Raman-based noninvasive metabolic profile evaluation of *in vitro* bovine embryos

Érika Cristina dos Santos
Herculano Martinho
Kelly Annes
Thais da Silva
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Marcella Pecora Milazzotto
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Érika Cristina dos Santos, Herculano Martinho, Kelly Annes, Thais da Silva, Carlos Alexandre Soares, Roberta Ferreira Leite, and Marcella Pecora Milazzotto*

Universidade Federal do ABC, Centro de Ciências Naturais e Humanas, Avenue dos Estados, 5001, 09210-580, Santo André, São Paulo, Brazil

**Abstract.** The timing of the first embryonic cell divisions may predict the ability of an embryo to establish pregnancy. Similarly, metabolic profiles may be markers of embryonic viability. However, in bovine, data about the metabolomics profile of these embryos are still not available. In the present work, we describe Raman-based metabolomic profiles of culture media of bovine embryos with different developmental kinetics (fast x slow) throughout the in vitro culture. The principal component analysis enabled us to classify embryos with different developmental kinetics since they presented specific spectroscopic profiles for each evaluated time point. We noticed that bands at 1076 cm$^{-1}$ (lipids), 1300 cm$^{-1}$ (Amide III), and 2719 cm$^{-1}$ (DNA nitrogen bases) gave the most relevant spectral features, enabling the separation between fast and slow groups. Bands at 1001 cm$^{-1}$ (phenylalanine) and 2892 cm$^{-1}$ (methylene group of the polymethylene chain) presented specific patterns related to embryonic stage and can be considered as biomarkers of embryonic development by Raman spectroscopy. The culture media analysis by Raman spectroscopy proved to be a simple and sensitive technique that can be applied with high efficiency to characterize the profiles of in vitro produced bovine embryos with different development kinetics and different stages of development.© 2016 Society of Photo-Optical Instrumentation Engineers (SPIE)

Keywords: Raman spectroscopy; metabolomics; kinetics; embryo; bovine.

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1 Introduction

In recent years, in vitro production (IVP) became the technique of choice for bovine embryos production leading to new possibilities in the bovine genetics market. However, despite the improvement of IVP process, the quality of the embryo and its ability to generate pregnancy are still lower than their in vivo counterpart.

Methods to identify the better embryos according to their development potential and viability to establish pregnancy, mainly based on noninvasive techniques, are being developed as a strategy to increase the IVP efficiency. The assessment of embryonic kinetic patterns, the levels of glucose, lactate, pyruvate, and amino acids in the culture media of IVF embryos, the oxygen consumption by the embryo, and secretome and metabolome patterns are some examples.

The metabolome is the systematic analysis of metabolites representing the functional phenotype of a cell and offers a unique opportunity to investigate the relationship between genotype and the resulting phenotype. The metabolites generated by the cell from the use of energetic precursor and compounds present in the medium are then secreted into the extracellular space, thereby altering the cellular microenvironment. Thus, the analysis of the culture medium can, directly or indirectly, indicate events that are occurring in the cell, which can be correlated with the embryo viability and development potential.

Similarly, embryonic viability can be related with development kinetics, although the results, so far, are still controversial. Mice embryos and bovine embryos fast with development seem to be more viable. However, studies show that the methylation state, the expression of imprinted genes and genes associated with stress, as well as the metabolic profile of the in vitro mice embryos with slow to moderate development rates are more similar to in vivo embryos.

Raman spectroscopy technique is a potential technique to determine the metabolic profile of IVP embryos. It is a noninvasive and nondestructive method, requiring a minimal previous preparation and small sample volume (few microliters), and it is also relatively cheaper when compared to other techniques. In addition, it is a high-resolution method to detect molecular vibrations, which allow the identification of the molecular structure and its components conformation, providing characteristic spectral profiles from the inherent biochemical composition of the analyzed sample.

