Apoptosis Markers in Follicular Fluid: A Comparative Study Between Infertile Women and Oocyte Donors

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Resumo

A infertilidade é uma condição clínica multifatorial que afeta milhões de casais em todo o mundo. A infertilidade feminina representa 30% dos casos e as causas mais comuns incluem idade materna avançada, disfunções ovulatórias, endometriose, anomalias uterinas e fatores ambientais ou genéticos. Como consequência, um número crescente de casais recorre às Técnicas de Reproduçao Medicamente Assistida, como a injeção intracitoplasmática de espermatozoides, que consiste na injeção de um espermatozoide no oócito. Durante esta técnica o liquido folicular e as células do cúmulos, ambos essenciais para o desenvolvimento e maturação do oócito, são descartados. No entanto, considerando a sua proximidade com o oócito, a análise do líquido folicular e das células do cúmulos torna-se particularmente promissora. Estudos recentes, sugerem que mulheres com disfunção ovulatória apresentam maior dano nas células do cúmulos, quando comparadas com mulheres férteis. Danos no DNA desencadeiam frequentemente a apoptose, um processo que elimina de forma controlada células danificadas. Assim, levantou-se a hipótese de que a apoptose constitua um dos mecanismos subjacentes que contribuem para a infertilidade feminina. Contudo, o papel específico deste processo na infertilidade femenina permanece pouco explorado.

Assim, o objetivo desta tese foi avaliar se a atividade da enzima desidrogenase lática (LDH) no líquido folicular poderia refletir danos celulares associado a apoptose ou outros processos de morte celular, sendo explorada como um marcador indireto da viabilidade celular em contexto de infertilidade feminina.

Inicialmente, procedeu-se à otimização do protocolo em que foram testadas varias diluições do líquido folicular (não diluído; 3:4; 1:2; 1:4; 1:5; 1:6) para garantir a sensibilidade adequada e a correta deteção dos níveis de LDH, bem como a normalização pela quantificação proteica através do método BCA. Após a otimização, o protocolo foi implementado em 32 amostras: 15 do grupo controlo (grupo 1) e 17 do grupo de inférteis (grupo 2).

As amostras não diluídas e as com menores diluições apresentaram saturação do sinal. As diluições 1:4, 1:5 e 1:6 foram as que apresentaram maior sensibilidade e nenhuma saturação, e por isso foram selecionadas. A normalização proteica revelou melhores resultados quando as amostras foram tratadas sem SDS, proporcionando uma quantificação mais fidedigna, devido à natureza extracelular das proteínas presentes no

líquido folicular. Aquando da análise dos níveis de LDH, não foram observadas diferenças estatisticamente significativas entre os grupos estudados, incluindo numa subpopulação de mulheres com disfunção ovulatória. Este resultado sugere que a medição da LDH, um marcador associado sobretudo à necrose celular, pode não ser suficiente para detetar processos apoptóticos no líquido folicular. A heterogeneidade clínica da população inférteis, assim como o pequeno número de amostras, poderá ter contribuído para a ausência de diferenças significativas. Este estudo evidencia as limitações da quantificação de LDH como único marcador de apoptose e enfatiza a necessidade de incorporar biomarcadores adicionais específicos para apoptose e stress oxidativo, para uma análise mais precisa da viabilidade celular e da fisiopatologia da infertilidade.

Palavras-chave: Infertilidade, líquido folicular, células do cumulus, apoptose.

Abstract

Infertility is a multifactorial clinical condition that affects millions of couples worldwide. Female infertility accounts for 30% of cases, and the most common causes include advanced maternal age, ovulatory dysfunctions, endometriosis, uterine abnormalities, and environmental or genetic factors. As a result, an increasing number of couples turn to medically assisted reproduction techniques, such as intracytoplasmic sperm injection, which involves injecting a sperm cell directly into the oocyte. During this procedure, the follicular fluid and cumulus cells, both essential for the development and maturation of the oocyte, are discarded. However, given their proximity to the oocyte, analyzing follicular fluid and cumulus cells becomes particularly promising. Recent studies suggest that women with ovulatory dysfunction exhibit greater damage to cumulus cells compared to fertile women. DNA damage often triggers apoptosis, a process that selectively eliminates cells that are damaged. Thus, it has been hypothesized that apoptosis is one of the underlying mechanisms contributing to female infertility. However, the specific role of this process in female infertility remains underexplored.

Thus, the aim of this thesis was to evaluate whether the activity of the enzyme lactate dehydrogenase (LDH) in follicular fluid could reflect cellular damage associated with apoptosis or other cell death processes, being explored as an indirect marker of cell viability in the context of female infertility.

Initially, the protocol was optimized by testing various dilutions of follicular fluid (undiluted, 3:4, 1:2, 1:4, 1:5, 1:6) to ensure adequate sensitivity and proper detection of LDH levels, as well as normalization through protein quantification using the BCA method. After optimization, the protocol was applied to 32 samples: 15 from the control group (group 1) and 17 from the infertile group (group 2).

Undiluted samples and those with the lowest dilutions showed signal saturation. The 1:4, 1:5, and 1:6 dilutions demonstrated the highest sensitivity without saturation and were therefore selected. Protein normalization yielded better results when samples were treated without SDS, providing more accurate quantification due to the extracellular nature of the proteins present in follicular fluid. When analyzing LDH levels, no statistically significant differences were observed between the studied groups, including in a subpopulation of women with ovulatory dysfunction. This result suggests that measuring LDH, a marker mainly associated with cell necrosis, may not be sufficient to

detect apoptotic processes in follicular fluid. The clinical heterogeneity of the infertile population, as well as the small sample size, may have contributed to the lack of significant differences. This study highlights the limitations of quantifying LDH as the sole marker of apoptosis and emphasizes the need to incorporate additional specific biomarkers for apoptosis and oxidative stress for a more precise analysis of cell viability and the pathophysiology of infertility.

Keywords: Infertility, follicular fluid, cumulus cells, apoptosis.

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List of Abbreviations

UP	UNIVERSITY OF PORTO
FCUP	FACULTY OF SCIENCE OF THE UNIVERSITY OF PORTO
ICBAS	SCHOOL OF MEDICINE AND BIOMEDICAL SCIENCES
AFC	ANTRAL FOLLICLE COUNT
AMH	ANTI-MUELLERIAN HORMONE
ART	ASSISTED REPRODUCTIVE TECHNOLOGIES
ATP	ADENOSINE TRIPHOSPHATE
BCA	BICINCHONINIC ACID
BMP15	BONE MORPHOGENETIC PROTEIN 15
BSA	BOVINE SERUM ALBUMIN
CCS	CUMULUS CELLS
COC	CUMULUS OOCYTE COMPLEX
CX	CONNEXINS
DMSO	DIMETHYL SULFOXIDE
DOR	DIMINISHED OVARIAN RESERVE
EVS	EXTRACELLULAR VEHICLES
FF	FOLLICULAR FLUID
FMR1	FRAGILE X MENTAL RETARDATION 1
FMRP	FRAGILE X MENTAL RETARDATION PROTEIN
FSH	FOLLICLE-STIMULATING HORMONE
EXPOI	FRAGILE X-ASSOCIATED PRIMARY OVARIAN INSUFFICIENCY
GDF9	GROWTH DIFFERENTIATION FACTOR 9
HAS2	HYALURONAN SYNTHASE 2
ICSI	INTRACYTOPLASMIC SPERM INJECTION

IVF	IN VITRO FERTILIZATION
LDH	LACTATE DEHYDROGENASE
LH	LUTEINIZING HORMONE
MTDNA	MITOCHONDRIAL DNA
OAT	OLIGOASTHENOTERATOZOOSPERMIA
OXPHOS	OXIDATIVE PHOSPHORYLATION
PBS	PHOSPHATE-BUFFERED SALINE
PCOS	POLYCYSTIC OVARY SYNDROME
PFK	PHOSPHOFRUCTOKINASE
PGCS	PRIMORDIAL GERM CELLS
PID	PELVIC INFLAMMATORY DISEASE
POI	PRIMARY OVARIAN INSUFFICIENCY
ROS	REACTIVE OXYGEN SPECIES
SDS	SODIUM DODECYL SULFATE
TGF-B	TRANSFORMING GROWTH FACTOR-BETA
UFI	UTERINE FACTOR INFERTILITY
WHO	WORLD HEALTH ORGANIZATION

Introduction

1. Infertility

Infertility is a clinical condition that affects millions of couples worldwide. According to the World Health Organization (WHO), infertility is defined as the inability to conceive after 12 months of unprotected sexual intercourse (Organization, 2024) (Bala et al., 2021; Carson & Kallen, 2021). Global statistics indicate that infertility affects approximately 186 million couples worldwide (Inhorn & Patrizio, 2015). Among these cases, around 30% to 40% are attributed exclusively to female factors, while male factors are solely responsible for roughly 20% to 30% of infertility cases and contribute to about 50% of all cases. (Vander Borght & Wyns, 2018). In approximately 10% to 20% of cases, the causes are mixed, involving both partners. In addition, it is estimated that 30% of infertile couples globally are diagnosed with unexplained or idiopathic infertility (Wasilewski et al., 2020). This condition is characterized by the absence of an identifiable cause despite comprehensive clinical evaluation, which complicates the diagnosis and treatment. However, prevalence varies significantly across countries, shaped by cultural factors, access to reproductive healthcare, and sexual education. Fertility can be influenced by a wide range of factors, including age, acute or chronic conditions, environmental toxins, occupational exposures, general lifestyle issues, infectious diseases, genetic conditions, and specific reproductive disorders that can affect men or women (Bala et al., 2021).

