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ASSESSMENT OF DNA DAMAGE IN CUMULUS CELLS OF INFERTILE WOMEN

Master's dissertation in Clinical Laboratorial Genetics, carried out under the scientific supervision of Professor Doctor Isabel Maria Marques Carreira, and Doctor Paula Maria Vieira Jorge, presented to the Faculty of Medicine of the University of Coimbra.

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AVALIAÇÃO DO DANO DO DNA EM CÉLULAS DO CÚMULUS DE MULHERES INFÉRTEIS

Dissertação no âmbito do Mestrado em Genética Clínica Laboratorial, realizada sob a orientação científica da Professora Doutora Isabel Maria Marques Carreira, e da Doutora Paula Maria Vieira Jorge apresentada à Faculdade de Medicina da Universidade de Coimbra.

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Resumo

A infertilidade afeta aproximadamente 15% dos casais e em todo o mundo, mais de 8 milhões dos nascimentos resultaram do sucesso de técnicas de reprodução medicamente assistida. Estudos recentes, utilizando o ensaio cometa para avaliar o dano do DNA nos espermatozoides, demonstraram que um elevado nível de fragmentação no DNA afeta a capacidade reprodutiva. Contudo, a aplicação do ensaio cometa na infertilidade feminina continua escassa.

O ensaio de cometa é um método simples, versátil e barato utilizado para detetar quebras na cadeia de DNA e locais *alkali-labile*. Uma das limitações desta técnica é a variabilidade dos resultados que parece ser influenciada por inúmeros fatores nas diversas fases deste procedimento. Assim, a otimização do ensaio cometa foi realizada em amostras de sangue total e todas as etapas desta técnica foram analisadas.

O *precoating*, a concentração de agarose de baixo ponto de fusão e Triton X-100, as propriedades de adesão das lamelas, o uso de um controlo positivo e a sua aplicação e o sistema de contagem mostraram-se críticos para o ensaio. Em amostras congeladas, a colheita de sangue com EDTA demonstrou um efeito protetor sobre as células. A otimização do protocolo garantiu que, dentro dos parâmetros testados, as melhores condições fossem cuidadosamente escolhidas. Assim, o ensaio do cometa foi implementado em células do cúmulos. Foi possível especular que uma maior fragmentação no DNA pode estar associada a um mecanismo de apoptose mais rápido nas células do cúmulos. A análise de ambos os tecidos em amostras de dadoras de ovócitos e de mulheres inférteis revelou que em alguns casos o dano está presente em ambos os tecidos, contrariamente ao que acontece em amostras de mulheres inférteis em que o dano é observado apenas nas células do cúmulos. Enfatizando a importância de estudar estes dois tecidos e apoiando a utilização das células do cúmulos como biomarcador da infertilidade feminina.

De acordo com a Associação Portuguesa de Fertilidade (https://apfertilidade.org/, acedido a 8 de julho de 2022) cerca de 10% das mulheres portuguesas sofrem de infertilidade idiopática, e sabe-se atualmente que variantes genéticas estão implicadas em cerca de 15% dos casos de infertilidade feminina. A insuficiência ovárica primária é uma das principais causas genéticas de infertilidade feminina e afeta aproximadamente 20% das mulheres com uma pré-mutação no gene *FMR1*. Contudo, os mecanismos patológicos e moleculares que regulam a sua influência na infertilidade não foram ainda elucidados. Considerando que a função do gene *FMR1* no ovário é pouco estudada foi realizada uma análise bioinformática das vias do gene *FMR1*, infertilidade ovárica e

resposta à fragmentação do DNA recorrendo a bases de dados. Este estudo revelou um único gene em comum, o *CREB1.* Este gene codifica para um fator de transcrição membro da família de proteínas que ligam o DNA “leucine zipper”. De acordo com a base de dados Harmonizome (https://maayanlab.cloud/Harmonizome/gene/CREB1), este gene apresenta 10 000 associações funcionais descritas, mas nenhuma relacionada com a (in)fertilidade feminina. Assim este resultado é sugestivo de existir uma relação do gene *CREB1* e a fertilidade feminina, algo que não foi descrito anteriormente, demonstrando a sua potencialidade para futuros estudos.

Palavras-chave: infertilidade feminina, células do cúmulos, ensaio cometa, dano no DNA.

Abstract

Infertility affects approximately 15% of couples, and worldwide, more than 8 million births have resulted from successful medically assisted reproduction techniques. Recent studies have used the comet assay to assess sperm DNA damage, demonstrating that a high level of fragmentation in DNA affects reproductive ability. However, comet assay in female infertility remains a challenge.

Comet assay is a simple, versatile, and inexpensive method used to detect strand breaks and alkali-labile sites. One of the limitations of this technique is the variability of the results inter- and intra- laboratories and therefore its optimization is necessary. Optimization of the comet assay was performed in whole blood samples and all the steps of this technique were reviewed.

Precoating, the concentration of low melting point agarose and Triton X-100, the strength of coverslips, the use of positive control and its application, and the counting system were shown to be critical for the assay. In frozen samples, blood collection with EDTA demonstrated a protective effect on the cells. Optimization of the protocol ensured that, within the parameters tested, the best conditions were carefully chosen. Thus, the comet assay was implemented on cumulus cells. It was possible to speculate that greater fragmentation in DNA may be associated with a faster apoptosis mechanism in cumulus cells. Analysis of both tissues in samples from oocyte donors and infertile women revealed that in some cases damage is present in both tissues, unlike in samples from infertile women where damage was observed only in the cumulus cells. Emphasizing the importance of studying these two tissues and supporting the hypothesis of using cumulus cells as a biomarker for female infertility.

According to the Portuguese Fertility Association (https://apfertilidade.org/, accessed July 8, 2022) about 10% of Portuguese women suffer from ideopathic infertility, and it is now known that genetic variants are implicated in about 15% of female infertility. Primary ovarian insufficiency is a major genetic cause of female infertility and affects approximately 20% of women with a pre-mutation in the *FMR1* gene. However, the molecular and pathological mechanisms regulating its influence on infertility have not yet been elucidated. Considering also that the function of the *FMR1* gene in the ovary is poorly studied, a bioinformatics analysis of *FMR1* gene pathways, ovarian infertility and DNA fragmentation was performed using databases. This study revealed a single gene in common in all three pathways, *CREB1*. This gene encodes for a transcription factor that is a member of the "leucine zipper" family of DNA-binding proteins. According to the Harmonizome database (https://maayanlab.cloud/Harmonizome/gene/CREB1), this

gene has 10 000 described functional associations, but none related to female (in)fertility. Thus, this result is suggestive that there is a relationship of the *CREB1* gene in fertility, something that has not been described before, demonstrating its potentiality for future studies.

Keywords: female infertility, cumulus cells, comet assay, DNA damage.

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“Há um tempo em que é preciso
Abandonar as roupas usadas,
Que já não tem a forma de nosso corpo,
E esquecer os nossos caminhos,
Que nos levam sempre aos mesmos lugares.

É o tempo da travessia: e,
Se não ousarmos fazê-la,
Teremos ficado, para sempre,
Á margem de nós mesmos."

-Fernando Pessoa.

