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Comparative Analysis of Culture Media and ATR-FTIR Metabolomics for Non-invasive Sex Determination in *In vitro* Produced Buffalo Embryos

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ABSTRACT

Background: Production of female offspring is vital for maximizing milk yield in the dairy industry, making embryo sexing essential. Metabolic differences between male and female embryos may be reflected in the culture medium (CM). This study explored non-invasive sex detection of *in vitro* produced buffalo embryos by analyzing metabolites in spent CM.

Methods: Buffalo ovaries were collected from a slaughterhouse and oocytes were matured and fertilized *in vitro*. Presumptive zygotes were cultured in either a sequential medium, Synthetic oviduct fluid (SOF) or a single-step medium, Potassium Simplex Optimized Medium (KSOM). After 5 days, morulae were placed in 25 µl of CM. On day 7, embryos and spent medium were collected and stored at -20°C. Embryo sex was determined using PCR. Spent media were analyzed using Attenuated Total Reflection - Fourier Transform Infrared Spectroscopy (ATR-FTIR) spectroscopy to detect sex-specific metabolites.

Result: This approach enabled non-invasive identification of embryo sex and revealed the impact of different culture media on sex ratio. The findings highlight the potential of spent CM analysis for predicting embryo sex, offering significant benefits for the dairy industry.

Key words: ATR-FTIR, Buffalo oocytes, *In vitro* fertilization, KSOM, Sex determination, SOF.

INTRODUCTION

Manipulating the sex of offspring in key agricultural species like buffalo enables targeted breeding and production strategies, with substantial economic benefits (Rao and Totey, 1999). Assisted reproductive technologies (ART) now make it possible to screen embryos for genetic traits before implantation, supporting more efficient herd management.

Traditional *in vivo* sex selection techniques, such as artificial insemination with sexed semen, are hindered by challenges like low conception rates and high costs. *In vitro* embryo sexing methods-including fluorescence *in situ* hybridization (FISH), polymerase chain reaction (PCR), single-nucleotide polymorphism (SNP) arrays and comparative genomic hybridization (CGH)-have been developed to address these issues, but these approaches remain invasive and labor-intensive (Fragouli *et al.,* 2008).

Metabolomic fingerprinting has emerged as a promising, non-invasive approach for embryo sex determination. Biological differences between male and female embryos-such as chromosomal complement, gene expression and epigenetic status (Alvarez *et al.,* 2010) lead to distinct metabolic signatures. Female embryos, for example, typically show lower mitochondrial metabolism and differences in carbohydrate and amino acid metabolism (Rubessa *et al.,* 2011). Advanced techniques like Nuclear Magnetic Resonance (NMR) spectroscopy, Mass spectrometry (GC-MS, LC-MS, HPLC-MS), FTIR, Near-infrared (NIR) and Raman spectroscopy are used to study the metabolic differences in embryo culture media.

Analysis of IVF culture media represents a significant advance, revealing distinct metabolomic fingerprints in CM between male and female embryos, especially when cultured in various media types (Harper *et al.,* 2012). This study uses ATR-FTIR-based targeted metabolomics to non-invasively analyze buffalo embryo culture media, aiming to determine embryo sex and improve breeding strategies.

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MATERIALS AND METHODS

In vitro production of buffalo embryos

The present study was conducted from 2019 to 2023 at the Centralized Embryo Biotechnology Laboratory and the Pharmacovigilance Laboratory, Tamil Nadu Veterinary and Animal Sciences University. The buffalo ovaries were

collected from Madras Corporation Slaughter house in normal saline maintained at 37°C within two hours of slaughter (Amran *et al.,* 2023). Cumulus Oocyte complexes (COCs) retrieval was done by slicing method and graded based on their cumulus cell investment and ooplasm homogeneity (Ammari *et al.,* 2022). Only COCs of grades A, B and C were utilized for *in vitro* maturation (Pitroda *et al.,* 2024) in TCM-199 maturation medium, supplemented with 10% FBS, 1 μg/mL Folltropin (FSH), 0.02 μg/mL LH, 1 μg/mL estradiol and 10 ng/mL EGF, for 24 hours at 38.5°C in a humidified atmosphere of 5% CO_2. Maturation was assessed by cumulus expansion and only oocytes with Degree 2 and 1 expansion were used for fertilization.