In women, this complexity is further amplified by the regulation of the reproductive system. Female infertility results from various factors that can compromise reproductive capacity. Among the leading causes are ovulatory disorders, such as polycystic ovary syndrome (PCOS) and primary ovarian insufficiency (POI) (Vander Borght & Wyns, 2018). Additionally, conditions such as endometriosis, uterine abnormalities, advanced maternal age, environmental factors, lifestyle factors, and genetic factors are also frequently associated with female infertility (Buck et al., 1997). Understanding these factors is crucial, as they can significantly impact a woman's ability to conceive and for proper treatment. The following chapter will explore these causes in detail, highlighting their mechanisms and potential implications for fertility.

1.1. Causes of Female Infertility

1.1.1. Pathophysiological Causes

A. Ovulatory Disorders

Ovulatory disorders are one of the leading causes of female infertility, accounting for approximately 25% of diagnosed cases (Carson & Kallen, 2021). These disorders occur when oocyte maturation or ovulation is irregular or absent. Polycystic Ovary Syndrome (PCOS) is a prevalent condition in this group, affecting 70% of women who experience anovulation. (Carson & Kallen, 2021). PCOS is a common endocrine disorder characterized by anovulation, hirsutism, and polycystic ovaries, and is frequently associated with insulin resistance, dyslipidemia, and obesity. Beyond its reproductive effects, PCOS also increases the risk of cardiovascular and metabolic conditions, including type 2 diabetes and metabolic syndrome (Azziz et al., 2016). Another important ovulatory disorder is Premature Ovarian Insufficiency (POI), characterized by premature ovarian failure before the age of 40 (Kirshenbaum & Orvieto, 2019). It is marked by the interruption of menstrual cycles (amenorrhea) for at least 4 months, accompanied by elevated gonadotropin levels. The prevalence of POI increases with age, affecting 1 in 1,000 women by age 30 and 1 in 100 by age 40 (Kirshenbaum & Orvieto, 2019). Although the cause remains undefined in most cases, possible etiologies include iatrogenic factors, infections, environmental factors, metabolic conditions (type 1 diabetes), endometriosis, autoimmune disorders, and genetic factors (Ishizuka, 2021).

B. Endometriosis

Another significant condition that can lead to female infertility is endometriosis. Endometriosis is a chronic, estrogen-dependent gynecological disorder characterized by the presence of endometrial-like tissue outside the uterine cavity, commonly affecting the ovaries, fallopian tubes, peritoneum, and other pelvic structures (Ata & Somigliana, 2024; Qi et al., 2025). It is one of the leading causes of infertility, affecting approximately 10% of women of reproductive age (Park et al., 2025). The mechanisms through which endometriosis leads to infertility remain unclear. This ectopic endometrial tissue responds to hormonal cycles similarly to the normal endometrium, undergoing proliferation and shedding, which leads to chronic inflammation, fibrosis, and the formation of adhesions and ovarian endometriomas. The disease is strongly associated with elevated local and systemic estrogen levels, which contribute to its progression (Park et al., 2025; Rathod et al., 2024). Endometriosis is a multifactorial condition involving oxidative stress, immune system dysregulation, genetic and epigenetic alterations, enhanced cell proliferation and invasion, and abnormal angiogenesis

(Dymanowska-Dyjak et al., 2024). These factors not only perpetuate the inflammatory environment but also disrupt the normal function of the reproductive organs, impairing ovarian reserve, oocyte quality, embryo implantation, and tubal transport, all of which contribute to infertility(Rathod et al., 2024).

C. Tubal

Tubal infertility is one of the leading causes of female infertility, accounting for approximately 30% of all diagnosed infertility cases in women (Dun & Nezhat, 2012). This condition results from damage, obstruction, or abnormalities in the fallopian tubes that prevent the proper transport of the oocyte to the fertilization site or hinder the migration of the embryo to the uterus. Tubal infertility may result from several clinical factors; among them, sexually transmitted infections such as *Chlamydia trachomatis* and *Neisseria gonorrhoeae* are the most prevalent causes (Dun & Nezhat, 2012; Ling et al., 2022). These pathogens are frequently asymptomatic and, when left untreated, can lead to pelvic inflammatory disease (PID), which promotes chronic inflammation and tubal fibrosis (Chua et al., 2017). PID is the leading cause of tubal pathology, responsible for more than 50% of cases, and can affect different regions of the fallopian tubes (Honoré et al., 1999). Other contributing factors include previous pelvic or abdominal surgeries, endometriosis, postoperative adhesions, and prior ectopic pregnancies—all of which may compromise tubal patency and impair normal function (Dun & Nezhat, 2012).

D. Diminished Ovarian Reserve

Diminished Ovarian Reserve (DOR) is often linked to a decrease in the number or quality of oocytes and is the leading cause of female infertility. This condition frequently results in reduced responsiveness to ovarian stimulation and lower success rates in assisted reproductive technologies (ART). Clinically, DOR is characterized by decreased antral follicle count (AFC) (which consists of the number of antral follicles in both ovaries) or decreased anti-Muellerian hormone (AMH) (Zhang et al., 2023). The main symptoms of DOR are menstrual changes including cycle disorder, shortened period, hypomenorrhea, and even amenorrhea or infertility (Zhu et al., 2023). DOR can have multiple etiologies, including genetic, autoimmune, idiopathic, and iatrogenic. With advancing age, both the quantity and quality of oocytes diminish. Beyond chronological aging, factors such as ovarian surgery, chemotherapy, radiotherapy, and genetic mutations can accelerate the depletion of ovarian reserve. All these factors can contribute to women's infertility.

E. Uterine Factors

Uterine factor infertility (UFI) is estimated to affect up to 1 in 500 women of reproductive age. However, the true prevalence remains unclear, as many cases go undiagnosed (Hur et al., 2019). The causes of UFI can be broadly classified into two categories: congenital (present from birth) and acquired (developed over time). The most common congenital uterine anomaly is the arcuate uterus, characterized by a shallow indentation (<1 cm) in the uterine cavity between the tubal ostia. Another frequent anomaly is the uterine septum, a fibrous or muscular partition dividing the uterine cavity (Hur et al., 2019). These malformations can interfere with embryo implantation and increase the risk of miscarriage, and they are estimated to affect around 4% of the general population. Acquired uterine abnormalities are more common and may emerge due to hormonal, inflammatory, or iatrogenic factors. These include endometrial polyps, uterine fibroids or myomas, adenomyosis and Asherman's syndrome (Bouko-Levy et al., 2023). These conditions can lead to impaired endometrial receptivity, menstrual irregularities, and subfertility. Together, these congenital and acquired conditions can significantly impact the ability of the uterus to support conception and maintain a healthy pregnancy.

1.1.2. Genetic Causes

The most well-known genetic cause of female infertility is the premutation in the *fragile X mental retardation 1* (*FMR1)* gene. *FMR1* premutation carriers, with 55 to 200 CGG trinucleotide repeats, are at an increased risk of developing Fragile X-associated primary ovarian insufficiency (FXPOI) (Fink et al., 2018). Approximately 20% of women who carry *FMR1* gene premutation experience POI, whereas this occurs in only 1% of women in the general population (Agustí et al., 2024). Women with the *FMR1* premutation exhibit increased mRNA levels in granulosa cells, which is associated with reduced FMRP synthesis. This negative feedback mechanism can induce ovarian damage mechanisms, which are clinically manifested as a diminished ovarian reserve (França & Mendonca, 2022). However, the exact molecular mechanisms by which the *FMR1* gene contributes to POI still need to be fully understood. In FXPOI and other cases of POI, disruptions in ovarian function due to genetic factors can induce further changes in the follicular microenvironment (Crawford et al., 2001). These include oxidative stress, inflammation, and hormonal imbalances, negatively affecting oocyte quality (Chen et al., 2023).

1.1.3. Environmental and Lifestyle Factors

A. Advanced Maternal Age

Advanced maternal age is a factor that has a significant impact on female infertility. As a woman ages, the quantity and quality of oocytes decrease, leading to lower fertilization rates and higher rates of spontaneous miscarriages. It is well established that maternal age is positively correlated with the decline in oocyte quality and an increased risk of chromosomal abnormalities in oocytes and embryos (Pathare et al., 2023).

B. Environmental Factors

Environmental factors significantly influence female fertility. Exposure to heavy metals can lead to hormonal imbalances and increased cellular damage in reproductive tissues (Manouchehri et al., 2022). Pesticide exposure results in adverse health effects, some of which include breathing problems, headaches, neurological problems, skin problems, immune system dysfunctions, cancer, and reproductive defects. Heavy metals can cause infertility in couples through various mechanisms, including egg impact. Additional exposure to radiation (ionizing or microwaves), heat, stress, mental disorders, cigarette chemicals, respiratory pollutants (such as lead), insecticides, pesticides, anesthetic gases, mercury, and cytotoxic drugs may also contribute to infertility.

C. Lifestyle Factors

Additionally, lifestyle factors such as smoking, alcohol consumption, chronic stress, and extreme weight variations directly affect oocyte quality and the functioning of the reproductive system (Buck et al., 1997).

2. Assisted Reproductive Technologies (ARTs)

All the previously described causes of infertility may lead couples to seek assisted reproductive technologies (ARTs). These comprise a set of medical procedures designed to increase the likelihood of achieving pregnancy in the context of infertility. Among the most commonly used techniques are in vitro fertilization (IVF), which consists of fertilizing the oocyte with the sperm in a laboratory setting, and intracytoplasmic sperm injection, in which a single sperm is directly injected into the oocyte. The latter technique was used in the context of the present study and will be addressed in more detail in the following chapter.