Considering the culture media analysis, Raman spectroscopy has been successfully described as a tool for the identification of the fertile potential of IVP human embryo. The evaluation of the metabolic profile of human embryos culture media by Raman spectroscopy, combined with the application of bioinformatics tools, is a fast and noninvasive way of assessing the reproductive potential of embryos and pregnancy rates. Also, different spectral profiles were obtained from embryos with good reproductive potential compared to embryos, which resulted in implantation failure. Furthermore, Zhao et al. reported a relation between morphology and the relative concentrations of sodium pyruvate and phenylalanine in culture media by Raman spectroscopy, which can be used to predict the embryo reproductive potential with an accuracy of 85.7% for...
clinical pregnancy. However, it is important to notice that the evaluation of embryos culture media by Raman spectroscopy had not been reported to other species than human. Although human and bovine embryos present several aspects of the metabolism in common, bovine embryos present differences regarding kinetics of development, lipid content, among others, which hinder the use of Raman results from human embryos for this species. Besides that, there is still a lack of noninvasive methods to evaluate the viability of bovine blastocysts prior to embryo transfer.

Therefore, the present study aimed to identify the metabolic profile of bovine embryos with different developmental kinetics throughout the in vitro culture (IVC) by the analysis of Raman spectroscopy from their culture media.

2 Materials and Methods

All procedures and protocols were performed in accordance with the Ethical Principles in Animal Research set forth by the Brazilian College of Animal Experimentation, with approval from “Ethic Committee in the Use of Animal” of Universidade Federal do ABC (protocol No. 008/2014).

2.1 Experimental Design

Bovine oocytes were in vitro-matured (IVM) and in vitro-fertilized (IVF). Presumptive zygotes were cultured individually (IVC) in an adapted well of the well system and classified according to their number of cells at 22 h after the onset of IVC in fast (four cells) or slow (two cells) embryos, generating two groups that were evaluated at the following time points: 22 h past the beginning of culture (hpc)—cleavage stage, 96 hpc—period next to embryonic genome activation, and 168 hpc—blastocyst stage. Groups were named as fast (FCL) and slow (SCL) cleaved embryos at 22 hpc, fast (FMO) and slow (SMO) embryos with >16 cells at 96 hpc, and fast (FBL) and slow (SBL) blastocysts at 168 hpc. The culture media from each embryo were collected and kept under −80°C for posterior analysis by Raman spectroscopy. A total of 150 embryos (25 embryos per group) were produced in vitro and Raman analysis was performed in pools of culture media from five embryos (five pools containing five embryos in each group). Raman spectra were collected per droplet of culture media from embryos in three different stages of development. Each droplet (10 μL) was placed in a petri dish and covered with mineral oil (3.5 mL). The spectra were performed by using a plan achromatic 50× objective glass (0.20 mm/NA = 0.50), the acquisition number × time of exposure was 3 × 20 s, and confocal aperture in 6.5 μm.

2.2 In Vitro Production

Embryos were produced following standard protocols for IVM and IVF. During the IVC, the zygotes were transferred individually into microdroplets of 20 μl of a culture medium of potassium simplex optimization medium (KSOM) (MR-106-D Millipore®) supplemented with 10% fetal calf serum (FCS), gentamicin, and nonessential amino acids. At 22 hpc, the KSOM medium was replaced by synthetic oviductal fluid (SOFaa) (supplemented with 5% FCS, essential and nonessential amino acids). Culture followed for 7 days at 38.5°C in 5% CO2 and saturated humidity.

2.3 Raman Spectroscopy

The Raman spectroscopy setup details and analysis were described elsewhere. Briefly, the equipment used on this study was the Triple T64000 Raman Spectrometer (Horiba Jobin-Yvon S.A.S., France) with microanalysis option and CCD detector 1024 × 256—OPEN-3LD/DR with quantum response of ~40%. The excitation laser was 532 nm (Verdi G5, Coherent Inc., United States) focused on a spot with 5-mW power. Two Raman spectra were collected per droplet of culture media from embryos in three different stages of development. Each droplet (10 μL) was placed in a petri dish and covered with mineral oil (3.5 mL). The spectra were performed by using a plan achromatic 50× objective glass (0.20 mm/NA = 0.50), the acquisition number × time of exposure was 3 × 20 s, and confocal aperture in 6.5 μm.