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Abbreviations

(ALS) Alkali-Labile Sites

(AMH) Anti-Müllerian Hormone

(CC) Cumulus Cells

(COC) Cumulus-Oocyte Complex

(CREB1) CAMP Responsive Element Binding Protein 1

(DAPI) 4′,6-diamidino-2-phenylindole

(DS) Double-Stranded

(FMRP) *FMR1* protein

(FSH) Follicle-Stimulating Hormone

(FXPOI) Fragile X-primary ovarian insufficiency

(FXS) Fragile X Syndrome

(FXTAS) Fragile X-associated Tremor/Ataxia Syndrome

(H_2O_2) Hydrogen Peroxide

(ICSI) IntraCytoplasmic Sperm Injection

(LH) Luteinizing Hormone

(LMPA) Low-Melting-Point Agarose

(NMPA) Normal Melting Point Agarose

(OECD) Organization for Economic Cooperation and Development

(PBS) Phosphate Buffered Saline, pH 7.4

(POI) Primary ovarian insufficiency

(RDF) Resource Description Framework

(ROS) Reactive Oxygen Species

(SB) Strand Breaks

(SSB) Single-Strand Breaks

(AU) Arbitrary Units

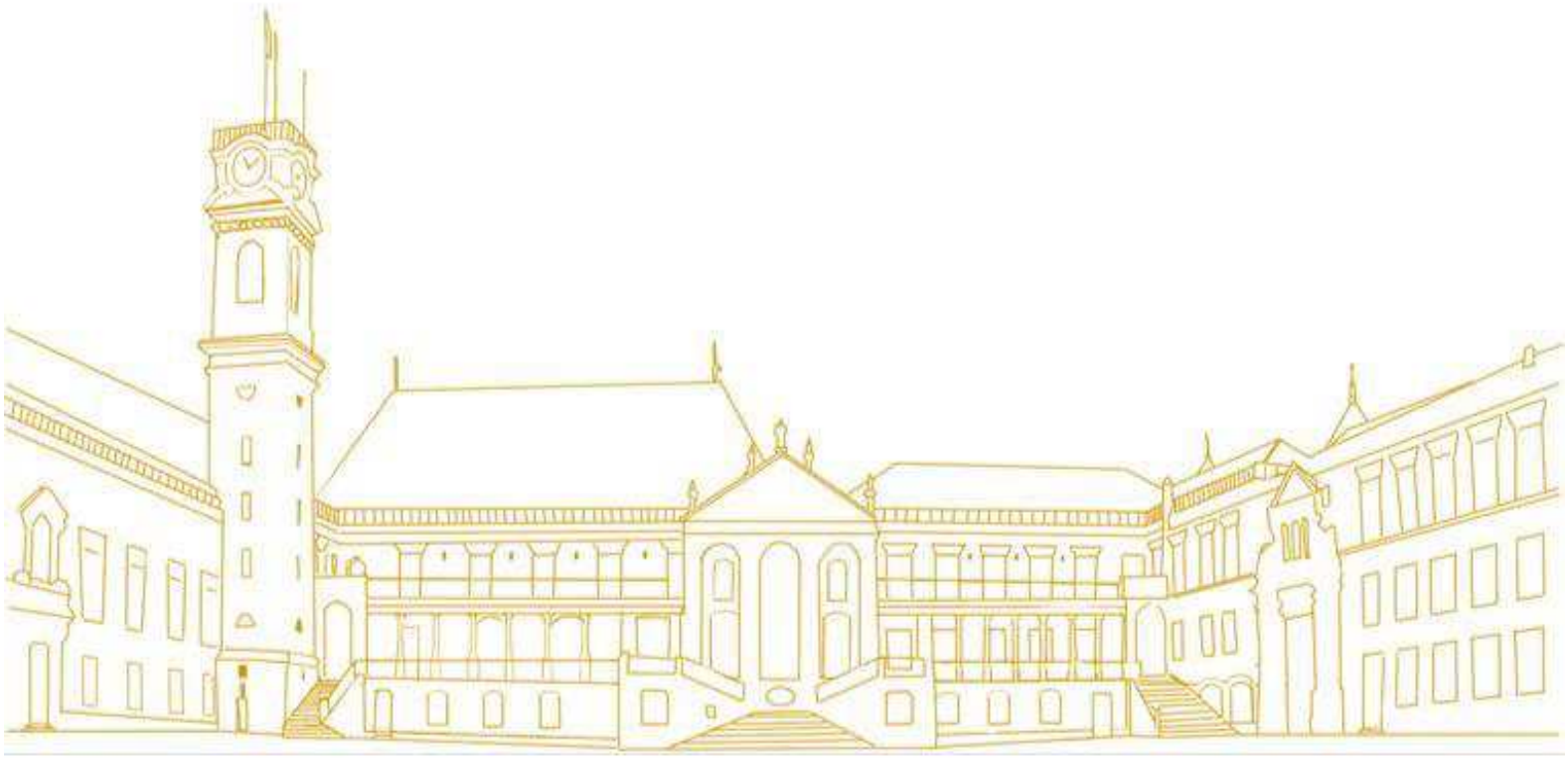
(XG) Relative Centrifugal Force

Thesis Organization

The present thesis is organized into five chapters. **Chapter 1** is intended to provide background information to set the context of this research and provides a comprehensive introduction to the literature review on the comet assay, female (in)fertility, genetic causes namely the interaction between the *FMR1* gene, female infertility, and cumulus cells. **Chapter 2** describes the material and methods used herein. Results obtained and discussion are presented in **Chapter 3**. **Chapter 4** draws the conclusions and future perspectives. Finally, **Chapter 5** provides the references of the articles cited.

CHAPTER 1.

INTRODUCTION



Chapter 1 | Introduction

1. Background

Infertility is a reproductive system disorder characterized by the inability to establish a clinical pregnancy after one year or more of regular unprotected intercourse, because of decreased ability to reproduce as an individual or with their partner [1]. Between 8 and 12% of reproductive age couples suffer from infertility concerns worldwide [2].

The success rate of the intracytoplasmic sperm injection, a widely used medically assisted reproduction technique, has been associated with the DNA breaks of the cumulus cells (CC), however, these results remain under discussion and controversy [3].

The comet assay is a simple technique, has been widely used to assess DNA fragmentation, though the variability and complexity of the results seem to affect clinical applicability [4].

Anticipating the use of the DNA damage, determined resorting to the comet assay, as a biomarker in female infertility, this thesis focuses on the optimization of the comet assay by testing some crucial factors and identifying key points aiming to overcome the results discrepancy. Following the establishment of a protocol to analyze DNA damage in CC and blood cells, the DNA fragmentation will be compared in these tissues using samples from infertile females and oocyte donors. Understanding the challenge of working with these cells this study aims to contribute to increase the knowledge of this developing research field.

In females, genetic causes of infertility include variants in genes that control numerous biological processes namely oogenesis, hormone signaling, development of female reproductive organs and preservation of ovarian reserve as well as chromosome abnormalities [5]. Despite the importance of the X chromosome in ovarian biology, only few X-linked genes have been associated to ovarian dysfunction [6]. One of the widely investigated gene is *FMR1* known to cause Fragile X- associated primary ovarian insufficiency (FXPOI; OMIM #311360), in 20% of females carrying 55–200 CGG repeats (premutation alleles) [6,7]. To the best of our knowledge the investigation of common links between *FMR1*, infertility and DNA integrity pathways has not yet been attempted. Aiming to boost the knowledge about increased risk for premature ovarian aging and infertility in *FMR1* premutation carriers we will analyze possible connection(s) between the different pathways using WikiPathways database. WikiPathways

(https://www.wikipathways.org/) [8] is an open, community created, expert curated database that integrates information from different databases (e.g., REACTOME). Identifier mapping and resource description framework (RDF) in the background allow connections between different pathways and interoperability with other resources. An international curation team ensures continuous updating.

2. Female infertility and reproductive treatments

Reproductive treatments and interventions can begin as early as one year or less, based on medical, sexual, and reproductive history, physical findings, age, and diagnostic tests results [1]. In 1992, intracytoplasmic sperm injection (ICSI), revolutionized the treatment of infertility in couples with a more difficult prognosis [9], consisting of micromanipulation that enables the injection of a spermatozoon into the ooplasm of a metaphase II oocyte [10]. It can be used when other medically assisted procreation techniques have failed, to solve many reproductive problems in female infertility, when there is less than optimal oocyte quality, and/or when a small number of oocytes can be collected [10]. The use of ICSI has been increasing and is now more commonly used than conventional in vitro fertilization in most of the countries [9].

Infertility can be caused by both male and female factors. Hypogonadotropic hypogonadism, hyperprolactinemia, disorders of ciliary function, cystic fibrosis, infections, systemic diseases, and lifestyle-related factors/diseases might adversely affect fertility in both genders. Besides, in female infertility, other factors can be involved like primary ovarian insufficiency (POI), endometriosis polycystic ovary syndrome, uterine fibroids, and endometrial polyps [2]. POI is a condition caused by the absence, non-functionality or early depletion of the ovarian reserve leading to infertility, instead of the gradual process of follicular atresia, typical of fertile women until menopause is reached. It is characterized by primary or secondary amenorrhea, increased serum levels of Follicle Stimulating Hormone (FSH) and decreased serum levels of anti-Müllerian hormone (AMH) [6,11]. FSH is the only hormone used to diagnose POI, but it presents high inter- or intra-cycle variability. On the other hand, AMH and antral follicle count have been considered more promising to assess ovarian reserve, given their high sensitivity and specificity in predicting ovarian response and inter-cycle reliability [11]. POI is one of the main causes of female infertility and numerous genes are implicated in this condition [6].

