Applications of MALDI Imaging Mass Spectrometry in reproductive medicine

Doctoral (PhD) thesis

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1. Introduction

1.1. General introduction

Infertility is a growing global concern, affecting approximately 17.5% of couples, with significant implications for mental health, financial stability, and societal dynamics. As fertility rates decline, especially in industrial Western countries, the importance of assisted reproductive technologies (ART), like in vitro fertilization (IVF), has increased, despite opposition from groups, particularly the Roman Catholic Church. In Hungary, infertility treatments received governmental support in 2020, leading to publicly funded IVF and screening services, yet the rising demand calls for more specialists.

The fertility rate in Hungary has shown a downward trend until 2003, with recent slight increases influenced by rising infertility rates among both genders. Male infertility, accounting for 40-45% of cases, necessitates concurrent testing with female partners. Factors contributing to male infertility include varicocele, blockages, infections, genetic issues, and external stressors. A holistic approach to treatment, focusing on patients' long-term fertility and overall well-being, is essential, even in cases where specific sperm abnormalities remain unexplained. Addressing these challenges is crucial in combating the rising infertility rates effectively.

1.2. Testicular tissue and spermatogenesis

Spermatogenesis, the process of producing haploid spermatozoa from spermatogonia, occurs in the seminiferous tubules of the testes and involves several stages: mitosis, meiosis, and spermiogenesis. After puberty, mitotic activity of stem cells continues throughout life, leading to the formation of primary spermatocytes which undergo meiosis to become secondary spermatocytes and eventually haploid sperm cells.

Sertoli cells play a vital role by providing structural and metabolic support, regulating tight junctions, and facilitating sperm release. They also preserve sperm health by protecting germ cells from the immune system and recognizing apoptotic cells. Key lipids, such as seminolipids, are crucial for spermatogenesis and membrane stability.

The epididymis, divided into caput, corpus, and cauda regions, is where sperm mature and gain motility. Changes in lipid content occur during this maturation phase.

Azoospermia, the absence of sperm in semen, affects 10-15% of infertile men and can be obstructive or non-obstructive. Sertoli cell-only syndrome (SCOS) is a common cause of non-obstructive azoospermia, characterized by the absence of germ cells, often linked to genetic disorders or environmental damage. Identifying biomarkers related to lipids and other constituents is essential for evaluating testis health and fertility.

1.3. Phospholipids in general

Phospholipids (PLs) are essential amphiphilic molecules that form the majority of cellular membranes and play crucial roles in cell signaling, metabolism, and pathology. They were historically recognized mostly as structural components, but are now known to also act as environmental sensors and regulators of metabolic processes.

The primary types of phospholipids include glycerophospholipids, such as phosphatidic acid (PA), phosphatidylcholine (PC), and sphingomyelin (SM), with glycerophospholipids being the most prevalent. Glycerophospholipids consist of a polar head attached to two fatty acids, and their structure can vary based on the composition and positioning of these fatty acids.

Sphingomyelins, a subclass of sphingolipids, are another significant type of phospholipid, containing sphingosine and a fatty acid. The diversity of phospholipids is influenced by factors such as the length and saturation of their fatty acid chains, head group identity, and the presence of unique structures like plasmalogens, which include ether or vinyl ether linkages.

Phosphatidic acid, a crucial precursor in phospholipid biosynthesis, impacts membrane properties and various signaling pathways. It can be produced through multiple pathways, including the acylation of lysophosphatidic acid and the breakdown of phosphatidylcholine. Phospholipid biosynthesis involves intermediates like diacylglycerol (DAG) and cytidine diphosphate-DAG (CDP-DAG), with specific enzymes facilitating these conversions.

Overall, phospholipids exhibit extensive molecular diversity and are vital for cellular structure and function.

1.4. Testicular lipid metabolism

Previous studies have investigated the role of testicular lipid metabolism in human reproduction, with particular emphasis on phospholipids and the endocannabinoid system (ECS) in the human testis. They highlighted the importance of phospholipids in steroidogenesis in Leydig cells and their potential effects on follicle-stimulating hormone (FSH) interactions. Research shows that phospholipase C treatment can reduce FSH receptor binding. Further studies suggest that exogenous cannabinoids may disrupt the reproductive male endocannabinoid system, a mechanism that is not yet fully understood in humans. Key components of the ECS, including cannabinoid receptors and enzymes, have been identified in testicular cells, emphasizing their role in spermatogenesis and Leydig cell function. Additionally, sperm in the testis show higher phospholipid content and different fatty acid profiles compared to ejaculated sperm, highlighting the critical role of lipids in sperm function and development.

