Metabolite Profiles of Human Follicular Fluid for Assessing Blastocyst Development during Intracytoplasmic Sperm Injection (ICSI) Treatment

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Abstract

Infertility constitutes a significant health issue worldwide, especially in Thailand, where the rising infertility impact has led to diminishing birth rates, highlighting the critical need for effective assisted reproductive technologies (ART). This preliminary study examined the metabolic content of follicular fluid (FF) associated with blastocyst development in patients undergoing intracytoplasmic sperm injection (ICSI) to explore potential biomarkers for embryo selection. Nine FF samples were collected and analyzed via liquid chromatography–tandem mass spectrometry (LC-MS/MS) to generate comprehensive metabolic profiles. A total of 117 metabolites were identified, and differential analysis via MetaboAnalyst 6.0 indicated that five metabolites nicotinamide, cytidine, glycerophosphocholine, N-acetyl-L-ornithine, and methylcysteine were significantly upregulated in embryos that successfully developed to the blastocyst stage, while these metabolites were not detected at significant levels in embryos that did not reach this developmental stage. No metabolites exhibited significant downregulation under these conditions. The subsequent Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis revealed five overrepresented pathways, with ether lipid metabolism ($p = 0.0074$) and nicotinate and nicotinamide metabolism ($p = 0.0076$) achieving statistical significance. The implementation of these findings indicates that distinct metabolic signatures in FF can serve as predictive biomarkers for embryo selection, thereby improving implantation rates and overall success in ART. However, the small sample size ($n = 9$) represents a limitation of this study and should be considered when interpreting the results. The analysis of these metabolite profiles elucidates the molecular pathways of embryo development and facilitates the progression of individualized fertility treatments.

Keywords: *Follicular fluid, Assisted reproductive technologies, Metabolomics, Blastocyst development, LC-MS/MS, Advanced reproductive age*

1. Introduction

Thailand is undergoing a major demographic transition, with its fertility rate falling below replacement level. The average number of children per woman has dropped to 1.53 from around 6 in previous generations, driven by lifestyle changes, higher education, women's workforce participation, and delayed marriage or parenthood. Consequently, annual births have declined from over one million to 618,193 in 2021 (Health, 2022).

Infertility, defined as the failure to achieve pregnancy after 12 months of unprotected intercourse, remains a significant global health concern (Zegers-Hochschild et al., 2017). Women aged 35 and older face reduced fertility (Broekmans et al., 2009). Assisted reproductive technologies (ART), including intrauterine insemination (IUI), in vitro fertilization (IVF), and intracytoplasmic sperm injection (ICSI), offer medical solutions to infertility. However, blastocyst development remains unpredictable, thereby affecting implantation and pregnancy outcomes.

The metabolic approach has gained increasing attention as a non-invasive and innovative strategy for evaluating reproductive potential through the use of specific biomarkers that reflect oocyte and embryo quality. This methodology offers valuable insights into the underlying pathophysiological mechanisms of infertility and presents promising implications for improving outcomes in ART (Fiscus et al., 2023). In particular, metabolomic profiling provides a sensitive and comprehensive method to detect subtle biochemical alterations linked to oocyte competence and subsequent embryo viability. Through such analysis, clinicians and researchers can achieve a more precise understanding of the factors influencing fertilization

success and early embryonic development, ultimately contributing to enhanced clinical decision-making in reproductive medicine.

Follicular fluid (FF), the biological medium surrounding the oocyte within the ovarian follicle, plays a crucial role in providing the nutritional and signaling molecules necessary for oocyte maturation and competence. Because FF is easily accessible during oocyte retrieval in IVF procedures, it serves as an ideal non-invasive sample for studying the metabolic environment of the oocyte (Revelli et al., 2009). The composition of FF reflects exchanges between blood plasma and follicular cells, representing a comprehensive snapshot of the microenvironment critical for folliculogenesis, oocyte development, and embryo formation.

Given the importance of the follicular microenvironment, metabolomic analysis of FF provides a powerful approach to identifying potential biomarkers capable of predicting reproductive outcomes. The present study applied liquid chromatography–tandem mass spectrometry (LC–MS/MS) to examine correlations between metabolite profiles in FF and the rate of blastocyst development in patients undergoing ICSI. This high-resolution analytical technique allows for the detection of novel metabolites that may serve as reliable indicators of embryo developmental potential. Ultimately, the findings from such studies aim to improve predictive models of IVF success, enhance individualized patient treatment strategies, and contribute to addressing the ongoing global decline in birth rates through more efficient reproductive interventions.