Fertilization was performed in 75 μl IVF-TALP medium supplemented with 10 μg/mL heparin in 35 mm Petri dishes. Motile sperm, prepared by swim-up from frozen semen straws were added to achieve a final concentration of 2 million sperm/mL and co-incubated with oocytes for 24 hours at 38.5°C and 5% CO_2. After fertilization, presumptive zygotes were transferred into pre-equilibrated 50 μl IVC droplets (10-15 embryos/droplet) for 5 days. Six replicates were performed.

Culture groups

Group 1: Two-step sequential medium (SOF), glucose-free for the first 3 days, then supplemented with 0.5 mM glucose.

Group 2: Single-step (KSOM).

After 5 days, embryos were cultured individually in 25 μl droplets of their respective media. On day 7, embryos and spent culture media were collected and stored at -20°C. DNA was isolated from embryos using Proteinase K, quantified and subjected to PCR using X-specific, Y-specific and satellite DNA primers for sex determination. Spent media from each embryo were labeled and stored for FT-IR analysis.

ATR-FTIR spectroscopy analysis

Spent culture media and blank controls were analyzed using a Golden-Gate ATR device (diamond crystal) on a Varian 620-IR FTIR spectrophotometer (Varian Resolutions Pro software v5.0.0.700). Targeted metabolites included glucose, glycine, glutamate, arginine, tyrosine, phenylalanine, tryptophan, threonine, methionine and valine.

Statistical analyses

Data were analyzed using one-way ANOVA to assess differences among groups. The Newman-Keuls test was used for multiple comparisons, controlling family-wise error rates. Analyses were performed using GraphPad Prism (v5). Results are expressed as mean ± SD. Statistical significance was denoted as *p<0.05, **p<0.01 and ***p<0.001, ensuring robust and reliable interpretation of experimental outcomes.

RESULTS AND DISCUSSION

Metabolomic profiling using ATR-FTIR spectroscopy was conducted to detect sex-specific metabolites in the spent CM of *in vitro* developed buffalo embryos (Fig 1 and Fig 2). Out of ten targeted metabolites, seven displayed distinct differences between male and female embryos, offering valuable insights into sex-specific metabolic profiles.

For embryos cultured in both SOF and KSOM, consistent patterns emerged. Female embryos showed significantly higher consumption of glucose, arginine, glutamine and methionine, as evidenced by greater depletion of these metabolites in their culture medium (Fig 3 and Fig 4). This suggests increased utilization of these nutrients by female embryos during development. These findings are consistent with previous human studies, where female embryos incorporated more glucose than males from the CM (Gardner *et al.,* 2011), with glucose uptake

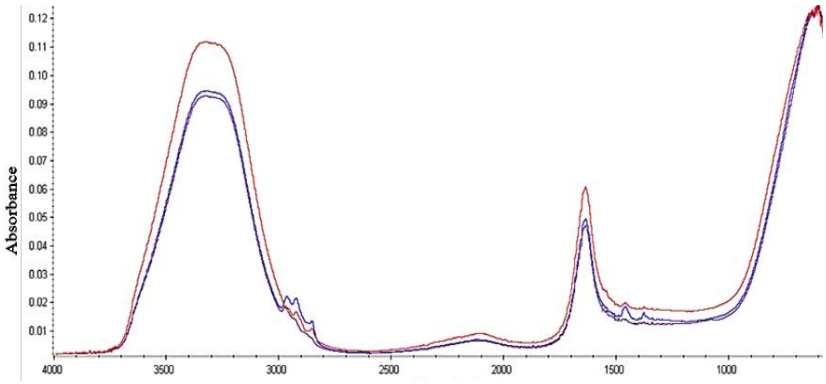


Fig 1: Average spectra profiles corresponding to spent media (SOF) of female embryos, male embryos and blank samples obtained by ATR-FTIR.

rates of 4.92 pmol/embryo/h for females versus 4.37 pmol/embryo/h for males (Gardner and Leese, 1987).

