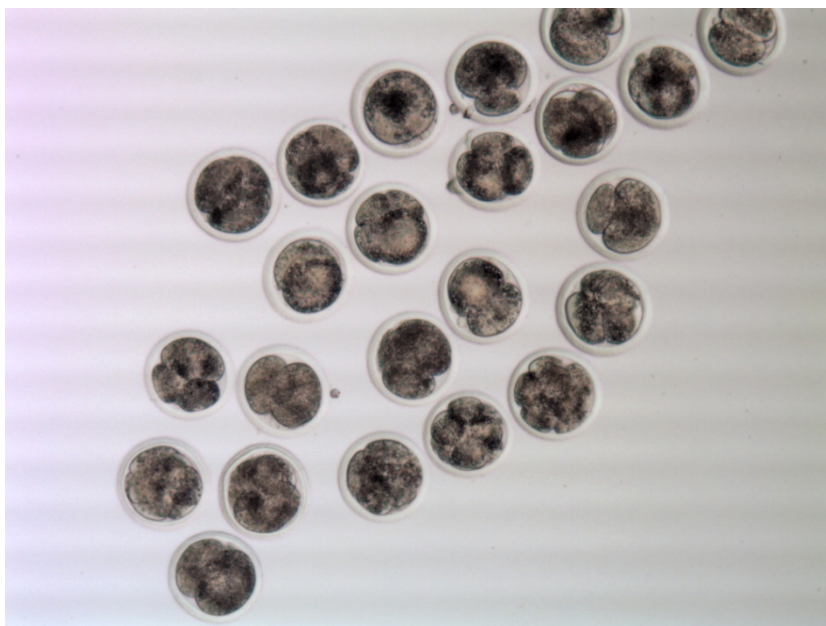
Figure 2. Example of zygotes after 48 hours of culture co-incubation.

In the second phase of the experiment, we compared 40 spent media drops from blastocysts: 20 from IVF and 20 from PA. The IVF embryo production followed the procedure described above while the PA embryos follow the chemical activation protocol. A total of 600 oocytes were fertilized and activated. The experiment was replicated four times.

4. Results

4.1. Experiment 1

A representative ¹H NMR spectrum of the medium profile after the embryo development is shown in Figure 3.
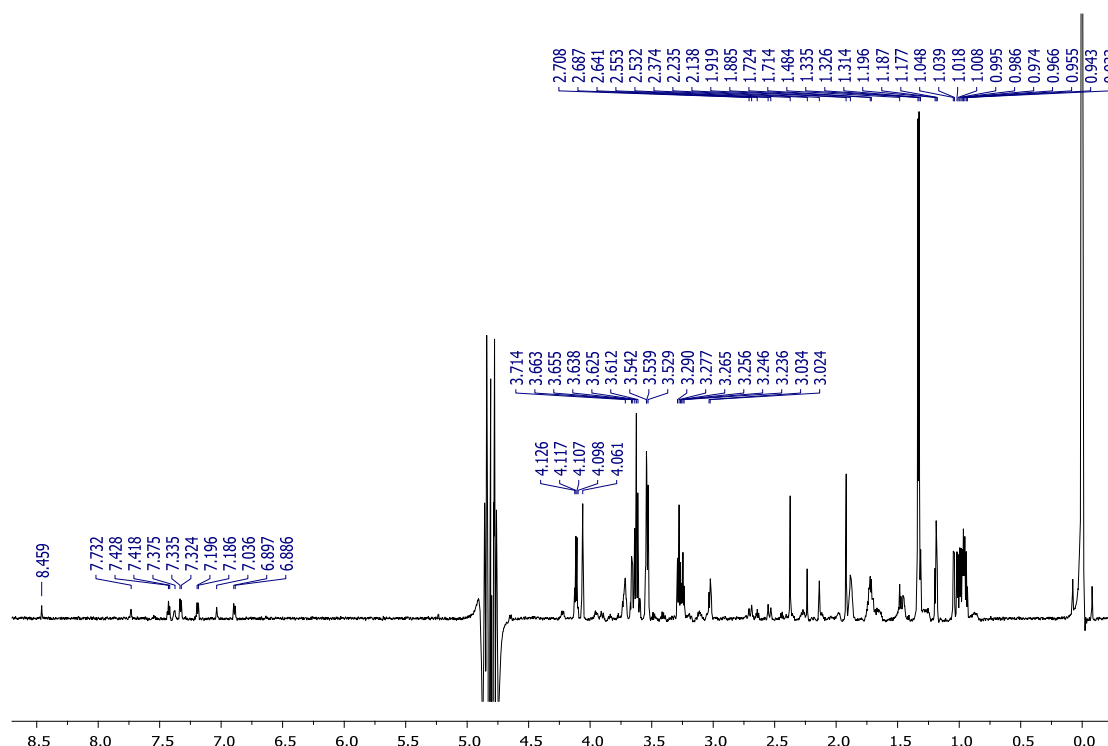
Figure 3. Example of ¹H NMR spectrum of embryo metabolites.