2. Objectives

This study aims to investigate the correlation between the metabolite profiles of FF and blastocyst development using LC-MS/MS analysis in patients undergoing ICSI, with the goal of identifying potential biomarkers to enhance ART success rates.

3. Materials and Methods

3.1 Study population

This descriptive cross-sectional cohort study investigated metabolomic markers associated with blastocyst development in women aged ≥35 years undergoing ICSI at Thammasat and Rajavithi Fertility Centers between September and December 2024. Participants were classified as successful (≥60%) or failed (<60%) based on Day 5 blastocyst rates (ESHRE Special Interest Group of Embryology and Alpha Scientists in Reproductive Medicine, 2017), which were calculated as the number of blastocysts formed on Day 5 divided by the number of normally fertilized oocytes (2PN). The required sample size was estimated using MetSizeR (Nyamundanda et al., 2013), based on 100 metabolite bins (p = 100), an expected proportion of significant features of 0.2, no covariates (ncovar = 0), the PPCA model, and a false discovery rate (target.fdr) of 0.10. The simulation indicated that 10 samples per group (total = 20) would provide adequate power for detecting significant metabolite differences. Exclusion criteria included systemic diseases and use of metabolism-altering drugs such as corticosteroids (Li & Cummins, 2022) or antipsychotics (Zhang et al., 2017). Ethical approval was granted by the Thammasat University Ethics Committee (MTU-EC-OO-0-101/67), and informed consent was obtained from all participants.

3.2 FF collection

FF was collected from 14–24 mm follicles (Levay et al., 1997) during oocyte retrieval and stored at −80°C (Rosen et al., 2008; Yang et al., 2023) FF from 1–3 follicles ≥16 mm containing MII oocytes was selected to avoid blood contamination. The metabolites in FF, spiked with the internal standard (DL-arabinose), were extracted using cold methanol (Yuan et al., 2012). The FF samples were centrifuged, and the supernatant was transferred into a new tube and dried using a SpeedVac without heat. The dried samples were kept at -80 °C until use.

3.3 Metabolite identification by LC-MS/MS

The dried sample was dissolved in LC/MS grade water and was filtered using a Phenex-RC filter (0.2 µm pore size). The filtered supernatant was transferred into a Chromacol vial for LC/MS/MS analysis.

(Yuan et al., 2012). Metabolite identification was analyzed by using a liquid chromatography Triple Quadrupole LC-MS/MS system (QTRAP® 5500). The metabolites compounds were separated using the HPLC with an amide column XBridge HPLC column (3.5 mm; 4.6 mm inner diameter (i.d.) × 100 mm length) and a mobile phase consisting of 50% buffer A [comprising 95% (v/v) water, 5% (v/v) acetonitrile, 20 mM ammonium hydroxide, and 20 mM ammonium acetate, pH 9.0] and 50% buffer B (100% acetonitrile), at a mobile phase of 400 µl/min for a duration of 23 minutes. The MS/MS section adjusted the spray voltage to 3,200 V, using nitrogen and argon as the auxiliary and collision gases, respectively. The metabolite detection parameters were set to a dwell time of 0.1 s for each single-reaction monitoring (SRM) transition, with a peak width of 1 m/z. The LC-MS/MS detection threshold was set at a signal-to-noise (S/N) ratio of 3:1.

3.4 Metabolomic analysis

Metabolomic data were processed using the free online web-based MetaboAnalyst 6.0 (https://dev.metaboanalyst.ca/ModuleView.xhtml), including fold-change analysis, volcano plots, and PLS-DA, followed by KEGG pathway enrichment. Other data were analyzed using SPSS version 29 (IBM, Armonk, USA). Quantitative variables were presented as medians with interquartile ranges. All statistical analyses were conducted at a 95% confidence level.

4. Results and Discussion

4.1 Results

4.1.1 Demographic data of the patients

Six infertile women provided nine FF samples. Three samples belonged to the successful group (blastocyst rate ≥60%) and six to the failed group (<60%). Three participants contributed to both groups because multiple oocytes were retrieved from each of them, and these oocytes showed variable developmental outcomes—some progressed to the blastocyst stage (≥60%), while others did not (<60%). There were no statistically significant differences in demographic characteristics between the two groups (Table 1). All participants were healthy, without chronic diseases or metabolism-altering medications. For ovarian stimulation, most received recombinant FSH (follitropin-alpha); others used follitropin-beta, combined with human menopausal gonadotropin (hMG).