FMR1 gene premutations are responsible for 3-15% of POI cases [6]. Approximately 20% of women with a premutation in this gene develop FXPOI [12].

Therefore, early identification of these cases is critical as it can have serious implications on reproductive capacity. The molecular and pathological mechanisms behind its influence on infertility have not yet been elucidated.

3. Comet assay

The comet assay also called single-cell gel electrophoresis and microgel electrophoresis was first introduced in 1984 by Ostling and Johanson as a microelectrophoretic technique for the detection of radiation-induced DNA damage in single mammalian cells [13]. Ostling and Johanson first demonstrated the migration of DNA fragments from nuclei under a neutral condition [13]. The technique was later improved by Singh et al., showing that an alkaline condition substantially increased the specificity and reproducibility of the assay [14]. Since then, the neutral comet assay is mostly used to detect double-stranded (DS) DNA breaks, whereas the alkaline comet assay is more sensitive to small DNA damage, including single and double-strand DNA breaks, alkali-labile sites (ALS), DNA-DNA, or DNA-protein cross-linking, and DNA single-strand breaks (SSB) associated with incomplete excision repair sites [15,16].

3.1. Standard comet assay

The alkaline comet assay is the most widely used method for measuring DNA damage in eukaryotic cells with the number of publications is rising each year [4]. The principle behind the comet assay assumes that in the nucleus DNA loops are attached to a nuclear matrix, forming supercoiled DNA structures, called nucleosomes. In presence of breaks in the DNA, it becomes relaxed. As a result, these loops, which are still bound to the nuclear matrix, are attracted to the anode, forming the characteristic "comet tail" seen under fluorescence microscopy (figure 1).

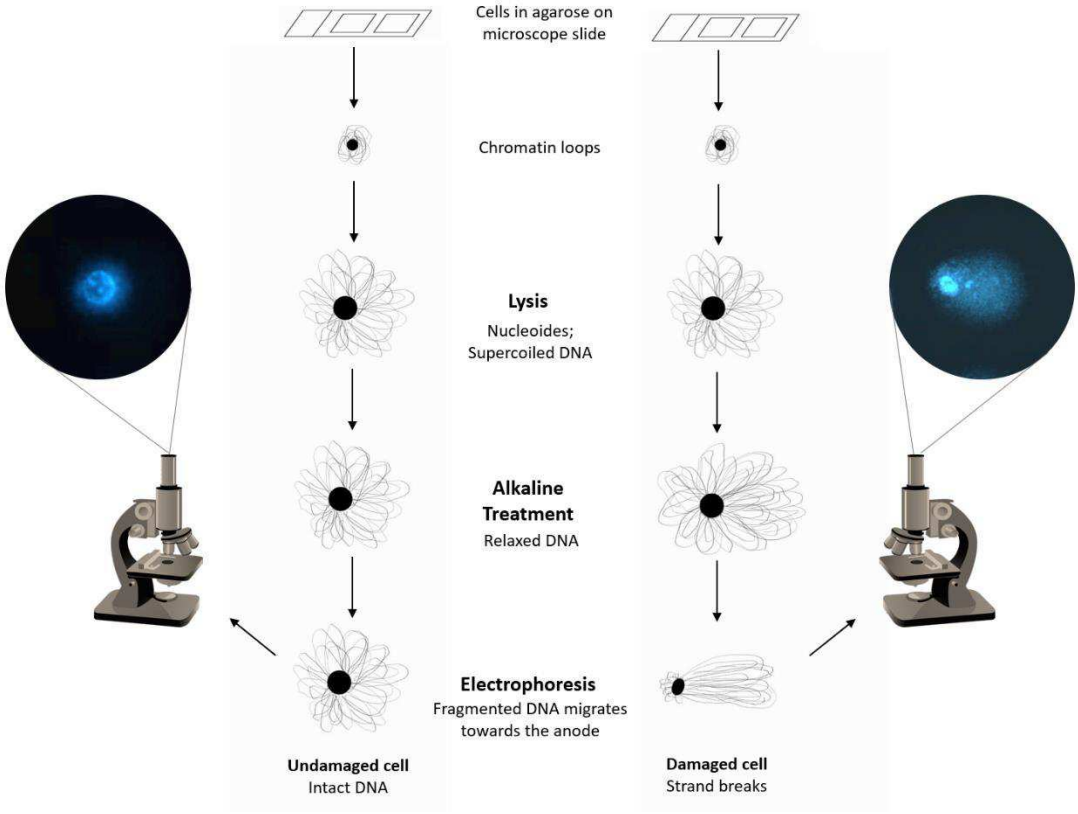


Figure 1 – Flowchart of the standard alkaline comet assay. The cells are embedded in agarose, and following the lysis step, and the alkaline treatment, proceed to electrophoresis. Finally, the comets are observed, analyzed, and classified under the florescence microscope. Based on Azqueta A. and Collins A. (2006) [17].

The comet assay protocol has eleven steps (figure 2):

1. **Treatment of the cells**, where the sample is diluted in PBS (Phosphate Buffered Saline), cells suspension is obtained and then ready for the next steps;
2. **Embedding cells**, the cell suspension is dipped in low melting point agarose (LMPA) to form the gel;
3. Both negative and positive controls should be used as reference. **Positive Control** (mock), the samples are exposed to a toxic agent, for example, hydrogen peroxide (H_2O_2). The presence of a positive control allows validation of several steps of the procedure;
4. **Lysis**, where membranes and other cellular constituents are removed, leaving the DNA without most of the histones, forming structures known as nucleoids;
5. **Alkaline treatment**, in the standard assay, the samples are transferred to the alkaline solution so that the protein denaturation of the nucleoid occurs, making the DNA more relaxed;
6. **Electrophoresis**, the relaxed DNA, still attached to the matrix, migrates towards the anode originating a "comet tail";

7. **Neutralization,** where the slides are dipped in PBS to wash and maintain pH of the samples;
8. **Fixation,** so that the slides can be stored longer for future analysis;
9. **Staining**, DAPI (4′,6-diamidino-2-phenylindole) is one of the commonly used fluorochromes.
10. **Quantitation**, comets are evaluated by fluorescence microscopy; and,
11. **Calculation of the percentage of DNA on the tail**, scoring of comets to evaluate the extent of the DNA damage can be done by image analysis, with a camera connected to a computer with the appropriate software, or by classifying comets visually. Comets are divided into five categories: 0 represents undamaged cells (comets with no tails) and categories 1-4 represent increasing levels of damaged cells (comets with increasing tail intensities and dragging), class 4 is used to classify cells when the DNA is all fragmented and cells are almost headless [18].