2.4 Data Analysis

Experimental data were plotted using the Origin 8.0 software (OriginLab, Northampton, Massachusetts). Spectra were acquired by Labscap 5.0 software and preprocessed to remove spikes by an algorithm, which compares different spectra and...
subtracts those data points that seems to be aberrant. The baseline correction and normalization by area of the spectra was performed by Fityk 0.9.8 software. Spectra were analyzed by principal component analysis (PCA), subroutines of the software MiniTab 16 (MiniTab Inc.). Vector normalization was made for the analysis done by PCA, and PCs were obtained from a covariance matrix. The following comparisons were made FCL × SCL, FMO × SMO, and FBL × SBL. For each comparison, analyses were performed in two spectral windows: 500 to 1800 cm\(^{-1}\) and 1800 to 3200 cm\(^{-1}\). The spectral features enabling discrimination were found by visual inspection of the PC scores in the loading plot (LP).

3 Results

The spectra of the analyzed groups (culture media from FCL, SCL, FMO, SMO, FBL, and SBL) and pure culture media (SOFaa) are shown in Fig. 2. The corresponding vibrational bands assignment is presented on Table 1.24

Figure 3 represents the results of PCA analysis of FCL × SCL (Figs. 3(a) and 3(b)), FMO × SMO (Figs. 3(c) and 3(d)), and FBL × SBL (Figs. 3(e) and 3(f)) in the 500 to 1800 cm\(^{-1}\) and 1800 to 3200 cm\(^{-1}\) ranges. Differences between fast and slow groups at different stages of development (CL, MO, and BL) in the two spectral windows evaluated (500 to 1800 cm\(^{-1}\) and 1800 to 3200 cm\(^{-1}\)) were better observed by PCA analysis. Table 2 shows the percentage of contribution of each PC to the data variability.

Figure 4 presents the results of LP analysis of FCL × SCL (Figs. 4(a) and 4(b)), FMO × SMO (Figs. 4(c) and 4(d)), and FBL × SBL (Figs. 4(e) and 4(f)) in the two spectral windows evaluated. In the first spectral range measured the PC scores of the CL group [Fig. 4(a)], in particular, 1001 cm\(^{-1}\) (phenylalanine), 1062 cm\(^{-1}\) (CH\(_2\)) and 1076 cm\(^{-1}\) (lipids) bands, contributed to a greater extent in the separation of the fast and slow groups. In the second spectral range measured [Fig. 4(b)], the region from 2700 to 2950 cm\(^{-1}\), especially, the 2791 cm\(^{-1}\) (DNA nitrogenous bases T, A, and G) and 2892 cm\(^{-1}\) (methylene group of polymethylene chain) had opposed profiles.

The first range evaluated of the MO group [Fig. 4(c)] presented a major contribution to separate the fast and slow groups in the 1001 cm\(^{-1}\) peak (phenylalanine). In the second spectral range evaluated [Fig. 4(d)], 2892 cm\(^{-1}\) (methylene group of polymethylene chain) and 2957 cm\(^{-1}\) (CH) peaks appear to have a greater contribution to the separation of fast and slow groups.

Finally, for the first range evaluated of the BL group [Fig. 4(e)], the greater contribution to separate the fast and slow groups was observed in the 1001 cm\(^{-1}\) (phenylalanine), 1076 cm\(^{-1}\) (lipids), and 1300 cm\(^{-1}\) (amide III) peaks. According the second range evaluated [Fig. 4(f)], the 2892 cm\(^{-1}\) (methylene group of polymethylene chain) peak showed a major contribution in the groups separation.

4 Discussion

Embryo selection, based on cleavage rate and morphology, has been developed in recent years aiming the improvement in embryo implantation and pregnancy rates. Although the morphological evaluation is fast and noninvasive, it can be considered a subjective and imprecise method.7,13,14 Recent studies suggest that the evaluation of metabolome by the culture media analysis can identify embryos with higher implantation potential, since during the IVP, the embryos consume and produce metabolites in the culture media that reflect their cellular activity and development potential.15,18,20 Similarly, embryo viability has been associated with the development kinetics of embryos.5,8 Therefore, the Raman spectroscopy technique, for culture media analysis, was used in this study to discriminate metabolic profiles of bovine IVF embryos with different development kinetics.