2.1. Intracytoplasmic Sperm Injection

Due to the variety of factors contributing to infertility, many couples resort to ART to overcome challenges in natural conception and improve their chances of achieving pregnancy. One of the most used ART techniques is Intracytoplasmic Sperm Injection (ICSI). This technique involves the injection of a single sperm into the oocyte's cytoplasm, ensuring fertilization. The process begins with ovarian stimulation using hormonal treatment to promote the development of multiple follicles, thereby increasing the chances of successful fertilization (Palermo, 1992). Once the follicles reach an appropriate size, the oocytes are retrieved through a procedure called follicular aspiration (Joris et al., 1998).

After collection, the cumulus cells and follicular fluid are usually discarded following the removal of the cumulus-oocyte complex (COC) through a washing and denudation process (Palermo, 1992). Then, a single sperm is carefully injected into the cytoplasm of each oocyte. After fertilization, the resulting embryos are cultured in the laboratory for several days, allowing their development to be closely monitored. The most viable embryos are then selected and transferred into the woman's uterus, where implantation may occur, potentially leading to pregnancy. During this procedure, the FF and the CCs are typically discarded, as they are not necessary for the clinical steps of assisted reproduction. However, for research purposes, both CCs and FF were collected.

3. Physiology of the Ovarian Follicle

The ovarian follicle is a highly dynamic environment, where granulosa cells, cumulus cells, and the oocyte interact to regulate follicular development and oocyte competence. FF serves as a medium for the exchange of nutrients, hormones, and signalling molecules, playing a pivotal role in creating optimal conditions for oocyte development. By analysing CCs and FF, it is possible to assess the molecular and metabolic environment within the follicle, which can offer valuable insights about follicular health, oocyte quality, and potential causes of infertility.

3.1. Structure and Development of the Ovarian Follicle

The ovarian follicle is a complex structure containing the developing oocyte and several cellular layers. It plays a central role in the female reproductive cycle, providing the necessary environment for oocyte development and maturation. Its structure and the interactions among its various components are essential to ensure oocyte competence from the beginning of folliculogenesis to ovulation (Telfer et al., 2023). Folliculogenesis refers to the process by which ovarian follicles develop and mature, starting during fetal development with the establishment of primordial follicles (Telfer & Andersen, 2021).

This process starts with primordial germ cells (PGCs) migrating from somatic cells to the gonadal ridge, where they create nests of oogonia (Figure 1). PGCs serve as the germline progenitors in all mammals, having the potential to develop into either oocytes or spermatogonia based on the surrounding gonadal environment and somatic cells (Biason-Lauber & Chaboissier, 2015). With differentiation, the oogonia undergo a defined period of proliferation before entering meiosis, which is arrested at prophase I in a resting state known as the diplotene (dictyate) stage (He et al., 2021). Then, these oocytes establish connections with somatic cells (granulosa cells), and an intact basal lamina encloses the unit to form the primordial follicle, creating a store of germ cells (the primordial pool). At this stage, primordial follicles are maintained in a state of growth arrest, characterized by low transcriptional and translational activity, which is essential for preserving chromosomal stability and prolonging reproductive life until they are activated and recruited for growth (Jamnongjit & Hammes, 2005).

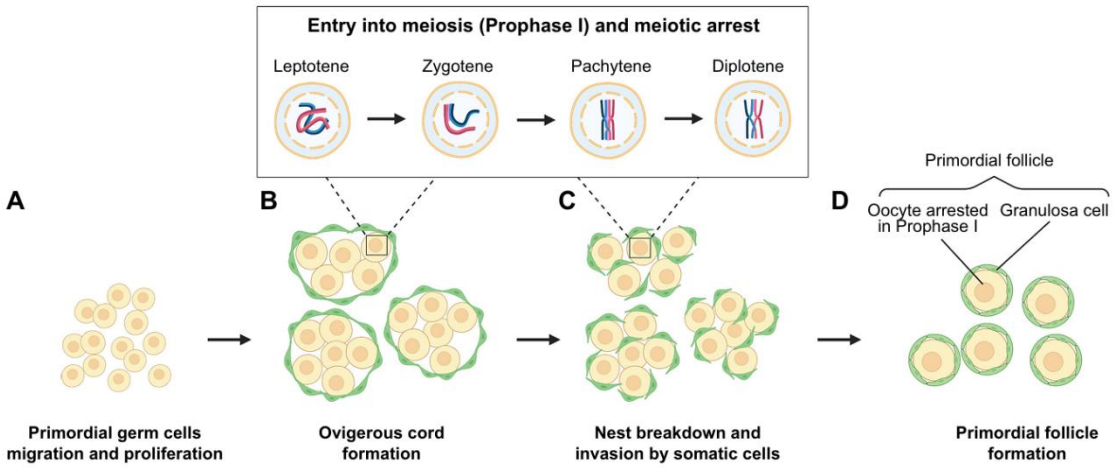


Figure 1- Early stages of ovarian follicle development.

The schematic illustrates primordial germ cell migration and proliferation (A), ovigerous cord formation (B), nest breakdown and invasion by somatic cells (C), and primordial follicle formation (D). The inset depicts oocyte progression through the stages of meiotic prophase I (leptotene, zygotene, pachytene, diplotene) and arrest. Adapted from Telfer et al., 2023, *Making a Good Egg: Human Oocyte Health, Aging, and In Vitro Development*. Used with permission under the Creative Commons Attribution CC-BY 4.0 license.

The activation of follicles begins as soon as the follicle population is established, and follicle growth increases before puberty (Figure 2). The early stages of follicle development are regulated by paracrine factors produced by the surrounding somatic cells. (Da Broi et al., 2018) In contrast, the later stages, typically composed of multiple layers, are controlled by gonadotropins, such as luteinizing hormone (LH) and follicle-stimulating hormone (FSH) (Jamnongjit & Hammes, 2005; Telfer et al., 2023). During puberty, the activation of the neuroendocrine axis promotes growth and hormone production of follicles, which is regulated by gonadotropin-releasing hormone from the hypothalamus in the brain.

The differentiation of granulosa cells marks the transition from primordial follicles to primary follicles, creating a single layer of cuboidal-shaped cells that enclose the oocyte. Subsequently, granulosa cells proliferate, creating multiple layers, while adjacent ovarian stromal cells develop into theca cells that encase the follicle (Rimon-Dahari et al., 2016). This process signifies the transition to a secondary/pre-antral follicle [25]. This step is crucial, as theca cells start to produce androgens that granulosa cells convert into estrogens (Telfer & Andersen, 2021). Regulated by a complex of paracrine factors, secondary follicles develop multiple layers of granulosa cells while the surrounding theca cells differentiate (Palma et al., 2012). This process also involves angiogenesis, resulting in the formation of blood vessels that are essential for further follicle development and ovulation (Fiorentino et al., 2023).

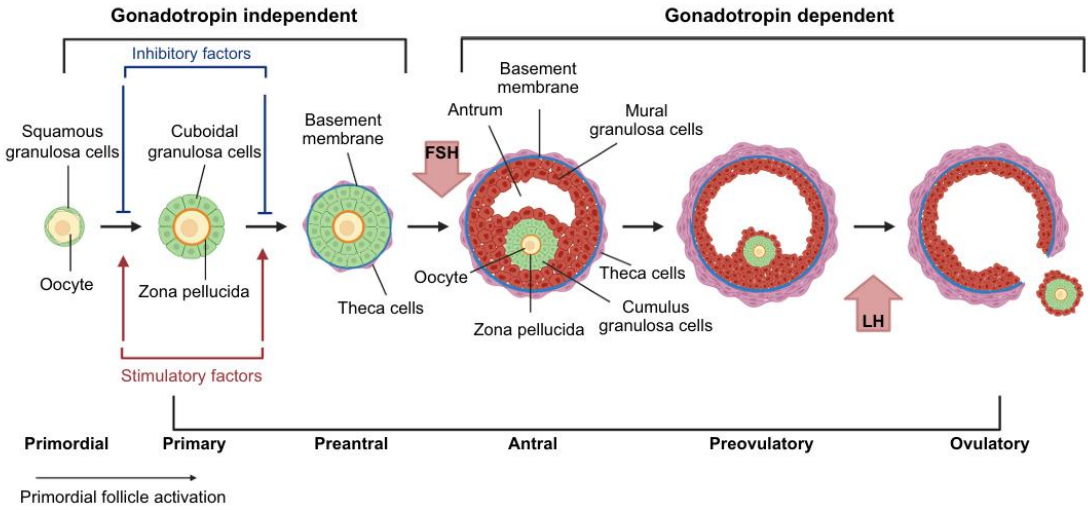


Figure 2- Follicle growth progresses from primordial to preovulatory stages.

Primordial follicles develop into primary follicles, then preantral follicles with multilaminar granulosa and thecal cells. Antral follicles form a fluid-filled cavity, releasing the oocyte-cumulus complex at ovulation via LH signaling. Early stages are FSH-independent, but later stages depend on FSH. Adapted from Telfer et al., 2023, *Making a Good Egg: Human Oocyte Health, Aging, and In Vitro Development*. Used with permission under the Creative Commons Attribution CC-BY 4.0 license

In this phase, the oocyte is enveloped by the zona pellucida, as granulosa cells and the oocyte produce mucopolysaccharides, which create a dense layer of glycoproteins and proteoglycans between the oocyte and granulosa cells (Fiorentino et al., 2023). At the same time, the oocyte increases in size and exhibits heightened transcriptional activity. It begins to release TGF-β family members such as Growth Differentiation Factor 9 (GDF9) and Bone Morphogenetic Protein 15 (BMP15) (Rimon-Dahari et al., 2016). These substances from the oocyte trigger the primary to secondary transition, contribute to the formation of TZPs, and encourage the proliferation and expansion of granulosa cells (Knight & Glister, 2003). GDF9 promotes the growth of human primordial follicles to the secondary stage, improves follicular survival, regulates steroid hormone production, and manages genes involved in cumulus cell expansion (Vitt & Hsueh, 2001). BMP15 is vital in the early stages of follicular development and is closely linked to the proliferation of granulosa cells. In the later stages, it affects FSH-driven differentiation of granulosa cells, controls cumulus metabolism and expansion, and enhances the developmental capacity of oocytes (Knight & Glister, 2003). In response to the cumulative effects of GDF9 and BMP15, granulosa cells undergo active proliferation and express receptors for FSH, estrogen, and androgens, which become more pronounced as follicles develop.