Table 1 Demographic data of patients

	Blastocyst development rate	Median	Range	*p*-value
Age (years)	≥ 60%	43	35-45	1.000
	< 60%	43	35-45	
Weight (kg)	≥ 60%	52	48.2-58.3	.714
	< 60%	54	48.2-58.3	
Height (cm)	≥ 60%	160	150-161	.905
	< 60%	160	150-161	
BMI (kg/m^2)	≥ 60%	20.3	18.6-25.9	.714
	< 60%	21.1	18.6-25.9	

4.1.2 Metabolite Profiles

A total of 115 metabolites were identified in the <60% blastocyst group and 114 in the ≥60% group. Venn diagram analysis revealed that 112 metabolites were shared between both groups. Three metabolites—thiamine phosphate, S-methyl-5-thioadenosine, and 1-methyl-histidine—were unique to the <60% group, while NADPH and coenzyme A were exclusively detected in the ≥60% group.

4.1.3 Fold Change (FC) Analysis

The fold-change analysis of FF metabolites between the ≥60% and <60% blastocyst development groups was performed. Fifteen metabolites were upregulated, and nine were downregulated. Prominently upregulated metabolites included cytidine, AMP, nicotinamide, GMP, CMP, methylcysteine, UMP, NADPH, coenzyme A, folate, phosphorylcholine, glycerophosphocholine, N-acetyl-L-ornithine, pyridoxine, and

arginine. Downregulated metabolites comprised S-methyl-5-thioadenosine, histidine, 1-methyl-histidine, urea, cystathionine, thiamine phosphate, guanine, thiamine, and S-adenosyl-L-methionine.

4.1.4 Volcano Plot Analysis

Volcano plot analysis was performed alongside fold-change analysis to identify key metabolites with both statistical significance and biological relevance. As shown in **Table 2** and **Figure 1**, five metabolites—nicotinamide, cytidine, glycerophosphocholine, N-acetyl-L-ornithine, and methylcysteine—met the significance threshold ($p < 0.05$) with positive $\log_2$ fold changes, indicating upregulation in the ≥60% blastocyst development group. No significantly downregulated metabolites were detected under these conditions.

Table 2 Significantly up-regulated metabolites identified from volcano plot analysis

Metabolite	Fold Change	Log2(FC)	Raw p-value	-log10(*p*)
Nicotinamide	3.6623	1.8727	0.0033414	2.4761
Cytidine	6.8036	2.7663	0.0048391	2.3152
Glycerophosphocholine	2.1511	1.1051	0.0074436	2.1282
N-acetyl-L-ornithine	2.1708	1.1182	0.029175	1.535
Methylcysteine	2.7825	1.4764	0.041307	1.384

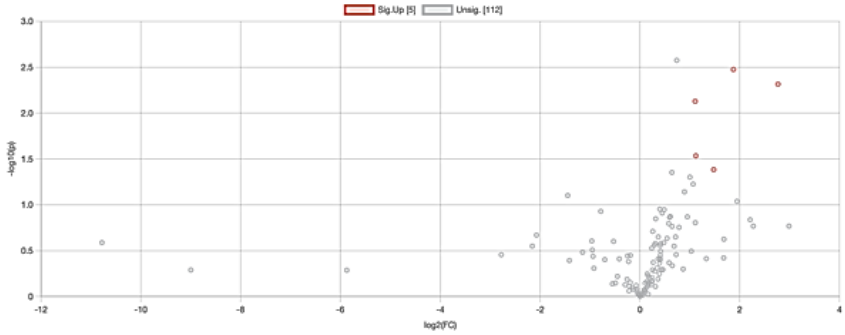

Figure 1 Volcano plots of FF metabolites between ≥60% and <60% blastocyst development rate groups. The figure illustrates the distribution pattern of all analyzed metabolites (n = 117). Significantly upregulated metabolites (n = 5) are shown in red, and non-significant features (n = 112) are represented in gray. The x-axis represents $\log_2$(fold change) [$\log_2$(FC)], and the y-axis represents $-\log_{10}$(p-value). Significance was determined based on [insert criteria, e.g., $p < 0.05$ and $|\log_2 FC| \geq 1$].

4.1.5 KEGG Pathway Enrichment Analysis

KEGG pathway enrichment analysis was performed to identify metabolic pathways associated with significant metabolite changes. The top five pathways were ether lipid metabolism, nicotinate and nicotinamide metabolism, vitamin B6 metabolism, purine metabolism, and pyrimidine metabolism (**Figure 2**). Among these, ether lipid metabolism ($p = 0.0074$) and nicotinate and nicotinamide metabolism ($p = 0.0076$) were statistically significant ($p < 0.05$), suggesting their key roles in regulating blastocyst development.