As we described above, the results are the ratio of the value obtained after the development of the embryos in the drops over the values of the medium before the introduction of the zygotes. This choice was made to minimize the protocol effect. A summary of the means and standard deviation of all variables is shown in Table 1.

To reduce the number of variables and see their interactions with each other, we computed the Pearson correlation, (Tables S1a and b). These results showed a direct correlation between citrate and six out of nine amino acids (lysine, leucine alanine, phenylalanine, histidine and tyrosine) but it is not correlated with any of the others sugars. Conversely, formate and acetate are not correlated with the other metabolites, but the myo-inositol behavior is directly correlated to pyruvate and lactate with no relationship to the amino acids. In the same way, pyruvate and lactate are correlated to myo-inositol.

Table 1. Summary of the means and standard deviation of variables of Experiment 1.

Variable	Mean	Std Dev	N	Range
formate	0.9476250	0.2015002	40	0.6700000
citrate	1.0088000	0.2085329	40	0.7990000
pyruvate	0.9908750	0.0450272	40	0.1640000
lactate	0.9839750	0.0395127	40	0.1500000
myo-inositol	0.9746000	0.0356880	40	0.1820000
acetate	1.0767500	0.0831162	40	0.3220000
lysine	1.0254000	0.1027803	40	0.4770000
methionine	1.0367750	0.0854432	40	0.3770000
leucine	1.0767250	0.2990775	40	1.1450000
alanine	0.9969000	0.1444865	40	0.6210000
valine	1.0606250	0.1205129	40	0.6060000
isoleucine	1.0581500	0.1645604	40	0.8130000
phenylalanine	1.1102500	0.1980694	40	0.7620000
histidine	1.0089000	0.2158400	40	0.7420000
tyrosine	1.0259500	0.1337502	40	0.5580000

When we compared the relationships between the amino acids, we found that behavior of lysine is correlated with leucine, histidine and tyrosine, but methionine is only linked with the alanine. Furthermore, leucine is correlated with alanine, phenylalanine, histidine and tyrosine. Alanine is linked with isoleucine, phenylalanine, histidine and tyrosine. Valine is only correlated with isoleucine, just as the histidine is linked with phenylalanine and the tyrosine with histidine. The PCA score plot shows that the cleaved group is spread almost all only in the first quarter (X = −4:0; Y = 0:+4) while the blastocyst group is uniformly distributed in the upper section of the entire chart (Figure 4).
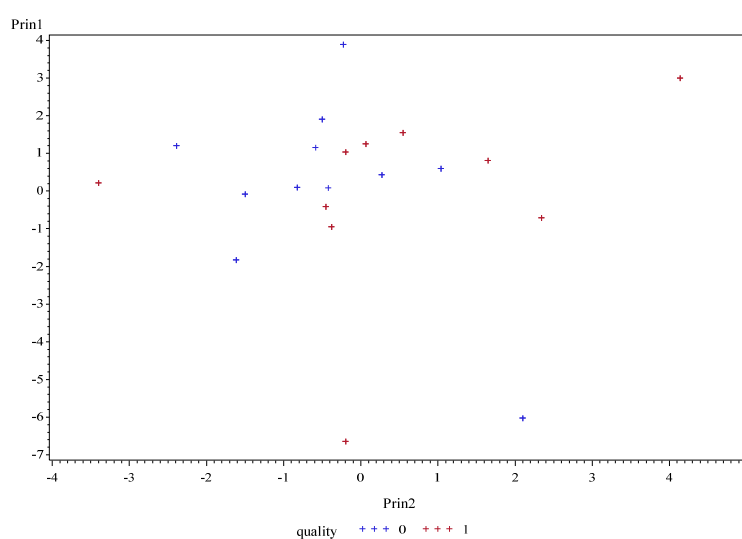
Figure 4. Plot of the first two principal components of experiment 1:0 or + = arrested embryos; 1 or + = blastocysts.