1.5. The role of lipids in the female reproductive tract

About 93% of arachidonic acid in the uterus is stored in phospholipids, primarily in phosphatidylcholine (PC), phosphatidylethanolamine (PE), and triglycerides (TAG). Arachidonic acid is released by phospholipase A2 and is converted into prostaglandins (e.g., PGI2, PGE2, PGF2α) by cyclooxygenase enzymes, which are crucial for ovulation, fertilization, implantation, and decidualization. Notably, prostacyclin (PGI2) promotes implantation and decidualization through the activation of peroxisome proliferator-activated receptor (PPARβ/δ), initiating cell proliferation and angiogenesis vital for early embryogenesis.

Additionally, lysophosphatidic acid (LPA) is crucial beyond implantation, influencing the spacing of blastocysts in the uterus. Its mechanism may involve myometrial contraction, with elevated expression of the G-protein-coupled receptor (LPAR3) during the peri-implantation period, suggesting an interaction with prostaglandin synthesis and COX-2 activity.

1.6. Mass Spectrometry

1.6.1. Background of Mass Spectrometry

Mass spectrometry involves three fundamental operations: generation of gas-phase ions from sample molecules (ion source), separation of ions based on their mass-to-charge ratio (m/z) (analyzer), and detection of these ions (detector). The primary components of a mass spectrometer include an ion source, masse analyzer, and detector, requiring a high vacuum (10^{-4}–10^{-7} mbar) achieved through a turbomolecular pump and roughing. Ions must travel through the spectrometer without contacting air to maintain their charge and trajectory. Positive ionization mode is standard for analysis, utilizing techniques such as Electrospray Ionization (ESI) and Matrix-Assisted Laser Desorption/Ionization (MALDI). The common analyzers include Time-of-Flight (TOF), ion trap (IT), and quadrupole-time-of-flight (Q-TOF), with detection performed by electron multiplier-type detectors.

1.6.2. Matrix-Assisted Laser Desorption Ionization Time-of-Flight Mass Spectrometry

MALDI ionizes sample molecules using a matrix material that absorbs laser energy. This process involves desorption of macromolecule-matrix complexes into high vacuum where they are accelerated, and the time-of-flight to the detector reflects their mass to charge ratio (larger ions travel more slowly than smaller ones). MALDI imaging mass spectrometry (MALDI-IMS) allows two-dimensional visualization of biomolecules without complex preparation or labeling, making it relevant for clinical diagnostics and research, particularly in cancer. Additionally, imaging mass spectrometry (IMS) enables the investigation of lipid localization in tissues, with special focus on lipid mechanisms in biological processes and molecular profiling.

2. Aims of the study

• The main goal of our research is to identify biomarkers and determine their local distribution, which could form the basis of a rapid diagnostic procedure specific to the spermatogenesis process.

• Using MALDI IMS, we investigate the distribution of phospholipids, comparing PC variations between normal and SCOS testicular tissues. We are developing a MALDI IMS protocol for analyzing testicular tissues, including histological preparation, matrix coating, and mass spectrometry measurements. Our measurements focus on lipid molecules crucial to spermatogenesis and pathological processes.

• One of the aims of our research is to explore possible correlations between the Johnsen scoring system and testicular phospholipid expression in SCOS patients.

• We plan to conduct MALDI-IMS analysis of naturally conceived and IVF embryos and uterine tissues, observing temporal and spatial changes in early embryonic lipids.

3. Materials and methods, instrumentation

3.1. Human testicular tissues

We analyzed azoospermic testicular tissues with histological results ranging from 2 to 6-10 on the Johnsen scoring system (1-10), meaning we performed histological evaluation of samples showing normal or mildly impaired spermatogenesis and those with Sertoli cell-only syndrome. A total of 12 samples were analyzed: three displayed normal/slightly impaired spermatogenesis (Johnsen scores 6-10) and nine exhibited Sertoli Cell Only Syndrome (SCOS, Johnsen score 2) as per histopathological examination.