The third stage of follicular growth, called the proliferative phase, is characterized by the creation of a fluid-filled cavity known as the antral follicle, which forms as the multilaminar follicle achieves a specific size. This phase marks the beginning of antral development, during which the follicle increasingly relies on endocrine regulation, especially by gonadotropins like FSH and subsequently LH.

During antral development, the follicle undergoes significant morphological and functional changes. FSH promotes the proliferation and differentiation of granulosa cells, which form two distinct subtypes: CCs, surrounding the oocyte and creating the COC, and mural granulosa cells, located at the follicle's periphery in contact with the basal lamina (Rimon-Dahari et al., 2016).

CCs establish bi-directional communications with the oocyte through gap junctions, facilitating the exchange of molecules such as cAMP, cGMP, and nutrients necessary for oocyte growth and maturation. These cells also drive cumulus expansion, a vital process for ovulation, influenced by factors including HAS2, PTGS2, and TNFAIP6, which are stimulated by the LH surge (Filatov et al., 2017). In contrast, mural granulosa cells primarily adopt an endocrine function, expressing high levels of FSH receptors and, during the pre-ovulatory phase, LH receptors. They are the main contributors to estrogen production through the aromatization of androgens provided by theca cells. At the same time, theca cells develop from the surrounding ovarian stroma and form the inner and outer thecal layers. These cells have LH receptors and steroidogenic enzymes, which are crucial for androgen production and offer structural support to the growing follicle (Filatov et al., 2017).

The dominant follicle is selected during the final stage of antral development. Initially, multiple follicles are stimulated by FSH, but as estradiol levels rise, negative feedback on the anterior pituitary decreases FSH secretion. This hormonal decrease leads to a functional selection process: only the follicle with the highest sensitivity to FSH, increased receptor expression, and strongest estrogen production survives atresia and continues to develop; this is the dominant follicle (Rimon-Dahari et al., 2016). The other follicles in the group undergo degeneration through a process of programmed cell death, known as apoptosis. The LH surge triggers a series of events that result in ovulation. (Filatov et al., 2017). LH influences mural granulosa and theca cells, facilitating luteinization and molecular changes that promote meiotic resumption, cumulus expansion, and follicular rupture, which results in the release of the COC into the fallopian tube. Furthermore, LH regulates essential genes related to nuclear maturation and oocyte competence through both direct intracellular signaling and paracrine

mechanisms (Filatov et al., 2017). The decrease of cGMP within the oocyte triggers PDE3A, reduces cAMP levels, and ultimately activates the MPF complex, enabling meiosis to progress to metaphase II (He et al., 2021). This precise and coordinated control among the oocyte, CCs, and mural granulosa cells is essential to ensure meiotic competence and the developmental potential of the oocyte after fertilization. The integrity of this communication network is a significant determinant of reproductive success.

3.2. Cumulus Cells

Cumulus cells (CCs) are specialized granulosa cells that differentiate to establish direct contact with the oocyte, together forming the COC (Turathum et al., 2021). This complex enables bidirectional communication between the oocyte and CCs, which is essential for the proper development and function of the COC. This communication is facilitated by cytoplasmic projections from the CCs that pass through the zona pellucida to form gap junctions with the oocyte membrane. Gap junctions are intercellular membrane channels composed of connexins (Cx), a family of integral membrane proteins that facilitate the direct transfer of low-molecular-weight molecules, such as ions, metabolites, amino acids, and intracellular signaling molecules, from the CCs to the oocyte (Del Bianco et al., 2024). CCs play multiple essential roles in ensuring reproductive success. In addition to providing physical support to the oocyte, they supply energy substrates and trophic signals that promote both nuclear and cytoplasmic maturation. One of the most extensively studied functions is the metabolic co-dependence between the oocyte and CCs.

Oocytes have a limited ability to uptake glucose and perform glycolysis, largely due to their low expression of phosphofructokinase (PFK), a key rate-limiting enzyme in the glycolytic pathway. Therefore, CCs metabolize glucose through glycolysis, generating adenosine triphosphate (ATP) and the products of glycolysis, including pyruvate and lactate. The glycolytic products are then supplied to the oocyte, which metabolizes them to generate ATP via oxidative phosphorylation, promoting oocyte competence. To facilitate the supply of glycolytic products from CCs [49], oocytes secrete paracrine factors, such as GDF-9 and BMP-15, which induce the expression of primary glycolytic genes (Del Bianco et al., 2024).

Cumulus expansion is a function of CCs that takes place in response to the LH surge. This expansion involves the synthesis and secretion of hyaluronic acid, primarily mediated by enzymes such as hyaluronan synthase 2 (HAS2). The process leads to the loosening and outward dispersion of CCs, facilitating the release and subsequent

capture of the oocyte by the fallopian tube (Massoud et al., 2024). Additionally, CCs are sensitive to changes in the follicular environment, particularly to the presence of reactive oxygen species (ROS). While ROS play a physiological role at low levels, their excessive accumulation is a major contributor to oxidative damage, primarily through their interaction with key cellular macromolecules such as DNA, proteins, and lipids, leading to processes like lipid peroxidation. This oxidative stress can severely impair mitochondrial function, resulting in reduced ATP production, structural damage, and activation of apoptotic pathways. In the context of reproduction, such disruptions negatively affect both oocyte and embryo development. Increased mitochondrial ROS can also damage mitochondrial DNA (mtDNA), which encodes essential components of the OXPHOS system. To counteract these harmful effects, cells have developed antioxidant defense mechanisms that aim to maintain redox balance and preserve cellular integrity (Kobayashi et al., 2024).

In recent years, CCs have garnered increasing attention as potential biomarkers of oocyte competence due to their close association with the oocyte and their accessibility following IVF procedures. Several studies have shown that the expression levels of specific genes in CCs are correlated with fertilization success, embryo quality, and implantation rates (Gebhardt et al., 2011).

In addition, techniques such as the comet assay have made it possible to identify changes in CCs that may compromise their function and, consequently, oocyte quality. These findings are particularly relevant in the context of female infertility, as altered cellular profiles can be observed in women with ovulatory dysfunction, advanced maternal age, or reproductive syndromes (Rodrigues et al., 2024).

CCs play a central role in the acquisition of oocyte competence, not only by providing physical and metabolic support, but also by reflecting the physiological state of the follicular microenvironment. Analysing these cells provides valuable insights into oocyte quality and represents a promising approach for diagnosing and treating infertility.

3.3. Follicular Fluid

Follicular Fluid (FF) is a nutrient-rich liquid that fills the follicular antrum and acts as a complex mediator of the microenvironment, facilitating communication between germ and somatic cells. It contains hormones, proteins, anticoagulants, electrolytes, ROS, antioxidants, and a diverse range of immune system cells, supporting essential reactions for oocyte growth. (Chen et al., 2023) . Additionally, compounds resulting from the biological activity of cells and local metabolic processes within the follicle are also present in the FF (Brinca et al., 2024).

FF has two origins: one part is derived from blood plasma, which crosses the blood follicle barrier, while the other is metabolites from the follicular wall, which are modified by theca and granulosa cells (Pan et al., 2024). The blood follicle barrier consists of a series of structures that regulate selective permeability, allowing the passage of small molecules. (Siu & Cheng, 2013) FF is considered a direct reflection of the metabolism and health of the oocyte and surrounding follicular cells. Alterations in the composition of FF may indicate pathologies that affect fertility (Pan et al., 2024).

FF plays several vital roles in supporting oocyte growth and maturation. The oocyte is the largest cell in the human body and requires large amounts of energy. FF provides glucose and fatty acids (energy substrates), which CCs and the oocyte metabolize to generate energy (Fiscus et al., 2024). FF's glucose and fatty acid metabolism balance is essential for maintaining energy homeostasis during oocyte maturation. Studies show that a well-balanced energy metabolism in the follicular environment is directly linked to improved oocyte quality (Warzych & Lipinska, 2020).

Another function of FF is to protect against oxidative stress. FF contains enzymatic and non-enzymatic antioxidants, such as glutathione, vitamin E, and catalase, which neutralize ROS. Oxidative stress is a harmful factor that can damage the DNA, proteins, and cell membranes of the oocyte. The antioxidant capacity of FF is crucial to ensure the integrity of the oocyte during its development (Zec et al., 2022).

Additionally, FF acts as a transport medium for hormones and growth factors that regulate oocyte maturation and CCs' function. Hormones such as FSH and LH, as well as paracrine factors like TGF-β, play fundamental roles in inducing cumulus expansion and preparing the oocyte for ovulation (Da Broi et al., 2018).