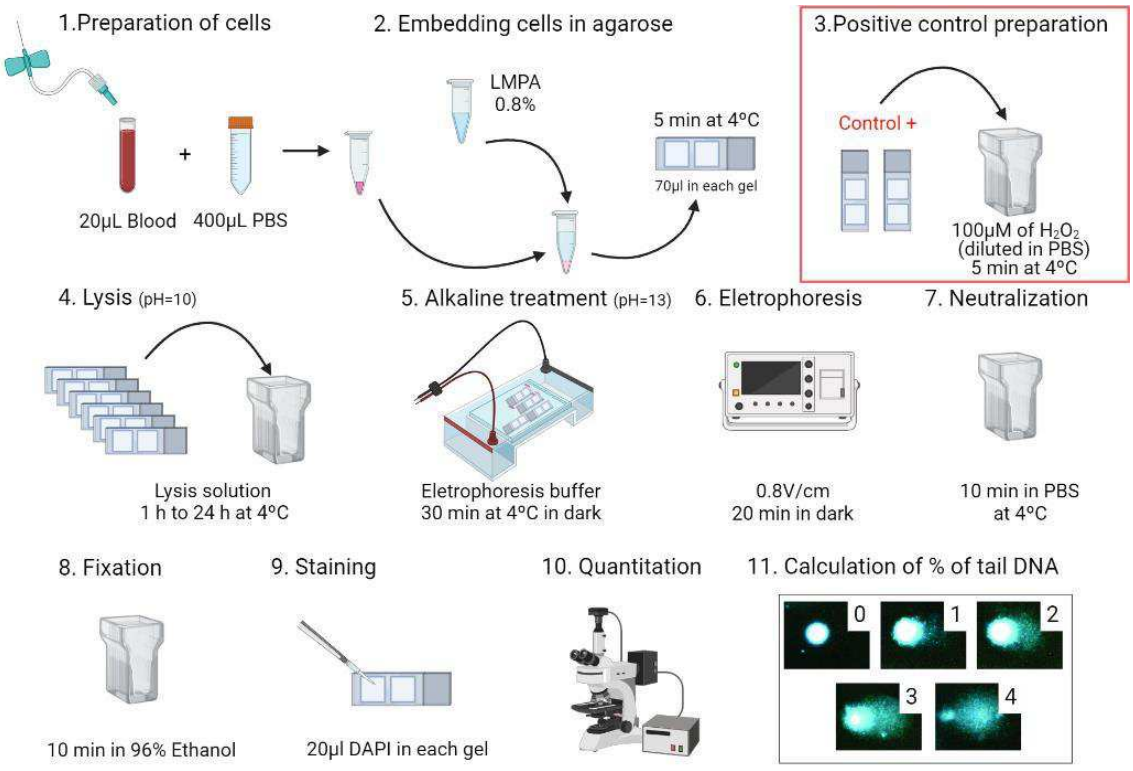


Figure 2 - Comet assay workflow where the eleven steps are graphically represented.

3.1.1. Advantages and disadvantages

In comparison with other genotoxicity tests, comet assay captivates by its fast results, simplicity, low cost, sensitivity for detecting low levels of DNA damage, and requires a small number of cells. Furthermore, it is versatile, as cells from a variety of

tissues can be studied, in a wide diversity of eukaryotic organisms, besides being relatively easy to perform and requiring simple and affordable equipment [4,18,19].

Comet assay has also some limitations, related to the challenges of obtaining absolute quantification, and unequivocal identification of the damage. In addition, different procedures have been used to measure DNA migration, consequently, there are differences in results between laboratories. Thus, the classification of comets is the greatest technical limitation [4,15].

In 1993, a collaborative multi-laboratory review of the comet assay was published by McKelvey-Martin et al. for the first time, demonstrating the principles, applications, development, and protocol of the assay [20]. However, it was only in 2000 that the initiative to develop a guideline for the comet assay in genetic toxicology, covering both *in vivo* and *in vitro* studies, was published. Between 2006 and 2012, formal validation was performed leading to the adoption of the mammalian *in vivo* alkaline comet assay by the Organization for Economic Cooperation and Development (OECD) [15].

3.2. Modified comet assay

The comet assay has been widely used to assess the effects of various endogenous and exogenous substances on DNA damage and repair. Therefore, various modifications have been made, from changing the pH of the electrophoresis buffer [21] to exposing cells to various chemicals to assess DNA repair capacity, to treating nucleoids with restriction enzymes (enzyme-modified comet assay) and even protein extracts to assess the effect of a given substance on DNA repair [22]. These modifications to the standard comet assay protocol show the adaptability and applicability of this technique [22]. Below some types of modified comet assays will be discussed.

3.2.1. Detection of oxidative damage

Enzyme-modified comet assay was introduced in 1993 by Collins et al. [11], this modification of the standard comet assay protocol is based on the use of a repair enzyme to increase the specificity and sensitivity of the assay. It allows the detection of oxidized bases, which are present as a result of oxidative stress [23].

Different DNA-damaging agents, such as ionizing radiation, for example, do not directly induce stand-breaks (SB) (not breaking the phosphodiester backbone). However, other lesions such as oxidized or alkylated bases, bulky adducts, and intra-

and inter-strand cross-links can be caused. These lesions are not detected by the standard comet assay and tend to have more serious consequences for the cell or organism when compared with SB [24]. To improve the analysis in these situations lesion-specific enzymes that convert altered bases to breaks have been widely used in genotoxicity testing with the in vitro comet assay [23], creating a tool to study the various DNA lesions induced by different chemicals and radiation. The combination of repair enzymes with the comet assay represents a huge advantage in all the fields in which the comet assay is used.

Several enzymes have been used, however the most used are formamidopyrimidine DNA glycosylase (Fpg) [25], which converts 8-oxoguanine and ring-opened purines to breaks, and endonuclease III (Endo III), which converts oxidized pyrimidines to breaks [23].

The enzyme-modified comet assay protocol adds one step to the standard alkaline comet assay: the incubation of the nucleoids with an enzyme, following lysis, and washing of the slides in an enzyme-specific reaction buffer [26]. In enzymatic treatment, depending on the enzyme, DNA is incised at the lesion sites, or the modified nucleotide is removed while leaving an AP site (apurinic/apyrimidinic site). In alkaline conditions, the AP sites are turned into SSB. In parallel with the enzyme incubation, a duplicate slide should be made with the enzyme reaction buffer only [24].

The optimal and the most efficient temperature is 37°C, so after adding the enzyme (or buffer), the slides are kept cold (e.g., placed on a cold metal plate), preventing activation of the enzyme before the slides are transferred to the optimal temperature and assuring the same incubation time in all gels. It is important to use cells with knowing lesions, to determine the optimum combination of enzyme concentration, and incubation time before utilization. The enzyme should be provided at an optimal concentration ensuring maximum detection of enzyme-sensitive sites without inducing non-specific breakdowns [26].

"Net enzyme-sensitive sites" are calculated by the difference in DNA migration (tail intensity) between reaction buffer-treated and enzyme-incubated slides [26].

3.2.2. Detection of global DNA methylation

The comet assay is an affordable and flexible method that can be easily adapted for the measurement of global DNA methylation [22]. DNA methylation is not only essential for maintaining the stability of the genome but also plays an important role in gene regulation [27].

This epigenetic event can be described as the chemical modification of DNA in which a methyl group is bound to the carbon-5 position of cytosine through the action of DNA methyltransferases without changing the original DNA sequence [22]. DNA methylation patterns can be established at a global or gene-specific level, based on regulatory needs [27].

Most of the CpGs in the genome are methylated, except for CpG islands, which are clusters of CpG dinucleotides frequently associated with the 5' ends of genes, remaining hypomethylated [28]. To prevent changes in DNA methylation and histone modification patterns, such as changes that disrupt important cellular processes including gene expression and DNA repair, for example, epigenetic mechanisms must be regulated [27].

The comet assay allows the measurement of global DNA methylation from a baseline modification using two restriction enzymes: *Msp*I and *Hpa*II. These two isoschizomer endonucleases recognize the same tetranucleotide sequence (5'-CCGG-3') but have differential sensitivity to DNA methylation. *Hpa*II is methylation-sensitive and thus inactive when any of the two cytosines are methylated, but it digests the hemimethylated 5'-CCGG-3' at a lower rate compared with the unmethylated sequences. On the other hand, *Msp*I digests 5'-CmCGG-3' but not 5'-mCCGG-3' [27]. Regarding the protocol, as in the enzyme-modified comet assay, a step is added after the lysis. The slides are laid in restriction enzyme reaction buffer before incubation with specific enzyme mixtures (with *Hpa*II or *Msp*I) at 37ºC [27].

In theory, the percentage of tail DNA after treatment with *Msp*I represents all 5′-CCGG-3′ sites in DNA, however, it is important to notice that when using *Msp*I and *Hpa*II only methylated cytosines beyond CpG islands are quantified because those enzymes recognize mainly sequences outside of CpG islands. Cytosines within these regions tend to be methylated while cytosines on CpG islands lean unmethylated [29,30]. The global 5'-CCGG-3' methylation can be calculated by the *Hpa*II/*Msp*I ratio.