Conversely, male embryos exhibited higher consumption of tyrosine, glycine and valine, as indicated by more significant depletion of these amino acids in their medium (Fig 5 and Fig 6). This suggests a preferential use of these metabolites by male embryos. Essential amino acid metabolism, particularly involving valine, leucine and isoleucine, is linked to the TCA cycle and energy production (Inoue *et al.,* 2021). Supporting this, recent studies have reported greater abundances of serine, glutamine, histidine, glycine, threonine, arginine, taurine, tryptophan,

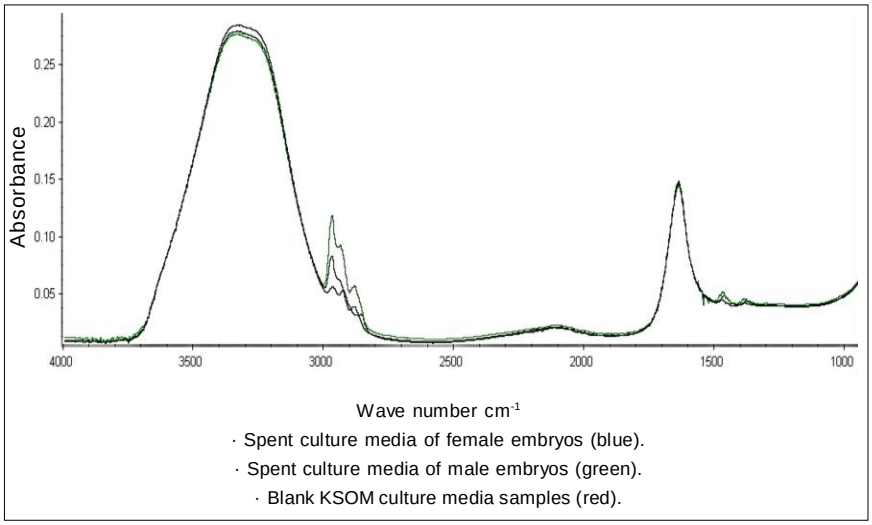


Fig 2: Average spectra profiles corresponding to spent media (KSOM) of female embryos, male embryos and blank samples obtained by ATR-FTIR.

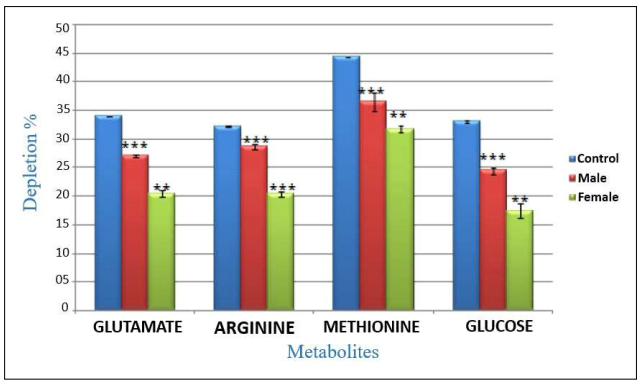


Fig 3: Metabolites depleted in female embryo cultured SOF spent media.

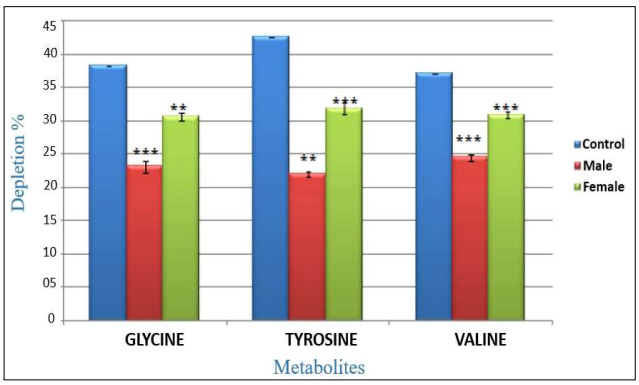


Fig 5: Metabolites depleted in male embryo cultured SOF spent media.

