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Non-Invasive Analysis of Gamete Metabolites During In Vitro Embryo Production Using Nuclear Magnetic Resonance

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***Abstract*— The in vitro production of bovine embryos has dramatically increased in recent years. From the first calves produced entirely from IVEP (in vitro embryo production)until today, the in vitro embryo production systems have been modified and improved. The media for embryo production generally are formulated with nutrient excess and furthermore it is known that excess nutrients can have negative effects on embryo development. In order to assess the actual metabolic needs of the gametes and embryo, we decided to evaluate energy consumption with a non-invasive technique, proton nuclear magnetic resonance (1H-NMR) during gamete co-incubation. In the first experiment we evaluated the behavior of cumulus oocytes complex (COCs), sperm and COCs plus sperm. We chose to evaluate pyruvate, lactate and alanine. The effect of the different groups was significant only for pyruvate ($p>0.05$) otherwise we did not find any difference in alanine and lactate concentrations. The concentration of pyruvate is significantly decreased in COCs and semen + COCs. After this experiment we re-formulated the IVF (in vitro fertilization) medium by changing the concentration of pyruvate and lactate (to 1/3 and 1/10 of standard concentrations) based on the metabolic results. The reduction of pyruvate and lactate had a positive effect on embryo production, which was evident by the increase ($P<0.05$) of advanced stage embryos in the modified vs. control media (39.6% vs. 30.4% respectively). These results suggest that this new formulation improves embryo development. These results can be used to change recommended concentrations of these substrates in IVF media.**

***Index Terms*— bovine, IVF, NMR, oocytes,Sperm.**

I. INTRODUCTION

The *in vitro* production of bovine embryos has dramatically increased in recent years, representing approximately 66% of embryos transferred in the world [1]. This is best illustrated by the worldwide statistics on IVP (*in vitro* production) embryo production and transfer in 2011. There were 453,471 IVP embryos produced and 343,927 transferred [2].In his review, Haslerstated that in the last 10 years the number of *in vitro* embryo transfer havecontinuously increased equaling the number of embryo transfers from *in vivo*-produced embryosannually[3]. *In vitro* fertilization has been adopted as a generic phrase that often is divided into several distinct parts 1) *in vitro* oocyte maturation, 2) *in vitro* fertilization, and 3) *in vitro* embryo culture. All three procedures usually are conducted in sequence to produce embryos exclusively *in vitro* (IVEP). Each of these steps is critically important in order to yield the highest quality, viable embryos, which will become calves after embryo transfer and implantation. From the first calves produced entirely from IVEP [4]until today,the *in vitro* embryo production systems has been modified and improved. The media for embryo production generally are formulated with nutrient excess, however, it is known that the excess nutrients can have negative effects on embryo development [5]. In order to assess the actual metabolic needs of the gametes, we decided to evaluate energy consumption. Metabolomic analysis is a well-established approach in biological systems, defined as "a non-targeted quantitative analysis of tissue and biofluids for low molecular mass organic endogenous metabolites"[6]. 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 in understandingbiological regulation and pathophysiological mechanisms [7]. Changes in steady-state concentrations and transient changes in intracellular metabolites resulting from processes such as cell signaling can also be readily investigated using metabolic techniques such as high-resolution nuclear magnetic resonance (^{1}H-NMR) spectroscopy. Over 70 compounds can be targeted using ^{1}H-NMR spectroscopy metabolomic approaches [8, 9]. Various applications of ^{1}H-NMR spectroscopy have been successfully used to analyze the metabolome in biological samples such as cell culture models [10] as well as in human blood, urine, and other body fluids [8, 9, 11]. Over past few years, ^{1}H-NMR spectroscopy has been applied to evaluate embryo culture media with high-quality results, in order to predict pregnancies [6, 12].We chose to evaluate the energetic substrates lactate and pyruvate, whichboth are important points of reference inmetabolic behavior. Embryos, immediately following fertilization, are reliant primarily on lactate and pyruvate as their energy sources [13-15]. Lactate production is used as an indicator of glucose metabolism, since the most glucose uptake can be accounted for by lactate production[16].