Some limitations of the methylation-sensitive comet assay have been described by Wentzel and Pretorius, including, limited sample throughput, insufficient enzyme digestion of nucleoids, and drying of agarose before enzyme digestion is complete [22]. However, these challenges and limitations are not specific to the methylation-sensitive comet assay, they can be found in other adaptations of the comet assay as well [22].

Recently modification of the alkaline comet assay, "EpiComet" has emerged allowing a single platform evaluation of genotoxicity and global DNA methylation using methylation-dependent endonuclease [5- methylcytosine (5-mC)] status of single-cell populations under user-defined conditions [31].

3.3. Applications of the comet assay

Over the past decade, comet assay has become one of the standard methods for assessing DNA damage, with applications in several research areas such as genotoxicity testing, human biomonitoring, molecular epidemiology, infertility, and ecogenotoxicology, as well as fundamental research in DNA damage and repair [4].

The assay is most applied in cultured mammalian cells, peripheral blood mononuclear cells, or disaggregated tissues [18] but can be used on any eukaryotic cell that can be obtained as a single cell suspension, such as whole blood or cells isolated, tissue cells biopsies that can be homogenized, buccal, and cultured cells. The assay allows the detection of intercellular differences in DNA damage and repair in the eukaryote cell population as well as in other organisms, such as invertebrates, bacteria, plants, yeast, and fungi [32]. It has been successfully used in many fields, for example, to measure DNA damage and/or repair in leukocytes [33], salivary gland tissue [34], bone marrow, liver, kidney, buccal cells [35], primary colon cells [36], different cancer cell lines like HT29 [37], HT29 clone 19A [36], LT97 [38], or HepG2 [39], bladder, lung, stomach, gill, hemolymph, digestive gland, and also different epithelial cells [40] like brain cells [41], and sperm cells [19,42]

Moreover, in human fertility the comet assay has been used to evaluate the success of ICSI technology by assessing DNA fragmentation in testicular, ejaculate sperm [43] and CC [3] samples.

3.3.1. Comet assay in infertility

The comet assay has been applied specially in male infertility. A PubMed search on July 5, 2022, using terms [comet assay] and [sperm] reveals more than 600 entries, while using the terms [comet assay] and [cumulus cells] only 28 publications were found. It is described that infertile men have higher DNA fragmentation than healthy ones [44]. Male infertility accounts for approximately half of all infertility cases, affecting 7% of the male population [45]. Despite a clear role of genetic causes in male infertility, at least 40% of all cases are classified as idiopathic [45].

The diagnosis of male infertility is based on the results of the semen analysis on microscopic assessment, including ejaculate volume, sperm concentration, motility, and morphology [46]. However, semen analysis to assess male fertility is still a challenge. Therefore, it has become necessary to develop tests to better investigate the pathophysiology and etiology of male infertility.

The study of sperm DNA damage was then considered a complementary diagnostic tool and biological marker of male reproductive health and infertility [47]. Compared to the other parameters DNA integrity distinguishes itself by being relatively independent of sperm quality providing a diagnostic and prognostic complementary tool, but distinct from conventional sperm analysis [48].

One of the major causes of pre-and post-implantation embryo loss in humans is sperm DNA fragmentation [49]. Sperm DNA fragmentation can be assessed by the Terminal Chromatin Structure Assay, or the Transferase dUTP Nick End Labeling assay [30]. However, these two methods detect primarily breaks in histone-associated chromatin whereas the comet assay has a broader use as it can detect breaks in both protamine and histone bound chromatin [30]. Furthermore, comet assay is the only technique that allows the quantitative measurement of DNA damage in individual cells and is adequate for testicular and oligozoospermic samples where cells are sparse, with a particular impact in a heterogeneous population like sperm [30].

Studies have attempted to understand if increased sperm DNA fragmentation affects reproductive outcome, however, the findings are contradictory, some studies indicate that fragmentation compromises reproductive success rate [50], while others have not found such effect [51]. Nevertheless, most of the publications report the association between DNA damage and lower fertilization, impaired embryo cleavage, and higher miscarriage rates [43]. Despite it is known that sperm quality is associated with chromatin remodeling errors during spermiogenesis and oxidative stress, the cause of sperm DNA fragmentation is still unclear [52].

The increasing use of the comet assay in male infertility has prompted the emergence of studies in female infertility as well, to establish a connection between DNA fragmentation and medically assisted reproduction techniques and fertilization success rate [3]. In agreement with reports in male infertility, the results are not yet unanimous hampering solid conclusions and clinical use.

4. Female infertility

More than 186 million people are affected by infertility. Although one of the most conditioning factors of infertility is advanced maternal age [53], other factors such as lifestyle, genetic causes, and the surrounding environment can also impact [54].

A women reproductive potential begins to decline around the age of 25-30 years, and the average age of last birth is 40-41 years in studied populations with natural fertility [55]. As age increases there is a decrease in fertility, this fact is related to the continuous

depletion of oocytes stored in the ovaries during foetal life, over time there is a reduction in the number of oocytes and at a later stage, expiration occurs at the onset of menopause [56].

The female age of a natural fertility population over time was analyzed by Eijkemans et al. showing that the fertility loss increases slowly from 4.5% at age 25 years, 7% at age 30, 12% at age 35, and 20% at age 38. Then increases rapidly to about 50% at age 41, almost 90% at age 45, and nearing 100% at age 50 [55]. Several diseases are known causes of female infertility such as endometriosis, polycystic ovary syndrome, and premature ovarian failure (POF). Tubal factors and ovulatory events and disorders can also contribute to female infertility [57].

The ability to become pregnant spontaneously decreases over time, leading many couple to resort to medically assisted reproduction techniques [58]. In 2017, according to the Portuguese Society of Reproductive Medicine (https://www.spmr.pt/), 2796 children were born in Portugal as a result of the use of medically assisted procreation techniques which represents 3.2% of the total number of children born in the country that year. As reproductive age advances the quality of oocytes decreases, additionally, premature recruitment of follicles is also affected, increasing ovulatory disorders, reducing the ovulatory frequency and the paired luteal phase, which leads to reduced conception rates [53] (pregnancy loss and spontaneous abortion) [56].

The success of medically assisted reproduction techniques is often compromised by inefficiency in determining the quality of the oocytes. This happens because the evaluation of oocyte quality is conducted by invasive methods that may impair their development. The granulosa cells divided into cumulus (CC) and mural cells, functionally interact with oocytes. Therefore, the study of CC might reflect the competence condition of the oocyte [59]. Assessing DNA damage in CC might reflect oocyte status, and directly female fertility.

4.1. Primary ovarian insufficiency (POI)

Primary ovarian insufficiency (POI) also known as POF, is one of the main causes of female infertility [6] and affects about 1-2% of women [5]. POI is a common endocrine reproductive disease characterized by the loss of ovarian activity before the age of 40 years [60] ovarian dysfunction, and decreased fertility, as demonstrated by abnormal ovarian reserve biomarkers and reduced ovarian response to hyperstimulation [61–63].

POI is characterized by being phenotypically, etiologically, and genetically heterogeneous, and associated with primary or secondary amenorrhea, decreased

estrogen production, and elevated gonadotropins [FSH and Luteinizing Hormone (LH)] levels and infertility [5]. Affects 1% of the general population and occurs in approximately 20% of women with an *FMR1* gene premutation (Premature Ovarian Failure 1 - POF1 OMIM 311360) [64]. Although the exact mechanism is not yet described it is possible that cellular alterations caused by the *FMR1* premutation impair follicular growth, ovulation rate and fertility.

4.2. *FMR1* gene and ovarian insufficiency

Numerous genes and signaling pathways are hypothesized to play critical roles in the control of follicular maturation and ovarian reserve [65]. Of these, *FMR1* gene is prominent, due to its expression in female germline, predominantly in granulosa cells [66,67].