The logistic analysis results, shown in Table 2, indicates that of all 15 metabolites that we evaluated, only four are different between groups. These are citrate, pyruvate, myo-inositol and lysine. The model created from this analysis correctly predicted the embryo quality 90% (90% of sensitivity and specificity) of the time.

Table 2. Model coefficients of Experiment 1.

| Parameter	DF	Estimate	Standard Error	Pr > ChiSq
Intercept	1	−0.104	0.455	0.8192
citrate	1	−3.2075	1.4295	0.0248
pyruvate	1	1.8945	0.8245	0.0216
myo-inositol	1	−3.5349	1.5174	0.0198
lysine	1	4.0708	1.3815	0.0032

4.2. Experiment 2

Table 3 presents the summary of the means and standard deviation from all of the variables. In this experiment we also evaluated the possibility correlations between the amino acids and sugars (Tables S2a and b). These results reveal that lactate is not correlated to any other metabolite. In contrast, the results show that formate is directly correlated with all amino acids and citrate. When we evaluate pyruvate it shows the same relationship with methionine, valine and isoleucine also have the same trend; they are strongly correlated. Moreover, citrate is correlated to myo-inositol and valine. The acetate fluctuations are correlated to lysine, alanine, histidine and phenylalanine.

Table 3. Summary of the means and standard deviation of the variables for Experiment 2.

Variable	Mean	Std Dev	N	Range
formate	1.0144359	0.3079948	40	1.4880000
citrate	1.1100256	0.3022427	40	0.9740000
pyruvate	1.1171026	0.4385306	40	1.4330000
lactate	0.9695385	0.0558621	40	0.3030000
myo-inositol	0.9855897	0.0464354	40	0.2250000
acetate	1.0353333	0.1995340	40	0.6750000
lysine	1.0580256	0.1529354	40	0.7010000
methionine	1.0185385	0.0888976	40	0.4200000
leucine	1.1896667	0.5729616	40	1.5210000
alanine	1.0520769	0.2775761	40	1.4710000
valine	0.9938205	0.2151197	40	1.3070000
isoleucine	1.0131282	0.2249035	40	1.4180000
phenylalanine	1.0461026	0.3218073	40	0.9870000
histidine	1.0325385	0.2910296	40	0.9550000
tyrosine	0.9976410	0.2562712	40	0.9180000

In the Figure 5, is possible to see that the IVF embryo plots are almost all in the upper half of the graph (X < 1; Y > 0); only 3 spots of 20 are out of that area. On the other hand, the PA spots are spread throughout the entire graph (the area over X > −3).
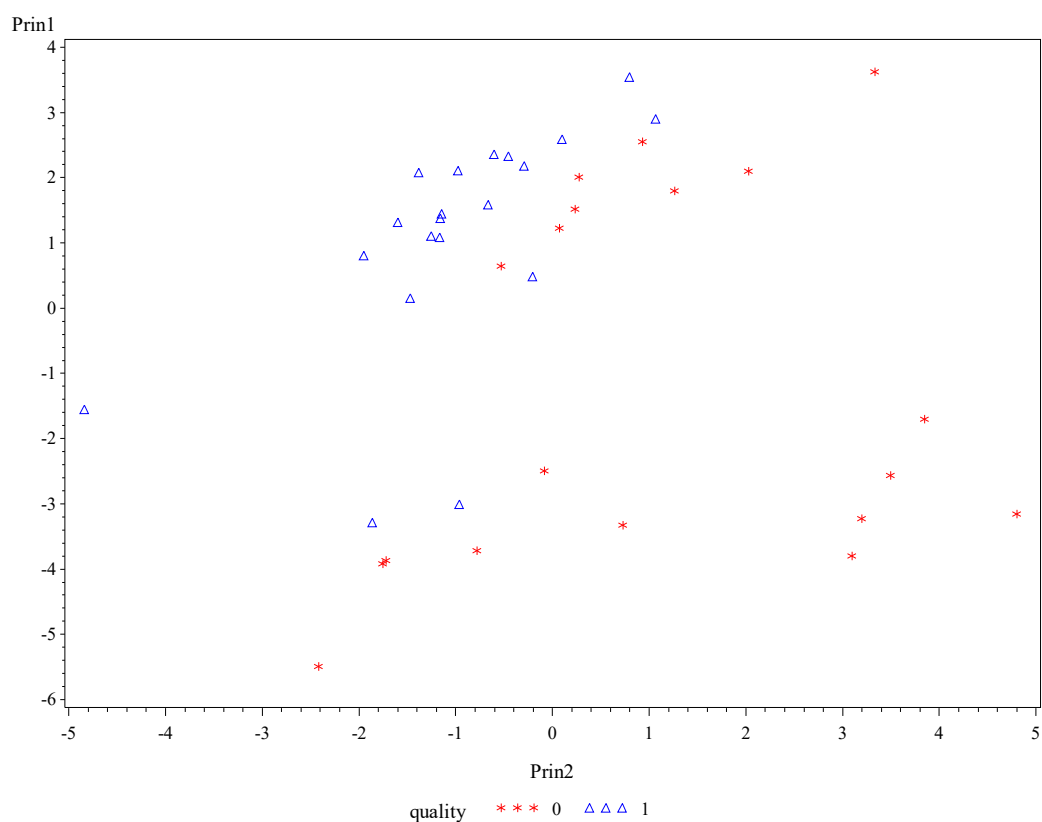
Figure 5. Plot of the first two principal components of the experiment 2:0 or * = PA blastocysts; 1 or △= IVF blastocysts.