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Lactate can be converted, in a reversible process, to pyruvate by lactate dehydrogenase (LDH)[17]. Pyruvate, the other hand, can be converted into acetate and enter in the Krebs cycle, which allows for the production of NADH and FADH2. We chose to assess alanine, as our reference molecule because alanine depletionis an important marker for oocyte maturation, which is an important parameter for embryo quality [18]. Also Pudakalakatti et al. found a direct relationship between the ratio of pyruvate and alanine concentration and the embryo quality [19].

Our research group focuses on *in vitro* fertilization, so the aim of our work was to evaluate the energy consumption during the co-incubation of gametes (IVF) and re-formulate the fertilization medium (increase or decrease the energy substrates) based on the metabolic needs of the gametes. Using magnetic resonance, we first evaluated the consumption of energy substrates then subsequently modified the fertilization medium to assess the impact on embryo production.

I. MATERIAL AND METHODS

A. Reagents and Media

Unless otherwise stated, all reagents were purchased from Sigma-Aldrich (*USA*). The IVF medium was Tyrode's modified medium[20] 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% of Bovine Serum (BS). The IVC (*In vitro* culture) medium consisted of Synthetic Oviduct Fluid (SOF) medium[21], with 30 µL/mL essential amino acids, 10 µL/mL non-essential amino acids, and 5 % BS.

B. *In Vitro Embryo Production*

The matured oocytes were purchased from DeSoto Biosciences (Seymour, *TN, USA*). *In vitro* matured COCs were washed and transferred, 25±2 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 Percoll discontinuous gradient (45–80%)[22]. After processing, pellets were diluted with IVF medium and added to the fertilization wells at the concentration of 1 x 10^6 sperm/mL. Gametes were co-incubated for 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 with 5% BS, washed twice in the same medium, and transferred, 30–50 per well, into 400 µL of SOF. Zygotes were incubated in a humidified mixture of 5% CO_2, 6% O_2, and 89% N_2 at the temperature of 39°C. The percentages of cleaved embryos and embryos reaching blastocysts 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 yield.[23]

C. *^{1}H-NMR spectroscopy*

Samples of media (60 µL) were thawed and added to 640 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 500-MHz Varian UNITY INOVA 500 NB spectrometer (Agilent Technologies, Santa Clara, CA) equipped with a 5mm Varian HCN PFG Z-probe at 23°C. Shimming of the sample was performed manually on the residual water signal.^{1}H-NMR spectra were recorded with a 90° radio frequency pulse (pulse width = 6.5 µs, transmitter power = 58 dB). Sixty-four scans acquired 22,000(K) data points with a spectral width of 5,500 Hz and a relaxation delay of 10 s. The residual water signal at 4.7 ppm was suppressed using the "presat" pulse sequence (saturation power = 10 dB, presaturation delay = 1.5 s).

Spectra were processed with MestReNova software, version 6.0 (Mestrelab Research, Santiago de Compostela, Spain). Prior to Fourier transformation, the FIDs were zero-filled to 64 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 following compounds were calculated by determining the ratio of the normalized integrals of the corresponding ^{1}H-NMR signals to the TSP signal integral (0 ppm, singlet, $Si(CH_3)_3$): sodium lactate (1.33 ppm, doublet, CH_3); sodium pyruvate (2.37 ppm, singlet, CH_3); alanine (3.67 ppm, quartet, CH)(Figure 1a). Signal assignments were made on the basis of previously published data[6, 12, 24].

D. Experiment 1

Five groups were compared covering all possible combinations in IVF medium: 1) Control (medium without cells), 2) Oocytes, 3) cumulus oocyte complexes(COCs), 4) Semen, and 5) gametes after co-incubation (Semen + COCs). Over two months, the media from the different groups were analyzed (7 replicates). The samples were collected after 20 hours of incubation. The final number of samples analyzed was 35. A total of 525 oocytes (175 per group for groups 2, 3 and 5) were analyzed. From each group, 100 µl of medium was taken and analyzed by ^{1}H-NMR spectroscopy.