4. Biomarkers

In human reproduction, considerable efforts have been made to identify biomarkers capable of assessing the follicular microenvironment, oocyte viability, and factors associated with infertility; however, a definitive and universally accepted biomarker has yet to be established (Revelli et al., 2009). Recent findings suggest that increased DNA damage and apoptotic activity in CCs may be linked to poor fertilization outcomes in infertile women (Rodrigues et al., 2024). DNA damage often triggers apoptosis, a process that selectively eliminates cells that are damaged. Thus, it has been hypothesized that apoptosis is one of the underlying mechanisms contributing to female infertility in infertile women. However, the specific role of this process in female infertility remains underexplored. Apoptosis naturally occurs during development and aging, functioning as a homeostatic process that maintains stable cell populations in tissues (Elmore, 2007). It also occurs as a defense mechanism, such as in immune reactions or when cells are damaged by disease or harmful agents. However, a wide variety of stimuli and conditions, both physiological and pathological, can trigger apoptosis. Considering these concepts, the evaluation of apoptosis in FF represents a promising approach to better understand the relationship between the oocyte microenvironment and its reproductive outcomes. In this study, the lactate dehydrogenase was chosen as a biomarker in FF due to its well-established role as an indicator of cell membrane integrity loss, commonly associated with necrosis. Although this marker isn't specific to apoptosis, its presence in follicular fluid may indicate ongoing cellular damage, including late-stage apoptosis or necrosis, thereby serving as an indirect marker of altered follicular health.

4.1. Lactate Dehydrogenase

Lactate dehydrogenase (LDH) is a cytoplasmic enzyme present in nearly all cell types (Technologies, 2023). Its detection in extracellular fluids is widely recognized as an indicator of compromised cell membrane integrity, which is a hallmark event in both apoptosis and necrosis. Due to these characteristics, LDH has become a valuable biomarker for assessing cell death in environments where intact cells are not available for direct analysis. The LDH assay is based on the enzymatic conversion of pyruvate to lactate (Zaib et al., 2022) with simultaneous oxidation of NADH to NAD^+. In this reaction, NADH reduces a water-soluble tetrazolium salt (WST), leading to the formation of an orange formazan dye. The amount of formazan produced is proportional to the concentration of LDH released into the medium, thus serving as an indirect indicator of cytotoxicity and cell death (Terry L Riss, 2013).

Aims

The present thesis focuses on evaluating whether the activity of the LDH in follicular fluid could reflect cellular damage associated with apoptosis or other cell death processes, serving as an indirect marker of cell viability in the context of female infertility.

Specific aims of the present work include:

1. To optimize the LDH quantification protocol in FF
2. To optimize the BCA protein assay for accurate normalization of LDH results, ensuring reliable comparison across samples.
3. To implement the optimized protocols in FF samples from infertile women and a control group (oocyte donors).
4. To compare LDH levels with reproductive parameters
5. To compare LDH levels between the two groups, including the analysis of a subgroup that is clinically relevant.

Methods

1. Study Population

This study included 32 women, comprising 15 from the control group (Group 1, with a mean age of 31.7) and 17 from the infertile group (Group 2, with a mean age of 35.8). All samples had been previously collected following ethical approval as part of a PhD project.

The group of infertile women was aged between 31 and 39 years and exhibited a predominantly multifactorial etiology of infertility. Endometriosis was the most prevalent condition, affecting 9 out of 17 participants (53%), and was often associated with other factors such as tubal factor (n=3), adenomyosis (n=2), diminished ovarian reserve (n=1), and von Willebrand disease (n=1). In two cases, endometriosis was also linked to repeated in vitro fertilization (IVF) failure. Ovulatory disorders were observed in seven participants (41%), appearing as an isolated cause in three cases and in combination with obesity, polycystic ovary syndrome (PCOS), and anti-thyroid antibodies (n=1), uterine abnormalities (n=2), or tubal factor (n=1). Tubal factor was identified in four women, but occurred as an isolated cause in only one case. Uterine factors were present in three participants, two of whom also exhibited ovulatory and oocyte-related abnormalities. Adenomyosis was found in two women, both in association with other factors. Oocyte-related issues were involved in four cases, never as a sole cause, and were commonly associated with uterine, tubal, or endometriosis-related factors. These findings highlight the multifactorial nature of female infertility in this cohort, underscoring the overlap of gynecological, endocrine, and immunological factors in the clinical profiles of the patients included.

Participants eligible for the study were non-smoking women undergoing ICSI) treatment. To mitigate age-related confounding variables, participants were paired by age during the study. Before the initiation of ovarian stimulation, baseline clinical and demographic information was collected, including age and ovarian reserve markers, day 3 FSH (in all participants), AMH, and AFC (in a subset of participants, as shown in Table 1).

Table 1 - Comparison of clinical and laboratory parameters between two groups of women undergoing controlled ovarian stimulation.

	Group 1 (n =15)	**Group 2** (n = 17)	**p-value**
Age (years)	31.7 ± 5.0	35.8 ± 2.5	0,01*
Markers of ovarian reserve			
Day 3 FSH (mUI/ml)	12.6 ± 10.0	6.5 ± 1.5	0,06
AMH (ng/ml)	12.4 ± 14.5	26.2 ± 20.2	0,02*
AFC	10.0 ± 3.9	7.2 ± 3.8	0,15
Response to ovarian stimulation			
Total dose of gonadotrophins (IU/ml)	2585.0 ± 620.3	3173.5.3 ± 1307.5	0,20
Stimulation duration (days)	10.1 ± 2.0	11.2 ± 2.7	0,21
Number of oocytes retrieved	16.0 ± 6.1	11.6 ± 10.4	0,14
Oocyte maturation			
Number of injected MII oocytes	9.4 ± 2.3	7.1 ± 6.4	0,08
Number of imature oocytes	2.5 ± 2.1	2.2 ± 2.9	0,41
Number of abnormal and degenerated oocytes	0.5 ± 0.6	1.7 ± 2.9	0,09
Fertilization success			
Number of oocytes with 2PN	5.1± 2.5	5.6 ± 4.8	0,63

Results are presented as mean ± standard deviation (SD). FSH- Follicle-Stimulating Hormone; AMH - *Anti-Müllerian Hormone; AFC - Antral Follicle Count; MII - Metaphase II oocytes;* Sample sizes used unless stated otherwise; Values are presented as mean ± S.D., where applicable.

Participation in the study was completely voluntary. All women were provided with clear and comprehensive information regarding the study's purpose, procedures, and any associated risks and benefits. Sufficient time was allotted for questions, and only after confirming that participants fully understood everything did they sign the informed consent form. It was underscored that their medical care would not be influenced by their choice to participate and that all collected data would be kept confidential and used solely for research purposes. Informed consent was obtained in the early stages of ovarian

stimulation to ensure it coincided with the timing of biological sample collection. On the day of follicular aspiration, FF and CCs were gathered from each participant. All samples were anonymized with coded identifiers and stored under suitable conditions for later analysis. The Ethics Committee of the ULSSA approved the study (approval number: 2020.119/097-DEFI/099-CE), which was conducted in accordance with both institutional and national ethical guidelines.

2. Sample Collection

Out of the 32 women, CCs and FF had been previously collected at the Center for Medically Assisted Reproduction of the Centro Materno-Infantil do Norte Dr. Albino Aroso (CMIN-ULSSA), which is part of the Santo António Local Health Unit (ULSSA) in Porto, Portugal.

These samples are typically discarded during standard clinical procedures; however, due to their relevance, they were collected. During the ICSI process, transvaginal follicular aspiration was performed under ultrasound guidance to retrieve mature oocytes. Following aspiration, FF was collected directly from the follicles and aliquoted into sterile tubes. The samples were centrifuged to remove cellular debris, and the supernatant was separated from the cellular pellet and stored at –80 °C until further analysis.

After oocyte retrieval, the oocytes were washed and denuded, and the CCs are then collected and preserved in DMEM/F-12 medium supplemented with GlutaMAX™ (Gibco™, Thermo Fisher Scientific, Waltham, MA, USA). For cryopreservation, the CCs were suspended in DMEM/F-12 with GlutaMAX™ supplemented with 20% dimethyl sulfoxide (DMSO) (Invitrogen, Thermo Fisher Scientific), transferred to Mr. Frosty Freezing Containers (Thermo Fisher Scientific), and stored at –80°C for 24 hours. Subsequently, all samples were transferred to liquid nitrogen for long-term storage until they were used in the experiment.

3. Sample Processing

On the day of the assay, CCs samples were taken from liquid nitrogen storage and gently thawed in a water bath at 37°C. FF samples were taken from -80°C storage and gradually thawed on ice at 4°C. Once fully defrosted, the protocol procedures begin.

4. Cell Quantification

Cell quantification was performed using a Neubauer chamber on CCs and FF collected from a single sample in both groups. The cell suspensions were diluted in a 1:1 ratio with 0.4% Trypan Blue (Sigma-Aldrich, St. Louis, MO, USA), resulting in a final 1:2 dilution. A 10 µL aliquot of this mixture was carefully transferred into a Neubauer hemocytometer for analysis under a light microscope (Olympus BX41). Cell viability was assessed based on Trypan Blue exclusion, where viable cells excluded the dye and remained unstained, while non-viable cells exhibited membrane permeability and absorbed the stain. The ratio of viable to non-viable cells was determined, providing a relative measure of cell viability cell counts were conducted across four quadrants of the counting chamber, and the total number of cells was estimated by applying the appropriate dilution factor and considering the calibrated chamber volume. This enabled the determination of cell concentration in the original, undiluted suspension.

5. Optimization of LDH Assay

To identify dilution(s) that preserved assay sensitivity while avoiding signal saturation or elevated background noise five FF samples (3 samples from the Group 1 and 2 samples from de group 2) were diluted with phosphate-buffered saline (PBS) at different dilution factors: 3:4, 1:2, 1:4, 1:5, and 1:6. The three best dilutions (1:4, 1:5, and 1:6) were subsequently selected for use in all subsequent experiments, as they allowed for the accurate measurement of enzymatic activity without compromising the assay's integrity.