PCA demonstrated that the culture medium of fast and slow developing embryos presents different spectroscopic profiles in the whole spectrum range analyzed (500 to 3200 cm\(^{-1}\)) at all stages evaluated (Fig. 3). Indeed, in humans, the analysis of culture media by Raman spectroscopy has been suggested as a high sensitive method to select embryos with higher viability. In this study, the metabolomic profiling of embryonic development was associated with implantation rates in IVF, predicting delivery or failed implantation with 80.5% accuracy.12

By LP analysis, we identified the bands that contributed to the discrimination among fast and slow groups. In the CL group, the DNA nitrogenous bases (T, A, G) seem to strongly contribute to the separation of these groups. There is, indeed, a correlation between the presence of free DNA in culture media and embryo quality.33 Genomic DNA (gDNA) and mitochondrial DNA (mtDNA) are detectable in human embryos secretome.
at the cleavage stage and a high proportion of mtDNA/gDNA in the secretome is associated with poor embryo quality and a high degree of fragmentation. Moreover, the assessment of the DNAmt/gDNA ratio in the day 3 of the embryo secretome in combination with the morphological classification has the potential to improve the success in the identification of viable embryos with higher development. Thus, further studies quantifying the DNA found in the culture media and the evaluation of mtDNA/gDNA ratio may provide important information on the relationship between development kinetics and embryo viability in the bovine species.

Furthermore, the results show that lipids greatly contribute to the separation of the CL and BL embryos from fast and slow groups. The relationship between lipid metabolism and embryo quality has been described in bovines and humans. Changes in the embryo lipid profile can be correlated with biosynthetic activity, membrane structure, and functional specialization of the embryos. These modifications can also be used to monitor the metabolic state during embryonic development and to evaluate the impact of the in vitro conditions on the embryo.

In our study, differences in lipid profile of culture medium of embryos at the stage of CL and BL coupled to differences in amino acid profile, such as phenylalanine, may represent blastomere metabolic changes related to, for example, the ability to use different energy substrates and consequently differences from the excess accumulation of this metabolism. Embryos with the capacity to develop to the blastocyst stage had a lower amino acid turnover than those that developmentally arrested. The highest consumption of amino acids that is also directed to the TCA cycle

Table 2. Percentage of variance of PC1, PC2 and PC3 in each spectral region for CL, MO, and BL groups.

<table>
<thead>
<tr>
<th>Groups</th>
<th>PC1 (%)</th>
<th>PC2 (%)</th>
<th>PC3 (%)</th>
<th>Raman shift (cm⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CL</td>
<td>94.8</td>
<td>4.8</td>
<td>0.4</td>
<td>500 to 1800</td>
</tr>
<tr>
<td>MO</td>
<td>99.8</td>
<td>1.2</td>
<td>0.1</td>
<td></td>
</tr>
<tr>
<td>BL</td>
<td>98.8</td>
<td>1.1</td>
<td>0.1</td>
<td></td>
</tr>
<tr>
<td>CL</td>
<td>99.7</td>
<td>0.2</td>
<td>&lt;0.01</td>
<td>1800 to 3200</td>
</tr>
<tr>
<td>MO</td>
<td>99.9</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
<td></td>
</tr>
<tr>
<td>BL</td>
<td>99.9</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
<td></td>
</tr>
</tbody>
</table>

Fig. 3. PCA of fast (FCL, FMO, FBL) and slow (SCL, SMO, SBL) groups: (a) PCA from 500 to 1800 cm⁻¹ region of CL groups; (b) PCA from 1800 to 3200 cm⁻¹ region of CL groups; (c) PCA from 500 to 1800 cm⁻¹ region of MO groups; (d) PCA from 1800 to 3200 cm⁻¹ region of MO groups; (e) PCA from 500 to 1800 cm⁻¹ region of BL groups; and (f) PCA from 1800 to 3200 cm⁻¹ region of BL groups.
may promote an increase in acetyl-CoA synthesis, a precursor of lipid metabolism. As a result of different preferred pathways, the fast and slow embryos of CL and BL groups could accumulate different energy precursors, one directing the exceeding glucose to glycogen synthesis while the other accumulating lipids. LP analysis also evidenced that the amide III bands contributed to a greater extent to the separation of the fast and slow groups at BL stage. The analysis of the amide III vibrational mode (1230 to 1300 cm$^{-1}$), denominated by the nitrogen–hydrogen bonds vibration and by the carbon–nitrogen stretching vibration, can be used for conformational analysis of proteins. Amide III analysis can also be used for quantitative analysis of proteins secondary structure, using the ratio of $\alpha$-helices and $\beta$-sheets bands at 1235 and 1275 cm$^{-1}$, respectively.