The normal number of repeats in the 5'UTR region of the *FMR1* gene FRAGILE X MESSENGER RIBONUCLEOPROTEIN 1 gene (*FMR1*, OMIM #309550) located on the X chromosome (Xq27.3), ranges between 5-44 CGG, in these cases the allele is normal and stable and there is no risk of expansion. A repeat number between 45-54 CGG is considered an intermediate allele, more unstable than the normal allele, but not associated with phenotypic changes. In situations where the number of repeats is in a range of 55-200 CGG (premutation), there is high instability, and an increased risk of expansion. In addition, the presence of premutation can cause other syndromes, increasing the risk of FXPOI in women and tremor/ataxia syndrome (FXTAS) in both sexes (less frequent in women) [68]. Finally, a full mutation occurs if the number of repeats is greater than 200 CGG. This causes hypermethylation of the *FMR1* gene promoter preventing RNA synthesis and, consequently, *FMR1* protein (FMRP) synthesis [69] being characterized as Fragile X syndrome (FXS; OMIM #300624). Among other tissues, FMRP is expressed in neurons and granulosa cells [5].

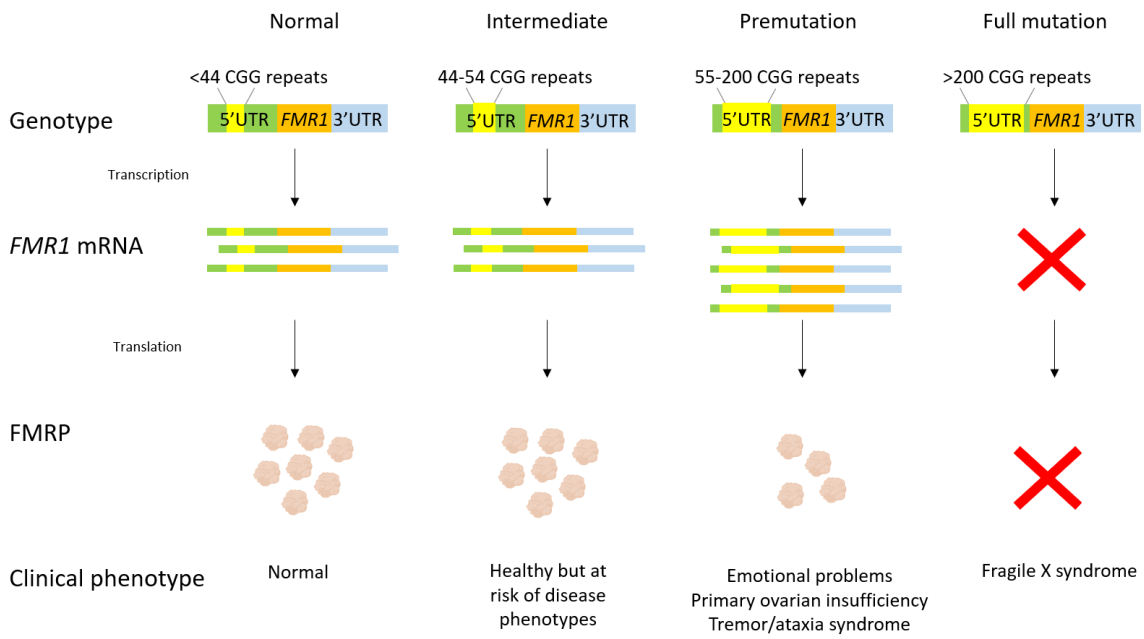


Figure 3 - Summary of expression patterns of the *FMR1* gene in normal, intermediate, premutation, and full mutation alleles. The CGG repeats are indicated in yellow. Adapted from Rosario R. and Anderson R. (2020) [70].

Women with *FMR1* premutation present increased mRNA levels in granulosa cells, associated with low FMRP synthesis. This negative feedback mechanism can induce ovarian damage mechanisms, which is clinically manifest as a diminished ovarian reserve [71].

It has been hypothesized that there is a functional link between *FMR1* gene and the phosphatidylinositol-3-kinase (AKT)/mammalian target of rapamycin (mTOR) signaling pathway [71]. This pathway is essential to folliculogenesis, as it maintains the primordial follicular pool and is functionally involved in primordial follicle activation, granulosa cell proliferation, oocyte-granulosa cell interaction and communication, and cell cycle proliferation [72,73].

4.3. Granulosa cells and infertility

Folliculogenesis, from recruitment to ovulation, is intended to develop and release a competent oocyte [74]. Intercellular communications established by the oocyte are essential for the survival and synchrony of the different follicular compartments [75]. The ovarian follicle has a highly segmented structure, featuring an antral region, where all cell types are present, being theca cells the most external to the gamete, and a basal region where different types of somatic cells are forming a contingent of granulosa cells.

Theca cells are separated from the follicle and therefore not physically interacting with it. The granulosa cells can be distinguished between those lining the antrum, designated mural cells, and those supporting the oocyte called CC [12,75] (figure 4).

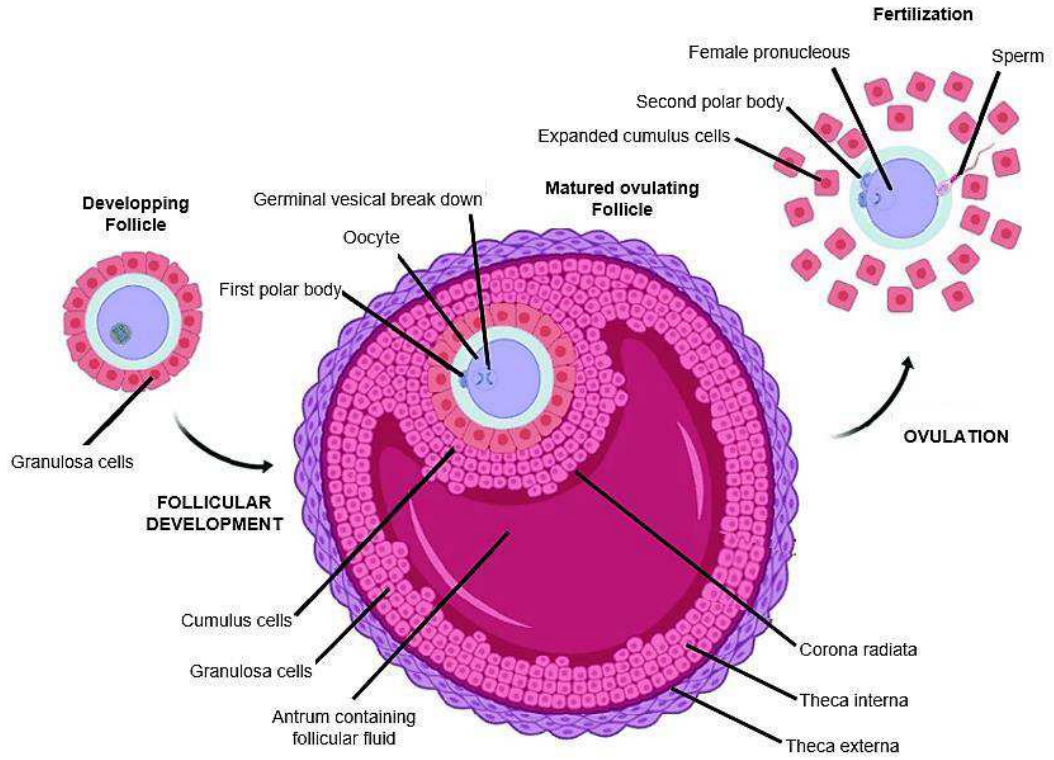


Figure 4 – Representation of cumulus cells during oocyte maturation and fertilization processes in mammals. Adapted from Estienne A. et al. (2020)[76].

Cumulus cells are derived from granulosa cells and closely related to the oocyte forming the cumulus-oocyte-complex (COC) [77]. CC can be easily recognized by their location once they directly surround the corona radiata cell layer in each oocyte [78].

The close relationship between CC and oocyte is established via gap junctions that facilitate communication between them, allowing the exchange of signals and nutrients to the oocytes [3]. Besides this contact, the oocyte itself is coated with glycoproteins [79]. For folliculogenesis and oogenesis to be successful all compartments must remain in synchrony throughout all phases: proliferation, growth, and differentiation, and inter-compartment communications need to be efficient, multi-directional, and tightly regulated [80]. The intra-ovarian communications are very complex being remotely controlled by the hypothalamic-pituitary-gonadal axis [81]. This communication is based on numerous growth factors, metabolites, and ions that act on the ovarian follicle using signal transduction from reception in the outer follicular compartments and transmission inward toward the oocyte. This signaling is then interpreted and output differently to ensure and

modulate delicate physiological functions, each of which seems to determine some aspect of oocyte quality [75].