6. Protein Extraction Optimization

Given that LDH is released into the extracellular environment upon cell damage, it became essential to normalize the results according to both total protein content and sample volume. For this purpose, the BCA assay was chosen as the normalization method due to its reliability and compatibility with protein extracts. Since this approach had not yet been applied to follicular fluid samples, it was necessary to optimize the extraction protocol. As part of this optimization, two conditions were tested: with and without the addition of Sodium Dodecyl Sulfate (SDS). Initially, the samples were centrifuged at 14,000 × g for 20 minutes at 4°C. Subsequently, the supernatant was carefully removed to ensure that only 25 µL of the remaining volume, representative of the cellular pellet, was left in the Eppendorf tube, following standard procedures commonly used in protein extraction protocols. The supernatant was reserved directly for further quantification without the addition of SDS. For the condition with SDS, 25 µL of 2% SDS solution (1:1 ratio) was added to the 25 µL pellet and incubated on ice for 20

minutes to enhance protein solubilization. Then, the samples were centrifuged, and the supernatant was collected for subsequent protein quantification.

7. Implementation of LDH Assay

The Cytotoxicity LDH Assay (Kit-WST, Dojindo, CK12) was performed in 32 FF samples (Control Group, *n* = 15; Study Group, *n* = 17). This assay is based on the ability of LDH released from damaged cells to convert lactate into pyruvate while simultaneously reducing NAD^+ to NADH. NADH then reduces a water-soluble tetrazolium salt (WST) in the presence of an electron mediator, resulting in the formation of an orange formazan dye. The amount of formazan dye formed is proportional to the amount of LDH released, indicating the level of cytotoxicity. For the assay, 100 µL of each FF sample was added to each well of a flat-bottom 96-well culture plate. Then, the samples were diluted in PBS and tested at the 3 stablished different dilutions: 1:4, 1:5, and 1:6. Then, 100 µL of the Working Solution was added to each well. The plate was protected from light and incubated at 37 °C for 30 minutes. After incubation, 50 µL of the Stop Solution was added to each well to stop the reaction. Absorbance was measured at 490 nm using a microplate reader (BioTek Synergy H1 Plate Reader).

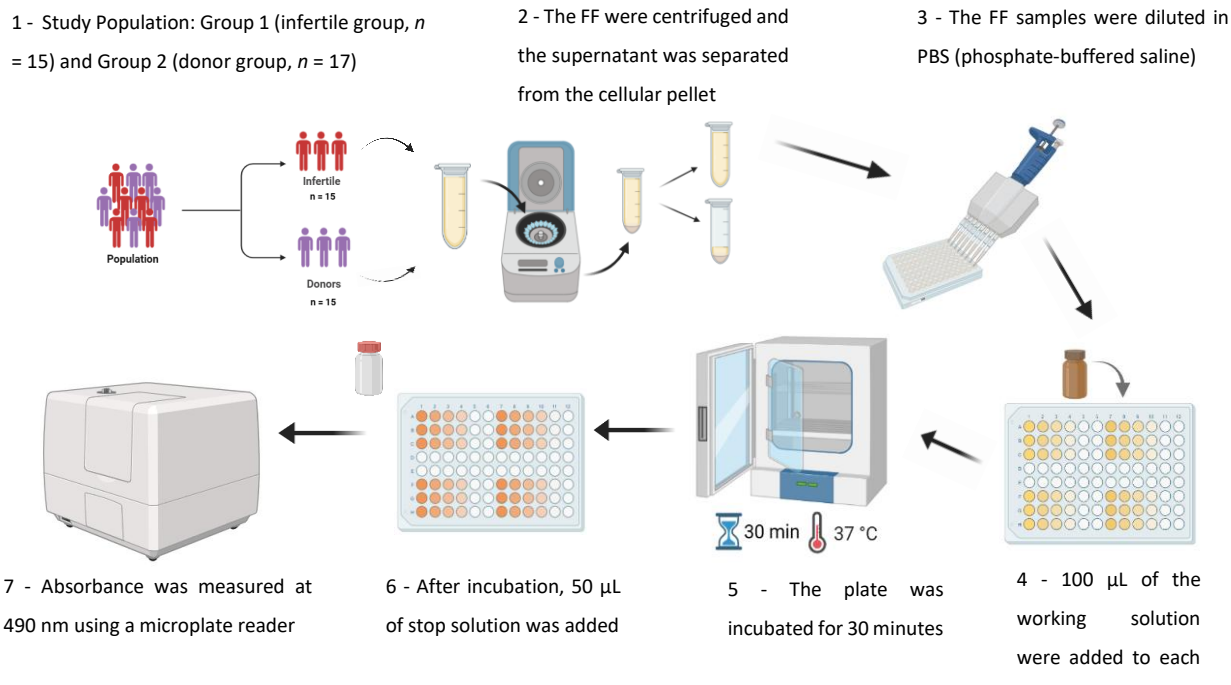


Figure 3 - Workflow of the LDH experimental protocol.

This protocol was applied for the analysis of follicular fluid (FF) samples from two groups: oocyte donors (n = 15) and infertile women (n = 17). (1) Samples were collected from both groups. (2) The FF was centrifuged to separate the supernatant from the cellular pellet. (3) The samples were diluted in phosphate-buffered saline (PBS). (4) 100 µL of the working solution was added to each well. (5) The plate was incubated at 37 °C for 30 minutes. (6) After incubation, 50 µL of stop solution was added. (7) Absorbance was measured at 490 nm using a microplate reader.

8. Protein Quantification

Total protein quantification of the samples was performed using the Pierce™ BCA Protein Assay Kit (Thermo Scientific, Cat. No. 23225), according to the manufacturer's instructions. This colorimetric assay is based on the biuret reaction and the reduction of Cu^{2+} to Cu^{+} by proteins in an alkaline medium, followed by the colorimetric detection of the Cu^{+} using bicinchoninic acid (BCA).

Briefly, a set of bovine serum albumin (BSA) standards was prepared by serial dilution from the stock standard provided with the kit (2.0 mg/mL BSA) to obtain a standard curve ranging from 25 to 2000 µg/mL. The working reagent was prepared by mixing Reagent A and Reagent B in a 50:1 ratio, as recommended by the protocol. A volume of 10 µL of each standard and sample (diluted as necessary to fall within the standard curve range) was added in triplicate into a 96-well flat-bottom microplate. Subsequently, 200 µL of the working reagent was added to each well. The plate was incubated at 37 °C for 30 minutes. After incubation, the absorbance was measured at 562 nm using a microplate spectrophotometer (BioTek Synergy H1 Plate Reader). A standard curve was then generated by plotting absorbance values of the BSA standards versus protein concentration, and the total protein concentration of each sample was determined by interpolation. All protein concentrations were used to normalize LDH values, allowing for correction of differences in total protein content between samples.

9. Statistical Analysis

To compare fertility-related outcomes, *t*-test were conducted. When the data failed normality, Mann-Whitney test were employed.

Pearson correlation coefficient was used to assess the linear correlation between LDH levels and fertility-related outcomes. All statistical analyses were performed using SigmaPlot version 14.0 (Systat Software® Inc., Chicago, IL, United States). A statistical significance level of 0.05 was used for all tests.

Results and Discussion

1. Cell Quantification

Cell quantification was performed on two representative samples from each of the two groups (Groups 1 and 2). The results showed that oocyte donors exhibited a significantly higher count of CCs (3,960,000 cells) compared to infertile women (180,000 cells). Consistent with these findings, no cells were detected in the FF from infertile women. Although the sample size was limited, this disparity suggests potential differences in follicular health or oocyte quality, given the critical role of cumulus cells in supporting oocyte development and maturation.

In addition, the variable number of cells in both CCs and FF, particularly in infertile women, raised concerns about the availability of sufficient cells to perform assays such as flow cytometry and immunohistochemistry, which were initially proposed to assess apoptosis and cellular markers. These techniques require a high number of viable and structurally preserved cells to ensure accurate and reproducible analysis.

Therefore, it was necessary to adapt our strategy to align with our objectives and sample limitations by focusing on the analysis of an extracellular biomarker.

To this end, the activity of LDH in follicular fluid was evaluated as a potential indicator of cellular damage associated with apoptosis or other cell death processes, being explored as an indirect marker of cell viability in the context of female infertility.

In this study, we selected LDH as a biomarker in FF due to the unavailability of CCs, which were separated and discarded during sample processing by centrifugation at the time of collection. Although LDH is traditionally considered a marker of necrosis, its release into the extracellular environment reflects a loss of membrane integrity, which may occur under conditions of excessive or dysregulated cell death, including late-stage apoptosis. Therefore, in the absence of direct access to CCs, LDH measurement in FF provides an indirect yet informative approach to assess cellular damage within the follicular environment, potentially offering insights into the apoptotic and necrotic processes associated with infertility.

2. Optimization of the LDH Protocol

To ensure the reliable measurement of LDH activity, 6 dilutions were tested in five FF samples (group 1, n = 3 ; group 2, n = 2). The undiluted FF, as well as the 3:4 and 1:2 dilutions, exhibited absorbance values at the microplate reader's maximum limit (4000), indicating signal saturation and rendering these conditions unsuitable for accurate measurement. Starting from the 1:4 dilution, absorbance values progressively decreased and fell within the optimal range for measurement. Among these, the 1:4, 1:5, and 1:6 dilutions demonstrated sufficiently high absorbance, clear differentiation between samples, and no saturation, establishing them as the most appropriate dilutions for the assay (Chart 1). Consequently, subsequent analyses were performed using these selected dilutions (1:4, 1:5, and 1:6).