E. Experiment 2

In the second phase of experiment, we re-formulated the IVF medium by changing the concentration of pyruvate and lactate acid(to 1/3 and 1/10 of standard concentrations) based on the metabolic results. The results showed no significant change inlactate for any group so we reasoned that we could reduce the concentration of lactate. Further, since the pyruvate consumption was less than 1/3 of the initial concentration, we decided to reducethat concentration as well. When we re-formulated the medium we increased the concentration of NaCl to balance the osmolarity (280 mOSM). We compared 2 groups: control IVF medium and new IVF medium. A total of 386 oocytes(204 and 182 for control and new IVF, respectively) were fertilized in the second experiment.

F. Data Analysis and Statistics

^{1}H-NMR spectroscopy: Spectroscopy data were subjected to analysis of variance using the Generalized Linear Model procedure (SAS, version 9, 1999). Independent variables

were the groups (Control, COCs, Semen, Oocytes, Sperm + COCs) and the concentrations of Lactate, Pyruvate, and Alanine. Data were normally distributed. Least square means post hoc test was used to perform statistical multiple comparisons. The *alpha*level was set at 0.05. All data were expressed as quadratic mean and mean standard errors.

Embryo production: All recorded parameters were subjected to a Student's t-Test to compare cleavage rate, blastocyst rate, the percentage of embryos cleaved, and the advanced embryos on the total number of embryos. The *alpha* level was set at 0.05. All data were expressed as quadratic means and standard errors of the means.

II. RESULTS

A. *Experiment 1*

In a preliminary experiment we evaluated the fetal bovine serum to determine if it would interfere with IVF medium analysis. Figure 1 shows that serum had almost no effect on the ^{1}H NMR spectra with a single peak at zero (0.0) in the spectrum.

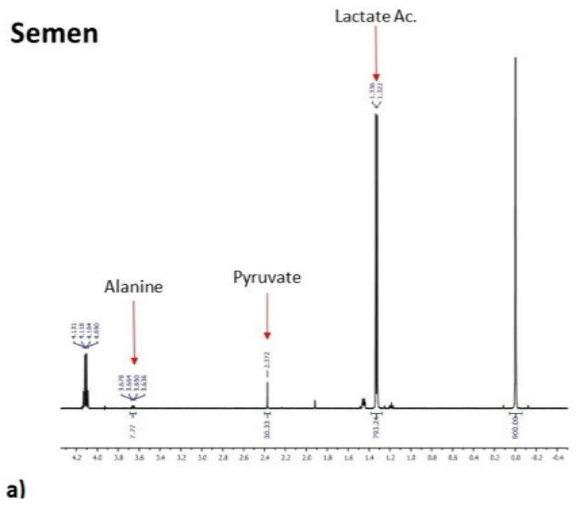
The effect of the different groupson concentration of metabolites weresignificant only for pyruvate (df=4; F= 3.72; P=0.017). The data of the post-hoc test are shown in Table 1. The concentration of pyruvate is significantly decreased in COCs andsemen + COCsin comparison to the control (P=0.004 and P=0.02, respectively). There was no difference in the concentration of lactate oralanine among groups (Table 1).

Table 1: Concentration (µM) of alanine, lactate and pyruvate in the different media samples.

	IVF	Control	COCs	Oocytes	Sperm	SEM
Ala	422.56	482.00	390.50	408.71	429.80	80.96
Lact	14903.23	14565.80	14164.02	13949.13	14503.58	457.97
Pyr	467.08^{Aa}	619.62^{Bb}	499.30^{c}	577.91^{bc}	607.61^{Bb}	34.55

^{A,B} Least square means in each column and row without common superscripts differ significantly (P< 0.01).

^{a,b} Least square means in each column and row without common superscripts differ significantly (P< 0.05).