The logistic analysis showed that of all components, only two are significant (Table 4): acetate and phenylalanine.

Table 4. Model coefficients of experiment 2.

Analysis of Maximum Likelihood Estimates

Parameter	DF	Estimate	Standard Error	Wald Chi-Square	Pr > Chi-Sq.
Intercept	1	−0.9158	0.6780	1.8244	0.1768
acetate	1	−4.5972	1.6942	7.3630	0.0067
phenylalanine	1	2.8137	0.9941	8.0104	0.0047

These two metabolites can help us to discern which embryos were made by PA versus those obtained by normal fertilization. The model set with these variables is trusted at 84.6%, which means that almost 9 times on 10, this model can predict the quality of the blastocyst.

5. Discussion

Morphological assessment is currently the primary technique for selection of viable embryos for uterine transfer during assisted reproductive techniques. However, this method has limited predictive power. At present, developmental rates and morphological characteristics such as percentage fragmentation, blastomere symmetry, and multi-nucleation are currently the main criteria used for the selection of embryos viability [21,23]. However, this approach is subjective and a relatively poor predictor of implantation.

A reliable, non-invasive embryo scoring system undertaken during early cleavage is needed to accurately predict, which embryos are either capable of reaching the blastocyst stage or possess full developmental potential. Such a system would be of considerable benefit for the advancement of embryo-based biotechnologies in domestic animals and assisted conception in humans. This is particularly the case for the embryos of domestic animals, which present special challenges for non-invasive morphologic investigations owing to the large amount of cytoplasmic lipid [24] .

Over recent years, there has been much interest in the use of metabolic parameters as markers of embryo viability, stemming from the fact that metabolism is intrinsic to early embryo health and is immediately disrupted when embryos are stressed [25]. Pyruvate and glucose uptake have been investigated in numerous studies as a marker of embryo viability [26,27]. Amino acid metabolism has also been investigated, and a decrease in amino acid turnover has been correlated with blastocyst formation and clinical pregnancy [28,29]. These studies led to the proposal that up-regulation of embryo metabolism is associated with a reduction in viability and they formed the basis of the quiet embryo hypothesis [30]. Studies to date exploring the link between the metabolite profile of spent media and embryo viability have primarily focused on Raman near-infrared (NIR) [31], and nuclear magnetic resonance (NMR) based metabolomic analysis. Raman spectroscopy [7] and NIR spectroscopy [32,33] were used to develop a diagnostic test for predicting delivery or failed implantation with initially promising results. Studies employing ^{1}H NMR based analysis of spent embryo culture media [20] have identified glutamate [8] and the pyruvate/alanine ratio as potential markers of embryo viability [34]. Other research groups did not find any improvement by adding the metabolism data to the morphological criteria, for example Hardarson et al. showed that using the NIR data did not provide additional, useful information to help select the best quality embryos [35]. These results and those of previous studies demonstrate that spent embryo culture media from viable and non-viable embryos differ in their metabolite composition thus making its assessment a potential non-invasive method for the selection of embryos.