The extent of COC interdependence was initially revealed as functional defects observed in the CC when the oocyte was removed from the complex [82]. This communication proved to be direct, at least in part, since stripping the somatic cells from the gamete had a significant impact on the competence and development of the oocyte. It is now firmly established that the CC should be in direct contact with the gamete to re-establish meiosis and provide metabolic support. The most frequently cited transferred molecules are amino acids, energy substrates, mainly lactate, pyruvate, phosphocreatine, the cyclic nucleotides cAMP and cGMP [75].

Cumulus cells are essential for oocyte proliferation, maturation which is regulated via FSH and LH [83], follicular growth rate, glycolysis promotion necessary for oocyte metabolism and acquirement of EGF molecule signaling capabilities [84]. The FSH and LH hormones are also important for the formation of structural passageways for sperm-egg interactions and the secretion of progesterone, during the fertilization of the oocytes, to induce acrosome reactions. Besides oocyte maturation, CC plays an essential role in ovulation via extracellular matrix expansion the ability to affect the rate of oocyte apoptosis, give rise to clone progeny after nuclear transfer, and positively enhance implantation [3]. In the mid-1990s it was shown that the use of CC improves embryo morphology in co-cultures and blastocyst formation [3].

Since this communication is bi-directional, the oocyte also contributes to cumulus cell function through paracrine signaling by secreting oocyte-secreted factors such as GDF9 and GDF9b/BMP15. Oocyte-derived GDF9b/BMP15 and fibroblast growth factors cooperate to promote glycolysis in CC and thereby supply energetic substrates to the oocyte [84]. This mutual interdependence is a fundamental aspect of oocyte development and fertility [75].

Schuettler J. et al. found FMRP expression in the oocytes in ovarian tissue sections, but a predominant expression of FMRP in the granulosa cells surrounding each oocyte [66]. Therefore, assessment of the DNA damage status of CC can be of interest for a prediction of the oocyte condition as well as the ICSI success [3].

Studies of DNA fragmentation in CC after a freezing cycle have been lacking. The presence of more studies on this subject will be essential for properly preserve the cells and ensure that the results are real and not artifacts due to the freezing method [78].

5. Objectives of the thesis

The present thesis focuses on evaluating DNA fragmentation in blood and CC samples as part of a major project that aims to determine the possible correlation between DNA integrity of CC, oocyte and ICSI success rate. Furthermore, it is intended to understand how premutation in the *FMR1* gene is associated with female infertility, POI, and DNA fragmentation.

The difficulties inherent in obtaining cells for the study of female fertility and of *FMR1* premutations carriers led us to consider the use of CC, since they are easy to obtain as by-products (rejects) of in vitro fertilization procedures (ICSI). Yet the use of these cells can be particularly challenging, and therefore it is important to ensure the optimization of the comet assay.

Specific aims of the present work include:

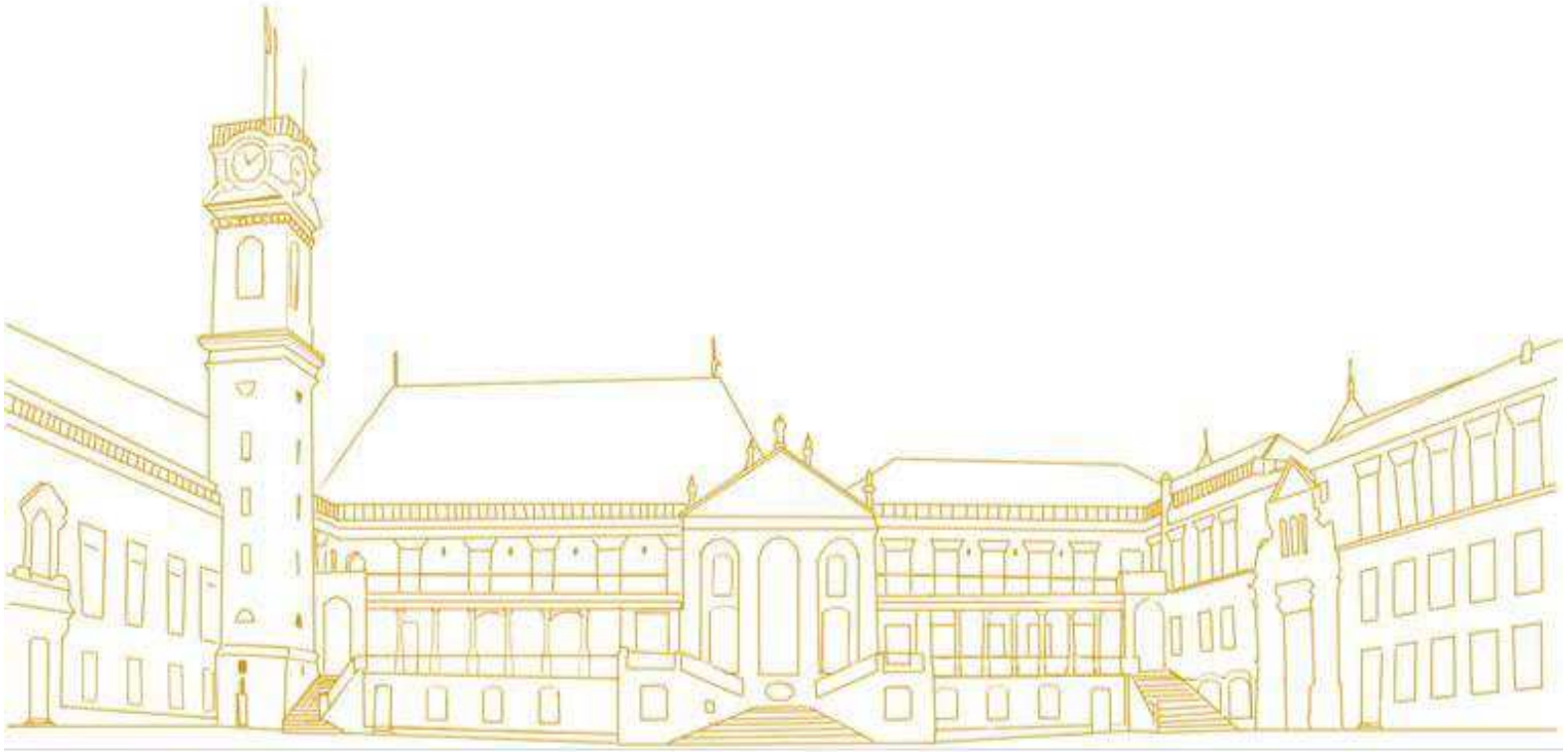
A. Optimization of comet assay protocol in whole blood samples, where several steps were carefully tested:
    1. Number of cells in suspension;
    2. Agarose (normal and low melting point) concentration;
    3. Slides quality;
    4. Lysis time;
    5. Positive Control preparation;
    6. Alkaline treatment period;
    7. Electrophoresis duration; and
    8. Counting and classifying comets methodologies.

B. Comparison by appropriate statistical analysis software between fresh and frozen whole blood samples.
C. Implementation of comet assay in CC by comparing with blood samples.
D. Assessment of DNA damage in samples from infertile females and oocyte donors.
E. Bioinformatic analysis of molecular interactions underlying DNA damage.

In the future we anticipate the use of comet assay to determine DNA damage and speculate that this can be used as a biomarker in female infertility and ICSI success rate.

CHAPTER 2.

MATERIAL AND METHODS



Chapter 2 | Material and methods

1. Pilot study

Blood samples (n=280) collected by venipuncture from infertile women and oocyte donors were kindly provided by the sample bank of Centro de Genética Médica Doutor Jacinto Magalhães, Centro Hospitalar Universitário do Porto.