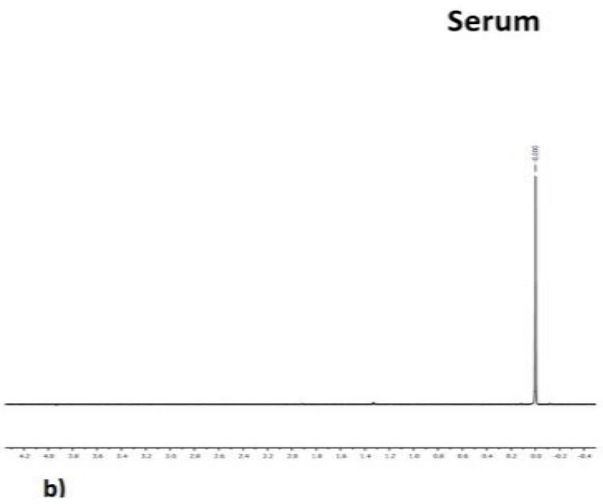


Figure 1:Representative proton nuclear magnetic resonance (1H NMR) spectrum of IVF medium with only the semen (Panel a) or only Fetal Bovine Serum (Panel b).

A. *Experiment 2*

There were no differences between conventional IVF and the re-formulated or modified IVF media regarding the cleavage rate (t= 0.238; df= 12 ;) and blastocyst rate (t= 0.834; df=12). However, when we compared the percentage of cleaved embryos (t= 2.218; df =12) and the advanced embryos (blastocysts) with the total number of embryos (t= 2.65; df =12), we found a statistical greater percentage of embryos cleaved (t= 2.218; df =12) and the advanced embryos (blastocysts) with the total number of embryos (t= 2.65; df =12), we found a statistical greater percentage of embryos from the modified IVF medium (P <0.05). The data are shown in Table 2 and in Figure 2.

III. DISCUSSION

The aims of our study were 1) to evaluate the various metabolic demands of gametes during *in vitro* fertilization, and 2) to use that information to re-formulate the IVF medium according to the identified needs of the gametes. Fertilization depends on the ability of the fertilizing sperm to 1) reach the ampulla, 2) penetrate the cumulus oophorus and the oocyte extracellular matrix, 3) fuse with the oolemma, and 4) decondense its nucleus into the ooplasm. To fertilize the oocyte, sperm must undergo several changes, collectively known as capacitation, to acquire hyperactivated motility and undergo release of the acrosomal contents or acrosomal exocytosis (AE). Assisted reproductive techniques such as artificial insemination, IVF, and intracytoplasmic sperm injection (ICSI) bypass cervical mucus, which affords clear advantages for genetic control, disease reduction, and economical production of food-producing animals through differential selection of motile spermatozoa and by acting as a physical barrier to non-motile cells [25]. The results of the first experiment show that when oocytes and sperm are put together, there is a statistically significant increase in consumption of energy substrates when compared with the control group, the medium without cells, which can be explained by two possibilities that are not mutually exclusive

of each other. The first possibility is that the biological consumption of energy substrates is statistically significant only when thereis a higher concentration of cells in the IVF media.

Table 2: Comparison of standard IVF (control) and modified IVF media.

Group	% CLEA	% TMBL/tot	% TMBL/cl	% BL/tot	% BL/cl	% *Advanced Embryos/TOT Embryos
Control	58.82 (120/204)	27.45 (56/204)	46.67 (56/120)	21.08 (43/204)	35.83[b](43/120)	30.36[b] (17/56)
Mod-IVF	59.89 (109/182)	29.12 (55/182)	48.62 (55/109)	26.37 (50/182)	44.04[a] (48/109)	39.62[a] (21/55)

[a,b] Least square means in each column and line with no common superscript differ significantly (P< 0.05).TMBL: embryos from tight morula to Hatched blastocyst; Bl: embryos from Blastocyst to Hatched blastocyst; Advanced Embryos: embryos from Expanded blastocyst to Hatched blastocyst; * Percentage of advanced embryos on the total of the embryos produced.