3.2. Animal uterine tissues

A total of 54 age-matched female CD-1 mice were randomly divided into two groups. To enhance ovulation, the female mice received hormone treatment (FSH, LH), after which they were mated. The first group of female mice (IVF group) was mated with infertile (vasectomized) males, while the second group (normal pregnant) was mated with fertile males of the same strain to induce pseudopregnancy and pregnancy, respectively. The mice were euthanized on specific days of pregnancy, and samples were collected from uterine tissues from both pregnant and pseudopregnant groups (natural and transferred embryos) at various embryonic stages.

3.3. Histological tissue sections

In case of human testicular tissues, the histological sections were prepared from samples obtained through surgical sperm retrieval at the Urology Clinic, stored at -80°C. The samples were embedded in a 3% carboxymethyl cellulose (CMC) solution, and 15 μm thick sections were cut using a Leica UV cryostat at -23°C for conventional pathological examinations. These sections were stained with Hematoxylin-eosin and then digitized using 3D Histech Pannoramic Desk digital slide scanner.

In case of animal uterine tissues for histological analysis, 4 μm thick FFPE (formalin-fixed paraffin-embedded) sections from mouse uteri were prepared using a Leica UV cryostat. Tissue sections were stained with Hematoxylin-eosin and analyzed with 3D Histech Pannoramic Desk digital slide scanner.

3.4. MALDI Imaging Mass Spectrometry

Imaging mass spectrometry (IMS) integrates histological information with mass spectrometric imaging technology. Fresh testis and uterus tissue samples were stored at –80° C before processing. Tissue was embedded in 2% carboxymethyl cellulose and sectioned at 15 µm using a Leica cryostat at –23° C, then thaw-mounted onto indium-tin-oxide-coated glass slides. Alpha-Cyano-4-hydroxycinnamic acid (CHCA) matrix was applied in 50 cycles with a 7 mg/mL solution made from 6:4 acetonitrile and 0.2% aqueous trifluoroacetic acid. Mass spectra were obtained using an Autoflex Speed MALDI TOF/TOF mass spectrometer in positive reflectron mode (m/z 400 to 3000). The lateral resolution was 50 µm, with a total of 300 laser shots per position. Data acquisition and evaluation were performed using FlexImaging 3.0 and FlexControl 3.4 software. Lipid identification utilized LIFT mode for PSD and CID fragmentation. Using the ClinProTools software, which is designed for the comparison of specific mass spectra, we performed basic statistical tests, discrimination analysis, and principal component analysis.

3.5. Lipid Identification by HPLC-MS/MS

This study utilized microdissections from the uteri of non-pregnant and pregnant mice to identify lipids. Lipid extraction from mouse uterus was performed using a modified Bligh and Dyer method. Tissue sections were homogenized with an extraction solution of chloroform/methanol/water (60/30/10 V/V%) containing 10 ng/ml BHT as an antioxidant. The process involved vortexing, ultrasound assistance, and multiple extraction phases to maximize lipid yield, followed by drying the extracts under nitrogen gas and reconstituting them in acetonitrile/isopropanol/water (65/30/5 V/V% in 0.1% formic acid).

Lipid profiles were identified using liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS). Analysis was conducted on a Dionex Ultimate 3000 HPLC system paired with a Q-Exactive mass spectrometer. The chromatographic separation utilized a Kinetex C-18 column with a flow rate of 150 µl/min and a sample injection volume of 5 µl. A binary gradient of methanol and water was employed to separate the compounds. MS and MS/MS measurements were conducted in positive ion mode.

4. Results

4.1. Histological results: human testicular tissues

The morphology of testicular tissue sections in normal functioning and SCOS conditions were examined. In the normally functioning testis, convoluted tubules showing complete spermatogenesis are found, with many type A and B spermatogonia (Johnsen score: 8-10). In the case of SCOS, normal, mature Sertoli cells and focal involuting Sertoli cells are present, with triangular nuclei (Johnsen score: 2).

4.2. Histological results: animal uterine tissues

In normal pregnancy, the implantation zones and embryos are symmetrically positioned at approximately equal distances. An equal number of embryos grow in both horns of the uterus, with a minimal number of spontaneous abortions.

In contrast, IVF embryos have an asymmetric shape, their positioning is more disorganized, their numbers differ between the two halves of the uterus, and the spontaneous abortion rate is high.