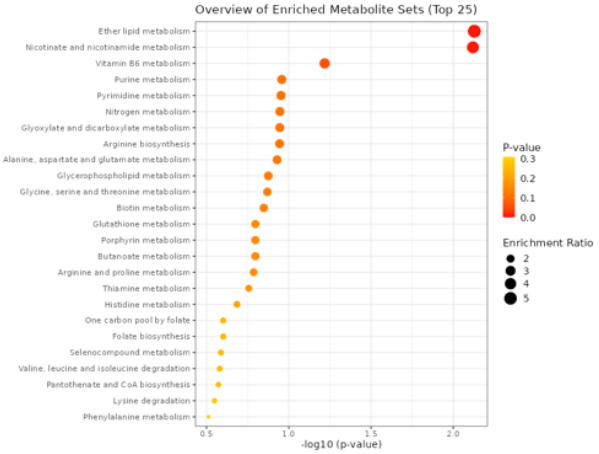

Figure 2 Overview of KEGG pathway enrichment analysis based on altered metabolites, as identified using MetaboAnalyst 6.0

4.2 Discussion

A total of nine samples from six patients were analyzed. No significant demographic differences were found between the ≥60% and <60% blastocyst development groups. Although the sample size was smaller than initially estimated due to strict eligibility criteria, the findings offer preliminary insights warranting validation in larger studies. All samples were free from blood contamination.

This study identified five metabolites nicotinamide, cytidine, glycerophosphocholine, N-acetyl-L-ornithine, and methylcysteine that were upregulated in the ≥60% blastocyst development group. These findings suggest potential biomarker roles for predicting embryo competence.

Nicotinamide, a vitamin B3 derivative and NAD^+ precursor, is crucial for redox balance, DNA repair, and mitochondrial function. Declining NAD^+ contributes to oocyte aging, whereas nicotinamide riboside restores mitochondrial activity, reduces oxidative stress, and promotes oocyte maturation (Li et al., 2023; Li et al., 2024b). Through SIRT1/AKT signaling, it prevents apoptosis and supports embryonic pluripotency, blastocyst formation, and implantation (El Sheikh et al., 2020; Guo et al., 2022; Son et al., 2013). Disrupted NAD^+ metabolism can lead to developmental defects, underscoring nicotinamide's role in reproduction (Bozon et al., 2025).

Cytidine, a cytosine-ribose nucleoside and precursor of cytidine triphosphate (CTP), is essential for RNA and DNA synthesis and maintaining nucleotide balance during blastocyst development (An et al., 2025; Yang et al., 2022). Through the pentose phosphate pathway, it links to glycolysis to supply energy and biosynthetic substrates (Ferrick et al., 2020; Gardner et al., 2023). Cytidine metabolism also supports epigenetic regulation via methyl-group provision for DNA and histone modifications essential in early embryogenesis (Eckert et al., 2007; Milazzotto et al., 2020). Enhancing cytidine metabolism under optimized culture conditions may improve embryo quality and ART outcomes (Eldarov et al., 2022; Funda et al., 2024).

Glycerophosphocholine (GPC) is a pivotal intermediate in phospholipid metabolism that ensures membrane synthesis, stability, and blastocyst development (Fernández-Bussy et al., 2015; Niu et al., 2023). It promotes phosphatidylcholine formation, maintains membrane fluidity, and regulates lipid droplet dynamics essential for embryonic energy storage and implantation potential (Aizawa et al., 2019; Desmet et al., 2020). Moreover, GPC contributes to epigenetic control through phosphocholine-derived intermediates affecting histone modification and zygotic gene activation (Li, 2023; Li et al., 2022). Elevated GPC levels in embryo culture media are associated with superior embryo quality, implantation success, and favorable ART outcomes (Funda et al., 2024; Shahzad et al., 2020).

N-acetyl-L-ornithine (NAO), an intermediate in the arginine pathway, supports trophoblast proliferation, differentiation, and antioxidant defense during early embryogenesis (Hussain et al., 2020; Leese et al., 2021). By modulating β-catenin and mTOR signaling, it regulates gene expression and development

(Li et al., 2024a). Arginine metabolites enhance embryo viability by improving metabolism and reducing oxidative stress (Gardner et al., 2023; Mancini et al., 2021). NAO profiling in spent media may aid embryo selection and improve implantation outcomes in ART (Correia et al., 2022; Lin et al., 2014).