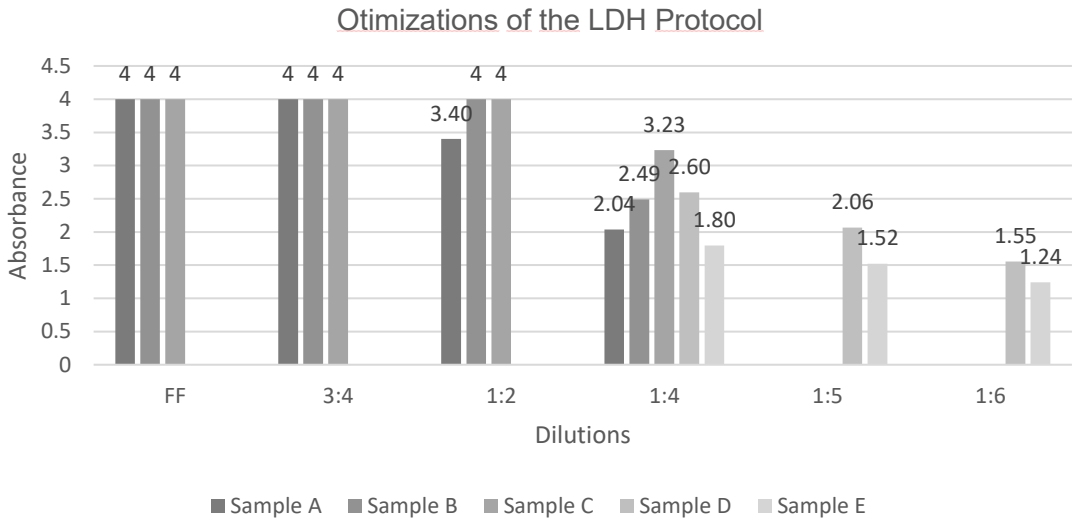


Chart 1- Optimization of the LDH protocol using follicular fluid (FF) samples from both experimental groups.

Five samples (Sample A and Sample B – infertile group; Sample 1, Sample 8 and Sample 9 – donor group) were tested under different dilution conditions (undiluted, 3:4, 1:2, 1:4, 1:5, and 1:6). Absorbance values were measured to determine the optimal dilution for reliale LDH activity assessment.

These findings align with previous reports by Huyser et al. (1992), who demonstrated the necessity of diluting FF with saline solution to mitigate interference from contaminants such as blood, which can significantly affect absorbance readings and biomarker levels, including LDH (Huyser et al., 1992). Implementing this dilution strategy allowed for more accurate and reproducible sample analysis. Thus, the choice of 1:4, 1:5, and 1:6 dilutions in this study was critical to ensure the precision and reliability of LDH activity quantification in FF.

3. Optimization of Protein Quantification

The absence of cellular content in FF made conventional normalization methods based on cell counts unusable. Although standard protein quantification methods often include an extraction step to solubilize cellular proteins, the lack of detectable cells in the FF samples raised questions about whether this step was relevant or necessary.

To address this, a parallel analysis was conducted:

- **Condition 1**: Samples treated with SDS (sodium dodecyl sulfate) extraction buffer for protein release;
- **Condition 2**: Untreated samples subjected to direct analysis without SDS.

The results demonstrated that the total protein content in FF was relatively similar in both conditions, regardless of SDS usage. In the samples where protein quantification was performed directly, without SDS treatment, the measured concentrations were slightly higher, with values of 5.05 µg and 4.77 µg, respectively. Conversely, when the same samples were treated with SDS for protein extraction, the measured concentrations were 4.49 µg and 4.15 µg, respectively (Chart 2). Although the differences are not drastic, this outcome suggests that SDS treatment, typically employed to solubilize proteins from cellular components, may interfere with protein quantification in cell-free biological fluids such as FF.

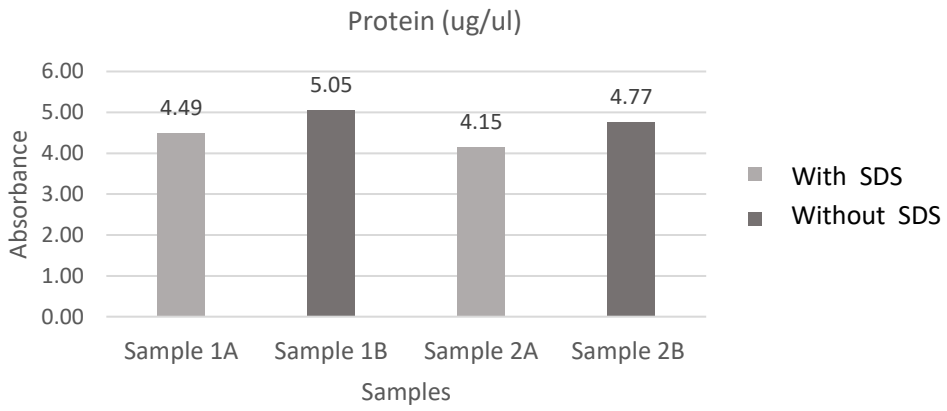


Chart 2- Comparison of total protein concentration (µg) in follicular fluid samples with and without SDS-based protein extraction.

Group 1: Sample 1; Group 2: Sample 2. Samples 1A and 2A were treated with SDS before quantification, while samples 1B and 2B were analyzed directly, without SDS. Protein concentration was measured by absorbance.

This outcome aligns with the biological characteristics of FF, where the absence of cells suggests that most proteins are already present in the extracellular environment, secreted by CCs or the oocyte. As a result, cell lysis is unnecessary for protein release.

Consistente with yhis findings, Svetlana Uzbekova et al. investigated the protein content of extracellular vehicles (EVs) present in bovine follicular fluid, highlighting that many of the proteins found in FF are actively secreted by follicular cells, including CCs and oocytes, through EVs. This suggests that these proteins are available in the extracellular environment without the need for cell lysis (Uzbekova et al., 2020).

Overall, the use of an SDS-based extraction protocol not only proved unnecessary but also led to a reduction in the measured protein concentration. Direct quantification of non-extracted samples is therefore the most suitable and accurate approach for assessing the native protein composition of the follicular environment.

Supporting this approach, Mittenbühler et al. demonstrated that direct analysis of extracellular fluids from muscle and adipose tissues, without extraction procedures, enables the identification of low-abundance bioactive proteins that are often lost in classical cellular extraction protocols (Mittenbühler et al., 2023). These findings highlight the importance of directly quantifying proteins secreted into the extracellular environment, especially in physiological contexts where cellular activity is reflected by protein secretion, such as in FF. Based on these results, all subsequent protein quantification procedures were performed using native, non-extracted samples.

4. LDH/BCA Implementation

After protocol optimization, the LDH/BCA ratio was applied to assess protein-normalized LDH activity in both the control group (Group 1, n = 15) and the infertile group (Group 2, n = 17). Each sample was analysed under the 3 preestablished dilutions (1:4, 1:5, and 1:6). The resulting LDH/BCA ratios for each sample are detailed in Table 2. This comprehensive approach allowed for a systematic comparison of LDH activity between groups and across dilution parameters.

Table 2 - Relative LDH activity (LDH/BCA) under three dilution conditions (1:4, 1:5, and 1:6).

Samples	LDH/BCA		
	1:4	1:5	1:6
Sample 1	0.54	0.43	0.30
Sample 2	0.42	0.31	0.27
Sample 3	0.42	0.34	0.29
Sample 4	0.43	0.39	0.33
Sample 5	0.39	0.33	0.27
Sample 6	0.29	0.26	0.22
Sample 7	0.37	0.32	0.27
Sample 8	0.57	0.46	0.37
Sample 9	0.55	0.48	0.39
Sample 10	0.43	0.37	0.29
Sample 11	0.33	0.31	0.27
Sample 12	0.34	0.27	0.25
Sample 13	0.40	0.34	0.27
Sample 14	0.58	0.48	0.42
Sample 15	0.29	0.22	0.20
Sample 16	0.33	0.26	0.23
Sample 17	0.39	0.30	0.26
Sample 18	0.28	0.24	0.23
Sample 19	0.49	0.41	0.34

Sample 20	0.39	0.32	0.30
Sample 21	0.42	0.32	0.32
Sample 22	0.42	0.33	0.29
Sample 23	0.49	0.41	0.32
Sample 24	0.71	0.56	0.43
Sample 25	0.29	0.24	0.19
Sample 26	0.27	0.22	0.20
Sample 27	0.37	0.33	0.28
Sample 28	0.37	0.33	0.28
Sample 29	0.34	0.29	0.25
Sample 30	0.41	0.33	0.27
Sample 31	0.44	0.35	0.30
Sample 32	0.42	0.37	0.31

Each value represents the ratio of LDH activity to total protein content (BCA assay) in follicular fluid samples from 15 donors and 17 samples from infertile women.

To contextualize these findings, it is essential to consider the physiological role of LDH and its behaviour in distinct cellular contexts. LDH is a cytosolic enzyme released into the extracellular environment upon loss of plasma membrane integrity, and it is generally associated with necrotic processes. During early and mid-apoptotic phases, plasma membrane integrity is largely preserved, and LDH release is minimal or absent. Thus, LDH assays seem to be more suitable for detecting necrosis rather than apoptosis (Chan et al., 2013).

Overall, the absence of elevated LDH levels in FF does not exclude the occurrence of apoptosis in the cells that originally constituted the follicular microenvironment. Indeed, previous studies have used LDH activity as an indirect marker of cell integrity and viability, though its utility is often enhanced when combined with other biomarkers [51], (Ansari et al., 2022).