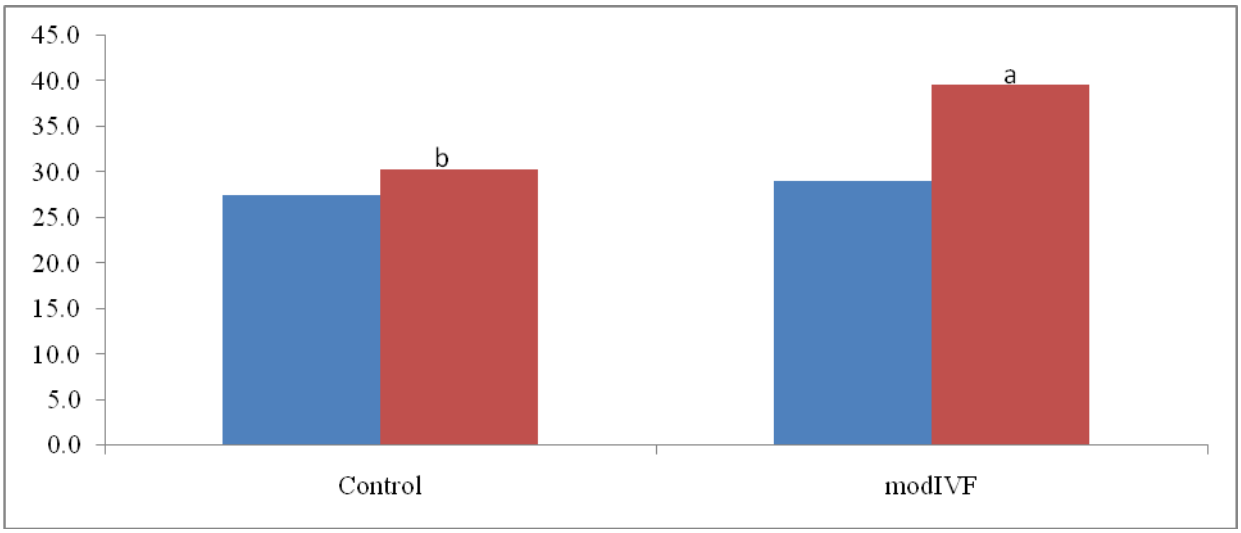


Figure 2: Percentages of blastocyst (blue) and advanced embryos (red) produced after IVF.
[a,b] Least square means in each column and line with no common superscript differ significantly (P< 0.05).

obtained in the second experiment in which reducing theconcentrations of energy substrates resulted in an increase in the percentage of advanced embryos. It is well known that sugars (specifically glucose) have significant toxicity impacts on the development of embryo [5, 26], especially in the early stages of development. A second explanation for the increase in the quantity of advanced embryos may be found in Parrish et al.[27] who observed that glucose had a negative effect on sperm, causing a delay in the acrosome reaction. It is possible that these other energy substrates may have similar effects on sperm. Therefore, decreasing their concentration may lead to less interference with the acrosome reaction and alterthe timing of sperm entry, resulting in a change in the timing of embryo development.

There are many studies that explain the relationship between metabolic behavior and amino acid fluctuations,which is the reason why we choose to evaluate amino acid profiles. Hemmings et al. demonstrated an increase in the alanine concentration is directly correlated with incomplete oocyte maturation [18]. Alanine was evaluated to determine if protein synthesis was occurring during fertilization. In the present study, we did not observe a significant decrease, or alteration in alanine concentration in the media. This result suggests that no protein synthesis occurred during incubation or co-incubation of gametes. Our data support that after oocyte maturation and up until the second or the third day of embryonic development uptake of substrates from the medium is minimal. This result is in agreement with Hougthon et al., where they saw that the amino acid depletion by embryos during the first days of development was higher in arrested embryos and concluded that the metabolism in the first divisions is minimal[28].

IV. CONCLUSIONS

In conclusion, the results obtained confirm that there is no protein synthesis during maturation and fertilization since the levels of alanine remained almost constant. The reduction of pyruvate and lactate had a positive effect on embryo production, which was evident by the increase (P<0.05) of advanced stage embryo development (from 30.4%to 39.6%respectively). These results can be used to change recommended concentrations of these substrates in IVFmedia. Future investigations involving embryo transfer and implantation studies should be performed to determine the quality of the embryos produced with the re-modulated IVFmedium.

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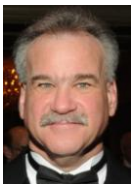

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