4.3. MALDI IMS results

4.3.1. Human testicular tissues

In this study, we utilized MALDI IMS to analyze human testicular tissues, focusing on lipid distributions. Tissue cryosections of 12 azoospermic patients with Johnsen scores of 2-10 were investigated by MALDI IMS.

Positive ion mode revealed prominent peaks for phosphatidylcholine (PC), sphingomyelin (SM), and phosphatidylethanolamine (PE) within the m/z range of 650 to 900.

The spatial distribution of lipids was assessed using mass spectrometric images, including protonated quasimolecular ions ($M+H^+$), along with sodium and potassium adducts ($M+Na^+$, $M+K^+$). Notably, the concentrations of SM (16:0) and (d18:1/16:0) were significantly higher

in samples with healthy spermatogenesis (Johnsen score 8-10) compared to those with SCOS (Johnsen score 2), where an increase in SM (d42:0) was observed.

Additionally, various types of PCs, including LPCs and diacyl PCs, exhibited differential distributions based on Johnsen scores, with higher levels found in samples showing unsuccessful spermatogenesis. Notable alterations in glycerophosphatidylcholine distributions were also identified. From the above, we can conclude a correlation between lipids and spermatogenic activity.

Plasmalogens, recognized as important ether phospholipids in spermatozoa, were highlighted for their crucial role in spermatogenesis and sperm functionality.

4.3.2. Animal uterine tissues

On embryonic day 6.5 of normal pregnancy, we found an increase in phosphatidylcholine (PC) 32:0 in uterine stromal cells at implantation sites except for the primary decidual zone (PDZ). PC 34:0, PC 34:1, and PC 34:2 showed higher expressions in uterine stromal cells at implantation sites, while PC 36:1 and PC 36:2 in the PDZ. In contrast, in transferred animals, the PCs showed an increase in glandular epithelia at interimplantation sites.

In normal pregnancy at day 8.5, specific phosphatidylcholines (PCs) demonstrated regional distribution, with PCs 32:0, 34:0, and 34:1 elevated in the mesometrial pole (M-pole), while PCs 34:2, 36:2, 36:4, 38:4, and 40:6 were more prominent in the antimesometrial pole (AM-pole). In transferred uterus samples, a similar trend was observed, but additional PCs showed higher expression in both poles.

By day 10.5, PC 32:0 was most abundant in the placenta, and several PCs (34:1, 34:2, 36:2, and 40:6) were elevated in the mesometrial decidua. Increased expression in transferred uterus samples indicated ongoing changes, with pronounced increases in PCs 36:2 and 38:4 at embryo absorption sites in the M-pole and AM-pole, respectively.

5. Discussion

5.1. Human testicular tissues

This study investigates lipidomic characteristics in normal and azoospermic human testicular tissues. Using MALDI TOF/TOF IMS, we analyzed the spatial distribution of phospholipids (PLs) including sphingomyelins (SM) and phosphatidylcholines (PC) in azoospermic cryosections.

The spatial expression levels of the investigated lipid species differed significantly between individuals with normal testicular function and those with SCOS. The results showed a significant correlation between lipid compositions and the Johnsen scoring system (1-10), which indicates the degree of spermatogenesis.

Key findings revealed down-regulation of certain monounsaturated and polyunsaturated diacyl-PC species in testicular samples with severe spermatogenic impairment (SCOS), alongside elevated levels of PC (16:0/20:4). SM (16:0) concentrations were notably lower in SCOS compared to normal spermatogenesis, suggesting disruption in SM biosynthesis pathways. Plasmalogens, which possess antioxidative properties, were also found to be down-regulated, indicating increased oxidative stress in SCOS samples.

Overall, our findings highlight the intricate relationship between lipid composition and male fertility, as indicated by Johnsen scores, emphasizing the potential of lipidomic profiling in diagnosing and understanding male infertility.

Our results may facilitate the identification of spermatogenic areas, potentially increasing the success rates of testicular sperm extraction (TESE) procedures.

5.2. Animal uterine tissues

In our study, PC34:1 and PC36:1, which are predominantly OA-containing, exhibited high intensities in different parts of the uterus during the peri-implantation period in both normal and IVF pregnancies. However, the accumulation of these lipids differed between the two groups. For instance, PC36:1 was observed in the mesometrial decidua in IVF pregnancy but was almost absent in normal pregnancy. This striking difference suggests the possible role of

OA-containing lipids in early embryonal life, which may play a pathobiochemical role in early embryonal development in IVF.