The choice of the LDH assay in this study was driven by the need to identify a marker that could be directly quantified in the FF, since the CCs were separated during sample processing. The FF does not contain cells, which made it impossible to apply other conventional apoptosis detection techniques that require cellular material, such as immunofluorescence or flow cytometry.

In addition to this limitation, factors such as the collection time and sample processing may have affected the stability of the LDH enzyme, potentially compromising the reliability of the results. Moreover, differences in the cellular composition of the FF, particularly the unknown proportion of CCs, mural granulosa cells, or other structures present, may influence the amount of LDH detected. Additionally, the clinical heterogeneity of the group of infertile women, which included women with different etiologies such as PCOS, endometriosis, POI, and idiopathic infertility. Each of these conditions is associated with distinct pathophysiological mechanisms, potentially leading to individual variations in LDH release. This clinical diversity can lead to individual variations, making it challenging to identify significant differences when the samples are analysed collectively. Therefore, analysing the infertile group as a homogeneous group may obscure relevant differences that would only emerge through a stratified analysis.

In summary, while the LDH/BCA assay did not reveal significant differences between oocyte donors and infertile women, this result does not rule out the presence of apoptotic or other forms of cell death in the follicular environment. The findings highlight the complexity of interpreting LDH data in FF and underscore the need for complementary biomarkers and stratified analyses to better understand the relationship between the follicular environment and infertility.

5. LDH Levels with Reproductive Parameters

With the aim of exploring a possible association between LDH levels in follicular fluid and different reproductive parameters, a correlation analysis was performed for two distinct groups: infertile women (LDH infertile) and the control group (LDH control). Table 3 presents the correlation coefficients (r), the values of statistical significance (p-value), and the sample size (n) for each analyzed variable.

Table 3 - Correlation coefficients between LDH levels in follicular fluid and reproductive parameters in the control and infertile groups

	Group 1		**Group 2**	
	R2	**p-value**	**R2**	**p-value**
Age (years)	-0.35	0.20	0.31	0.23
Markers of ovarian reserve				
Day 3 FSH (mUI/ml)	0.04	0.94	-0.33	0.21
AMH (ng/ml)	-0.17	0.55	0.02	0.94
AFC	0.21	0.55	0.25	0.48
Response to ovarian stimulation				
Total dose of gonadotrophins (IU/ml)	-0.32	0.25	-0.33	0.20
Stimulation duration (days)	-0.22	0.44	0.20	0.43
Number of oocytes retrieved	0.44	0.10	0.40	0.13
Oocyte maturation				
Number of injected MII oocytes	-0.59	0.16	0.11	0.70
Number of imature oocytes	0.38	0.16	0.07	0.80
Number of abnormal and degenerated oocytes	**-0.01**	0.98	0.16	0.57
Fertilization success				
Number of oocytes with 2PN	-0.55	0.20	0.11	0.70

The correlation coefficients (r), statistical significance values (p-value), and sample sizes (n) are presented for each analyzed variable. No statistically significant correlations ($p > 0.05$) were identified between LDH levels and the evaluated parameters.

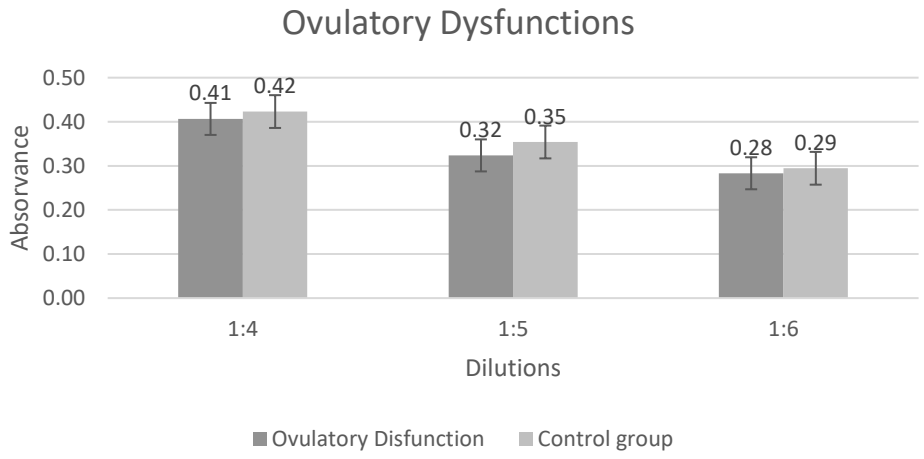
As shown in the table, no statistically significant correlations ($p > 0.05$) were observed between LDH levels and the analyzed parameters in either group. This includes classic indicators of ovarian reserve, such as age, day 3 FSH, AMH, and antral follicle count (AFC), as well as variables related to ovarian stimulation, oocyte maturation, and fertilization rate.

Despite some apparent moderate correlations, such as the number of MII oocytes injected ($r = -0.59$) and the number of oocytes with 2PN ($r = -0.55$) in the control group, these did not reach statistical significance. The same applies to the infertile group, where, although r reached 0.43 for the fertilization rate, the p-value (0.13) indicates no significant correlation. These results suggest that LDH levels alone are not clearly associated with the main reproductive markers, at least within the sample and methodology used in this study. However, the hypothesis that LDH, in combination with other markers or under different experimental conditions, may have clinical or biological relevance cannot be ruled out.

6. Ovulatory Dysfunctions

To address the clinical heterogeneity within the infertile cohort, the LDH assay analysis was repeated using a subset of five samples (Samples 21, 22, 29, 30, and 31) from women with ovulatory dysfunction. Absorbance values under the 3 dilutions (1.4, 1.5, and 1.6) were compared between this subgroup and the control group (Chart 3).

Absorbance values for five selected samples (21, 22, 29, 30, and 31) are shown. These samples were selected due to



Charts 3 - LDH/BCA values in follicular fluid samples from women with ovulatory dysfunction.

LDH/BCA values were analysed in relation to a specific clinical condition (ovulatory dysfunction), allowing a more accurate interpretation of LDH activity levels. Bars represent mean values ± standard deviation.

The control group exhibited slightly higher absorbance values across all dilutions compared to the ovulatory dysfunction subgroup. The absence of significant differences in LDH release between the groups may indicate that there are no changes in cellular necrosis levels in the analysed samples. It is important to note, that the limited sample size (n=5) may have reduced the power to detect subtle, yet biologically relevant, differences. Overall, results suggest that the ovulatory dysfunction present in the women included in this study does not appear to be associated with increased necrosis in CCs. Nonetheless, the absence of elevated LDH does not rule out the possibility of apoptosis, as early apoptotic events typically do not result in significant LDH release due to preserved plasma membrane integrity. Consequently, the LDH assay is insufficient for detecting apoptosis. This limitation highlights the necessity of incorporating additional, more specific biomarkers (e.g., activated caspase-3 or Annexin V staining) to fully evaluate cell death mechanisms within the follicular microenvironment (Awonuga et al., 2023; Qi et al., 2023).

The follicular environment in infertile women may exhibit elevated levels of reactive oxygen species (ROS), disturbances in hormonal signalling, and inflammatory mediators. These factors can negatively impact oocyte quality and the functionality of somatic cells, even in the absence of overt cell death. Indeed Agarwal et al. (2012) reported that Oxidative stress is implicated in PCOS, a common cause of ovulatory dysfunction, and that this stress may disrupt follicular maturation and reduce oocyte competence [65].

Under the conditions and sample size analysed, ovulatory dysfunction was not associated with increased necrosis in CCs, as assessed by the LDH assay. However, the assay's insensitivity to apoptosis and its inability to capture other potential alterations, such as oxidative stress or inflammation, highlight the need to integrate apoptosis-specific markers and oxidative stress. Such approach will enable a more comprehensive characterization of the cellular and molecular alterations occurring in the follicular environment of women with ovulatory dysfunction.

Conclusion

This study aimed to indirectly assess apoptosis levels in cumulus cells by analyzing follicular fluid from infertile women and oocyte donors.

To this end, the activity of lactate dehydrogenase (LDH) in follicular fluid was evaluated as a potential indicator of cellular damage associated with apoptosis or other cell death processes, being explored as an indirect marker of cell viability in the context of female infertility.

Initially, cellular characterization revealed the absence of CCs in the FF samples from infertile women, preventing direct cellular analyses. In contrast, donors showed a significantly higher count of CCs, indicating greater follicular integrity.

Alternatively, LDH activity was quantified as an extracellular marker of necrosis. The activity was normalized to total protein concentration (BCA assay). The results did not show significant differences between groups, including the subgroup with ovulatory dysfunction, suggesting that necrosis is not a predominant event in these physiological conditions.

However, the use of the LDH assay demonstrated limitations, notably its lack of specificity for apoptosis and sensitivity to handling conditions. Therefore, there is a need for more specific techniques for detecting apoptosis, such as active caspase marking (e.g., caspase-3), Annexin V.

In conclusion, although LDH quantification provides some information about cell integrity, it does not provide an adequate assessment of apoptosis in FF. Future studies should incorporate molecular, biochemical, and imaging approaches for a more robust characterization of cell death associated with infertility.

In conclusion, although the quantification of LDH activity in follicular fluid provides some information about cellular integrity, it is insufficient to accurately assess apoptosis due to its lack of specificity and susceptibility to external factors, such as sample handling. However, apoptosis alone may not fully explain the observed alterations in these patients. Therefore, it is essential to consider alternative mechanisms that could contribute to infertility. One such hypothesis is endocrine dysregulation. Hormonal imbalances, particularly involving gonadotropins or androgens, may impair follicular development and cumulus-oocyte communication, potentially contributing to the absence or dysfunction of cumulus cells observed in some infertile women.

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