Based on this information, it can be suggested that there is a correlation between the embryo development kinetics and its ability to promote changes in the structure of essential proteins. Interestingly, the changes in this spectral region were only evident after the activation of the embryonic genome and hence after mRNA synthesis by the embryo. Therefore, these changes may result from failures in gene expression or genomic and epigenetic alterations occurred in the early stages of development.

It is interesting to note that the LP analysis showed specific profiles in the vibration bands of phenylalanine and the methylene group of polymethylene chain, which greatly contributed to the separation of the fast and slow groups in all evaluated stages. Phenylalanine is an essential amino acid that is not produced by the organism but obtained through the diet or in the case of IVP embryo, by supplementation of the culture medium. This amino acid has an important role in the preimplantation embryo development, especially in protein synthesis, energy supplement, and pH adjustment of the medium. The relationship between phenylalanine concentration in the culture medium and the embryonic viability has been described in other species. These concentrations are used as biomarkers of clinical pregnancy in humans and blastocyst formation in pigs. Furthermore, in vivo bovine blastocysts showed higher rates of phenylalanine, valine, and threonine when compared to in vitro blastocysts. Thus, the fact that phenylalanine is related to embryonic viability and also with development kinetics, as showed in the results of this study,
suggests that viability has indeed an important relation with kinetics.

Finally, there is no data in the literature that relate the methyl-


group of the polymethylene chain (methylene with CH2 bond) with embryonic development. However, studies indicate that the analysis of the intensity proportion of CH3/CH2 bands can be related to molecules conformational changes. Analysis of different types of brain malignant tumor cells showed an increase in the spectrum bandwidth that included the peaks at 2935 and 2880 cm⁻¹, which correspond to the groups methyl (CH3 bond) and methylene (CH2 bond), respectively. The decrease in intensity of the CH2/CH3 bands ratio, when compared to normal or benign cell types, also suggests changes in molecular conformation of malignant cells. The similarity between embryonic and tumor cells regarding plasticity, high adaptability in the cell growing tissue, morphological correlations, among others, could indicate that the molecular conformation disorders evidenced by the CH3/CH2 bands ratio could also be valid for the identification of the embryo metabolic status.

Therefore, we suggest that the phenylalanine and methylene group of the polymethylene chain bands, which present opposing profiles at all stages of embryonic development, can be considered Raman spectroscopy biomarkers of bovine embryos development kinetics. Table 3 shows a summary of the biological function of the important bands for fast and slow embryos described on this study.

5 Conclusion

The use of Raman spectroscopy for the analysis of IVP bovine embryos culture media has the potential to be applied as a diagnostic method for noninvasive evaluation and characterization of embryos. Some metabolites already described as biomarkers of embryonic viability appear to contribute in major proportion to the separation of the groups with different development kinetics at the stages evaluated, suggesting a relationship between kinetics and viability of bovine IVP embryos.

Acknowledgments

The authors would like to thank the Brazilian agencies FAPESP (Grant No. 2012/10351-2), CAPES, and UFABC for financial support; and Multiuser Central Facilities of UFABC for the experimental support.

Table 3  Raman bands assignments in fast and slow embryo and their biological function or interpretation.

<table>
<thead>
<tr>
<th>Frequencies (cm⁻¹)</th>
<th>Assignment</th>
<th>Biological function/interpretation</th>
</tr>
</thead>
<tbody>
<tr>
<td>2719</td>
<td>DNA nitrogenous bases</td>
<td>Identification of embryos with high potential for development</td>
</tr>
<tr>
<td>1076</td>
<td>Lipids</td>
<td>Embryo quality</td>
</tr>
<tr>
<td>1300</td>
<td>Amide III</td>
<td>Conformational analysis of proteins</td>
</tr>
<tr>
<td>1001</td>
<td>Phenylalanine</td>
<td>Embryo viability</td>
</tr>
<tr>
<td>2892</td>
<td>Polymethylene chain</td>
<td>Modifications to the molecular conformation</td>
</tr>
</tbody>
</table>

References