The polyunsaturated fatty acid-containing PCs demonstrated both spatial and temporal differences during the peri-implantation period in both groups. It is noteworthy that the greatest discrepancies between the normal and IVF groups were observed at embryonic day 8.5 in the intensity of these PC lipids (PC36:2, PC36:4, PC38:4, and PC 40:6).

Our results underscore the significance of lipid composition in the peri-implantation period and its potential influence on IVF outcomes.

Our method may facilitate a deeper understanding of the mechanisms of implantation processes and enhance reproductive success rates through targeted lipidomic approaches.

6. Summary and novel findings

- We have developed a MALDI IMS protocol for the analysis of testicular tissues, through which we identified and localized characteristic phospholipids in the testes, enabling us to infer whether spermatogenesis is pathological or normal.

- Based on thecurrent literature, we were the first to demonstrate a correlation between the Johnsen scoring system and phospholipid expressions in testicular tissues of patients suffering from Sertoli cell-only syndrome (SCOS).

- To the best of our knowledge, we are the first to utilize MALDI IMS to detect and compare significant changes in phosphatidylcholines during early embryonic development in an IVF model.

7. Acknowledgements

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8. List of publications

8.1. Publications related to the thesis

- MALDI imaging mass spectrometry reveals lipid alterations in physiological and Sertoli cell-only syndrome human testicular tissue sections

  https://doi.org/10.3390/ijms25158358

  Authors: Alexandra Sulc, Péter Czétány, Gábor Máté, András Balló, Dávid Semjén, Árpád Szántó, László Márk

  Journal: International Journal of Molecular Sciences, 2024

  Impact factor: 4,9

  Citation: 0

- Investigation of phosphatidylcholine by MALDI imaging mass spectrometry in normal and IVF early-stage embryos

  https://doi.org/10.3390/ijms25137423

  Authors: Stefania Gitta, Éva Szabó, Alexandra Sulc, Péter Czétány, Gábor Máté, András Balló, Tímea Csabai, Árpád Szántó, László Márk

  Journal: International Journal of Molecular Sciences, 2024

  Impact factor: 4,9

  Citation: 0

8.2. List of other publications

- Application of Mass Spectrometry Imaging in Uro-Oncology: Discovering Potential Biomarkers

  https://doi.org/10.3390/life12030366

  Authors: Czétány Péter, Stefánia Gitta, András Balló, Alexandra Sulc, Gábor Máté, Árpád Szántó, László Márk

  Journal: Life, 2022

  Impact factor: 3,2

  Citation: 3

- Histological Study of Some Echium vulgare, Pulmonaria officinalis and Symphytum officinale Populations

  https://doi.org/10.1177/1934578X1100601017

  Authors: Nóra Papp, Tímea Bencsik, Kitti Németh, Kinga Gyergyák, Alexandra Sulc, Ágnes Farkas

  Journal: Natural Product Communications, 2011

  Impact factor: 1,37

  Citation: 4

9. Presentations

- Biospektroszkópia-tömegspektrometria 1:0

  Márk László, Sulc Alexandra

  XV.Környezetvédelmi Analitikai és Technológiai Konferencia és 63. Magyar Spektrokémiai Vándorgyűlés

  Balatonszárszó, 2024. március 6.-8.

- Spermtyper és Andromics: Andrológiai minták multimodális analitikai vizsgálata

  Gitta Stefánia, Sulc Alexandra, Márk László

  Magyar Andrológiai Társaság XV. Kongresszusa,

  Kecskemét, 2023. november 23.-25.

- Molekulák térben és időben: képalkotási tömegspektrometria alkalmazása a humán reprodukció kutatásában

  Márk László, Sulc Alexandra, Gitta Stefánia

  Magyar Andrológiai Társaság XIV. Kongresszusa,

  Kecskemét, 2022. szeptember 22.-24.

- Lipidomikai változások SCOS hereszövetekben

  Sulc Alexandra, Bánkúti Stefánia, Czétány Péter, Balló András, Máté Gábor, Szántó Árpád, Márk László

  Magyar Andrológiai Társaság XVI. Kongresszusa

  Eger, 2024. november 23.