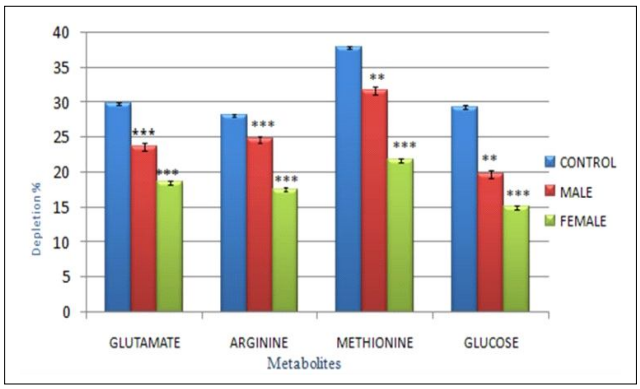


Fig 4: Metabolites depleted in female embryo cultured KSOM spent media.

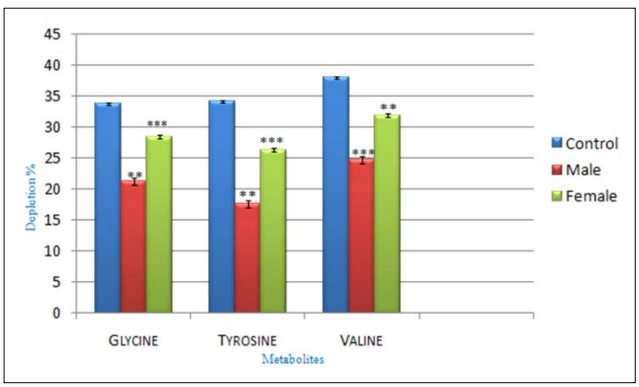


Fig 6: Metabolites depleted in male embryo cultured KSOM spent media.

phenylalanine, isoleucine, leucine and agmatine in the allantoic fluid from male versus female sheep fetuses (Halloran *et al.,* 2021). Similarly, in pigs, umbilical uptake of arginine, methionine, lysine, proline and phenylalanine is higher in male fetuses (Brown *et al.,* 2022).

Both SOF and KSOM systems consistently showed significant sex-related differences in seven metabolites, with tyrosine, glutamate and glucose emerging as strong, reliable biomarkers for non-invasive embryo sexing.

Embryos at days 5-7 provided a robust platform for metabolomic comparisons, which correlated well with PCR-based sex determination results. Male and female bovine embryos differ not only in chromosomal complement but also in transcriptional activity and epigenetic status (Alvarez *et al.,* 2010). Sexual dimorphism in embryonic transcription influences carbohydrate and amino acid metabolism, with female embryos exhibiting lower mitochondrial metabolism (Rubessa *et al.,* 2011). Since metabolic products are secreted into the CM as part of the embryonic secretome, differences in CM composition can reflect both embryo sex and viability. In summary, these findings highlight significant sex-specific differences in metabolite utilization during early embryonic development. The differential consumption of glucose by female embryos aligns with earlier reports, while distinct amino acid utilization patterns suggest unique metabolic requirements between male and female embryos.

CONCLUSION

This study demonstrates the potential for non-invasive sexing of buffalo embryos using ATR-FTIR-based metabolomics in two culture systems: sequential and single-step. Both systems revealed significant differences in seven metabolites, with tyrosine, glutamate and glucose serving as effective sex markers. The consistency across media confirms these metabolic differences are intrinsic to embryo sex. Notably, KSOM favored female embryos, while SOF favored males, suggesting culture media can influence sex ratios. These findings offer a promising, non-invasive approach for embryo sexing, with implications for optimizing assisted reproductive technologies and improving *in vitro* embryo culture protocols.

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Conflict of interest

All authors declare that they have no conflict of interest.

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