In this study, we found that the behavior of citrate, myo-inositol, pyruvate and lysine have a great impact on the completion of zygote development. It is known that the speed of development is a useful parameter to evaluate the viable embryos [36,37]. The faster developing embryos begin aerobic metabolism first, which could explain why the values of pyruvate and citrate are lower in the blastocyst than in the cleaved embryos. It is also known that myo-inositol has a positive effect on blastocyst development [38,39]. These sugars (pyruvate, citrate and myo-inositol) have a great impact on the blastocyst development. Our present results show that for each additional unit of citrate found in the medium there is a 9.6% increased possibility that the embryo will become a blastocyst. In the same way, for each additional unit of pyruvate in the medium there is a 12% increased likelihood to produce a blastocyst. In contrast, for each additional unit of myo-inositol in the medium there is a 7.2% lower probability of having a blastocyst produced. The only amino acid

that was shown to have an important role in the identification of the future blastocyst is lysine. Our results show that each additional unit of lysine in the medium increases the chances of producing a blastocyst by 6.9%. Our finding on the importance of lysine on embryo development is in agreement with Houghton et al. and Lydie Nadal-Desbarats et al. [20,29]. Those studies showed that in the first days of development, the future blastocysts produce higher level of lysine. However, the lysine effect shown in our experiments is in contrast with the results of Brison et al.[28], but in that case they used a porcine model.

In the second phase of our study we evaluated the discriminating parameters for the quality of the embryos. Our results showed that in the PA blastocysts, the levels of acetate are higher than in the IVF blastocysts, and otherwise these showed a positive trend for the citrate. These two parameters suggest that in the IVF embryos, glycolysis starts before it does in the PA embryos. In the present study, the only amino acid indicative of embryo quality is phenylalanine. This observation is in accordance with that of Zhao et al. [40], who showed that high level of phenylalanine is correlated with a high reproductive potential. In our study, the IVF blastocysts also have higher levels of this amino acid than PA embryos. When we estimated the magnitude of these effects, we found that for each additional unit of acetate in the medium, there is a 36.2% lower probability of finding an IVF blastocyst, on the contrary for each additional unit of phenylalanine in the medium, there is a 22.1% increased likelihood of having a viable blastocyst.

In the last portion of the present study, we combined the results of these two experiments and reformulated our medium for embryo culture. Our idea was to extrapolate the optimal parameters to evaluate, after 48 hours of culture, whether the zygote will become an embryo and what will be the quality of the embryo produced. The results of this calculation showed how acetate and phenylalanine are important parameters to evaluate the quality embryo: for each unit more of acetate there is minus 38.6% chance that this will be a good blastocyst and at the same time for each unit in more phenylalanine there is 26% that it will be a blastocyst of good quality. Compared with the other results, this logistic analysis has less sensitivity and specificity (Table 5) however, these parameters are able to distinguish on day 3 eight blastocysts of ten, defining also the quality of such embryos: either PA or IVF.

Table 5. Classification table of the prediction of embryo quality.

Classification Table

Prob Level	Correct		Incorrect		Percentages				
	Event	Non-Event	Event	Non-Event	Correct	Sensitivity	Specificity	False POS	False NEG
0.500	16	15	4	4	79.5	80.0	78.9	20.0	21.1

In summary if we evaluate acetate and phenylalanine we are able to predict the embryo quality, using this formula:

$$x = \frac{1}{1 + e^{1.39 - 5.06*ACETATE + 3.49*PHENYLALANINE + 0.95*BADANDGOOD}}$$

It is possible to forecast, on day 3 if a zygote will become a blastocyst and the quality of that blastocyst. All zygotes that gave a value over 0.5 have a greater possibility of being good quality blastocysts, alternatively, for values under 0.5, if there is a blastocyst produced, there is a higher chance that it will be a poor-quality blastocyst.

Future experiments will be required to evaluate the relationship of these results with pregnancy rates. However, the present results show that we can determine on day 3 whether an embryo will become a blastocyst and ascertain the blastocyst quality. This is a very powerful tool to objectively determine embryo quality. These results should have significant impact on the production of high quality bovine embryos from IVF.

Acknowledgments

Conflict of Interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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