Methylcysteine supports one-carbon metabolism by supplying methyl groups essential for epigenetic regulation of embryonic gene expression (Reyes Palomares et al., 2024). Folate-dependent pathways and nutrients such as betaine, 5-MTHF, methionine, choline, folate, and vitamin B12 enhance methylation, mitochondrial function, and embryo viability (Cai et al., 2023; Golestanfar et al., 2022; Kudo et al., 2015). Elevated homocysteine, especially in women with the MTHFR C677T genotype, correlates with poor IVF outcomes (Chen et al., 2021). Thus, methylcysteine metabolism is crucial for embryonic development and may improve ART efficiency (Ikeda et al., 2017; Sun et al., 2018; Syring et al., 2023).

Collectively, these results demonstrate that the metabolic composition of FF, particularly the identified metabolites, plays a crucial role in supporting blastocyst development. Understanding the mechanistic contributions of these metabolites may provide valuable insights into embryo viability and lead to improved strategies in ART.

KEGG analysis identified ether lipid metabolism ($p = 0.0074$) and nicotinate and nicotinamide metabolism ($p = 0.0076$) as key pathways in early embryogenesis. Ether lipid metabolism supports energy supply, membrane synthesis, and trophoblast differentiation through plasmalogens and signaling molecules such as LPA and S1P (Aikawa & Hirota, 2024; Kong & Gao, 2024; Zhao et al., 2023). Upregulation of plasmalogen biosynthetic genes promotes trophoblast and placental development (Calderari et al., 2023; Lipinska et al., 2023). Nicotinate and nicotinamide metabolism further maintain the redox balance critical for blastocyst formation. This pathway supports blastocyst development by driving NAD synthesis essential for energy metabolism, DNA repair, and epigenetic regulation (Lees et al., 2020; Meng et al., 2021). NAD produced via the nicotinamide mononucleotide (NMN) and nicotinate mononucleotide (NaMN) pathways sustains mitochondrial function, while precursor supplementation (NR, NMN) improves oocyte and embryo quality (Li et al., 2023; Liang et al., 2024; Ramírez-Martín et al., 2025). Through sirtuin activity and Wnt/β-catenin signaling, nicotinamide promotes pluripotency, differentiation, and implantation, underscoring NAD metabolism as a potential target to enhance ART outcomes (An et al., 2025; Milazzotto et al., 2022).

Previous studies have investigated fatty acid biomarkers in follicular fluid associated with oocyte developmental competence. The present research differs in its primary objective, aiming to examine the metabolite profile of follicular fluid in relation to blastocyst development rates. This approach extends understanding from oocyte-level competence to later stages of embryonic development and may provide additional insights into metabolic factors influencing embryo quality beyond fertilization (O'Gorman et al., 2013). Both FF and spent embryo culture media contain metabolites that reflect the physiological status of the developing gamete or embryo, but they provide information from different stages of development. Metabolic profiling of FF, as reported in previous studies, primarily reflects the microenvironment of the oocyte and its developmental competence, whereas analysis of spent culture media focuses on metabolites secreted or consumed by the embryo, thus representing its metabolic activity and implantation potential. Together, these approaches capture distinct yet complementary aspects of reproductive metabolism, contributing to a more comprehensive understanding of factors associated with successful embryo development (O'Gorman et al., 2013; Wallace et al., 2014).

This study has several limitations. The small sample size (nine samples from six patients at a single center) and the homogeneous cohort of women over 35 years limit generalizability. The cross-sectional design prevents causal inference, and only follicular fluid metabolites were analyzed, without accounting for endometrial, genetic, or sperm factors. Potential confounders such as BMI and lifestyle were not fully controlled. In addition, only blood-free samples were included, and clinical outcomes like implantation or live birth were not assessed. Larger, multicenter longitudinal studies are needed to validate these findings.

5. Conclusion

In summary, this study demonstrates that specific metabolites—nicotinamide, cytidine, glycerophosphocholine, N-acetyl-L-ornithine, and methylcysteine—are distinctly elevated in embryos that

successfully progress to the blastocyst stage. KEGG pathway enrichment analysis further identified ether lipid metabolism and nicotinate and nicotinamide metabolism as the most significantly enriched metabolic pathways that are involved in blastocyst development. These findings underscore the potential roles of membrane lipid remodeling and NAD^+ biosynthesis/redox balance in supporting early embryonic development. However, due to the small sample size, these results should be interpreted with caution, and further validation in larger, independent cohorts is necessary to confirm these associations.

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