For the second part of the study, cumulus cells (CC) samples (n=15) from women with fertility problems and oocyte donnors were provided from voluntary donations. Briefly, during intracytoplasmic sperm injection (ICSI) procedure an ovarian puncture occurs to collect the oocyte. The oocyte is then washed, denudated, and the CC are then collected and preserved in DMEM/F-12 medium with GlutaMAX™ supplement (Gibco™, Thermo Fisher, Waltham, Massachusetts, United States) (such samples are usually discarded as part of medically assisted reproduction procedure). Cryopreserved CC samples are maintain in DMEM/F-12 medium with GlutaMAX™ supplement (Gibco™) and 20% DMSO (Invitrogen™, Thermo Fisher Scientific, Massachusetts, United States), then the samples are then placed in Mr. Frosty™ Freezing Container (Thermo Fisher Scientific). After 24 h samples are moved to liquid nitrogen. Before comet assay is performed, cryopreserved samples must be thawed at 37ºC.

2. Comet assay

The Comet Assay was performed under alkaline conditions, pH≅13.

2.1. Treatment of the cells

2.1.1. Blood cell suspension

Samples containing 20µL of blood were diluted in 300µL of refrigerated PBS (Sigma-Aldrich®, St. Louis, Missouri, United States, 1x, pH=7.4), to obtain a cell suspension and placed on ice. In the case of frozen samples these were previously thawed at 4°C.

2.1.2. Cumulus cells

Cumulus cells suspension is transferred to a microtube, centrifugation is performed (1200 xg for 5 min) and the medium is removed without compromising the cellular pellet.

Then, 200µL of PBS (Sigma-Aldrich®) are added and centrifugation is performed in same conditions. The manipulation of these cells is performed in a culture room. In case the cells were contaminated with blood is necessary to do one or two extra washes in PBS (Sigma-Aldrich®) by repeating centrifugation (1200 xg for 5 min). After washing, the supernatant is removed and 200µL of PBS (Sigma-Aldrich®) are added to the pellet then the mixture is homogenized to ensure that the cells are detached, and the sample is kept in an ice bucket until the next step.

2.2. Precoating

Normal melting point agarose GRS Agarose LE (NMPA) (GRiSP, Porto, Portugal) was dissolved in water to a final concentration of 1% and placed in a water bath at about 60°C. After 10 min, the slides were dipped in agarose, excess agarose was removed with absorbent paper, and the slides were left to dry horizontally, at room temperature, overnight. After drying, the slides were identified at the bottom right of the frosted corner so that we know that precoating has already been performed.

To determine the most appropriate slide for the assay and the classification of the comets, precoating was performed on five different types of slides (table 1).

Table 1 - Tested blades with 26 x 76mm and respective characteristics.

Slide	CAT/VWR	Brand	Dimensions	Thickness tolerances
1	NA	MGF-slides	26 x 76mm	1mm- 1.2mm
2	VBS656/A	Bio-sigma	26 x 76mm	1mm- 1.2mm
3	630-2012	Thermo Scientific	26 x 76mm	1mm
4	NA	CETI	26 x 76mm	1mm
5	VBS656/B	Bio-sigma	26 x 76mm	1,1 ± 0,1 mm

NA- not available.

2.3. Embedding cells

Ultra-Pure DNA Grade Agarose - Low Melt Preparative Grade Agarose (LMPA) (Bio-Rad laboratories, Hercules, California, United States) was diluted in PBS (Sigma-Aldrich®), to final concentrations of 0.6%, 0.8%, 1% and 1.5% and placed in a 37°C water bath for 7 min. A volume of 150µL of each concentration was added to 20µL of the cell suspension.

After homogenization this mixture divided and two gels with 70µL were place in each slide. Above each gel, a coverslip was placed, and the slide is immediately

refrigerated during 5 min. The coverslips were then removed, and the slides were kept in cold (4ºC).

2.4. Positive control sample

In each assay, at least two slides with the same sample were exposed to hydrogen peroxide (H_2O_2) (to artificial cause damage) and the remaining slides were kept at 4ºC. H_2O_2 was chosen as the standard genotoxic substance to establish the positive control and perform the methodological adaptation. In *in vitro* genotoxicity tests, an ideal concentration should cause toxic effects and consequently DNA damage and low cytotoxicity, so that there is no compromise of cells viability.

H_2O_2 from two different stock solutions 30% H_2O_2 (Pareac, Castellar del Vallès, Catalunha, Barcelona), and 0.9M H_2O_2 (Alvita, Porto, Portugal) was diluted in PBS (Sigma-Aldrich®), to obtain a dose-response relationship *in vitro* exposure. The stock solution of 30% H_2O_2 (Pareac) was used to test different concentrations of 10%, 15%, and 20% of H_2O_2 during incubation times of 5, 10, 15, and 20 min. The stock solution of 0.9M H_2O_2 (Alvita) was used to prepare 100µM H_2O_2 and used at 5 min incubation time. Exposure to 0.9M H_2O_2 (Alvita) was done in two different ways: directly on the gel and covered with parafilm or a coverslip (more uniform) and incubating in a staining jar in the dark and at 4°C. After exposure, the slides were dipped in PBS (Sigma-Aldrich®) to proceed with protocol.

2.5. Lysis

Slides were placed in a staining jar with lysis solution[1] 2.5M NaCl, 0.1M EDTA, 0.01M Tris, 10M NaOH, and 1% Triton X-100 (pH 10) during different test conditions (1 and 24 h), in the dark at 4ºC.

2.6. Alkaline treatment and electrophoretic separation

Slides were placed in the electrophoresis tank in the dark at 4°C, arranged with the frosted edge towards the negative pole of the tank. The tank is kept equilibrated, during different test conditions (30 and 40 min) for protein denaturation of the nucleoid to occur, after immersion of the slides in alkaline solution 0.3M NaOH and 0.01M EDTA

[1] The lysis solution can be stored at 4°C for one month, and pH should be adjusted before each assay, following the addition of Triton X-100.

(pH 13) the tank is removed from the dark and cold bench and set at 25V for 20 min. Electrophoresis were performed under light and dark conditions.

2.7. Neutralization, Fixation and Staining

Slides were washed in PBS (Sigma-Aldrich®) for 10 min at 4°C, fixed with 96% ethanol for 10 min, and left to dry at room temperature overnight.

After staining with 20µL of DAPI (1 µg/mL) and covered with a 22x22mm coverslip, slides were kept in the dark until visualization.

2.8. Comets Visualization and Classification

Slides, containing two gels, were visual analyzed under a fluorescence microscope (Nikon Eclipse E400, Japan, magnification ×400) until 100 comets/gel. When it is not possible to classify 100 comets an extrapolation of the value is carried out. Samples with less than 20 comets are not considered. The comets to score are randomly selected among distinct regions of the slide, excluding the edges and places with deformities (*e.g.* air bubbles, overlapping or anucleate comets). Comets were classified into five classes from 0 to 4:

- Class 0 corresponds to intact comets, without any damage;
- Class 1 where minimal damage was observed;
- Class 2 comets with medium nucleus and higher fragmentation, compared to the class one;
- Class 3 to comets with intense damage; and,
- Class 4 to maximum damage.

The score (between 0 and 400 AU) results from the sum of the multiplication of the class by the number of comets associated with each class (for each gel) (equation 1). The score of the slide or damage index results from the average of the scores of the two gels. Samples with more than 100UA were categorized as having damage.

Visual selection of mononuclear leukocytes instead of polymorphonuclear leukocytes is possible for the human eye during scoring; however, this may be challenging for an automated system.

$$\text{Score (AU)} = (0 \times \text{a}) + (1 \times \text{b}) + (2 \times \text{c}) + (3 \times \text{d}) + (4 \times \text{e})$$

Equation 1 - Formula for calculating the score of a gel. In the end between 0 and 400 AU are obtained. The letters a,b,c,d,e correspond to the number of times that comets of class 0,1,2,3,4 (represented in blue) are present, respectively.

2.9. Statistical analysis

Statistical analysis was performed with SigmaPlot version 14.0 (Systat Software®Inc.,Chicago, IL, United States). The distribution of data was evaluated by Shapiro-Wilk test. Student's t-test or Mann Whitney U test were used for continuous comparisons. $P<0.05$ was considered to indicate a statistically significant difference. The assays were performed in duplicate in independent experiments.

2.10. Bioinformatics methods

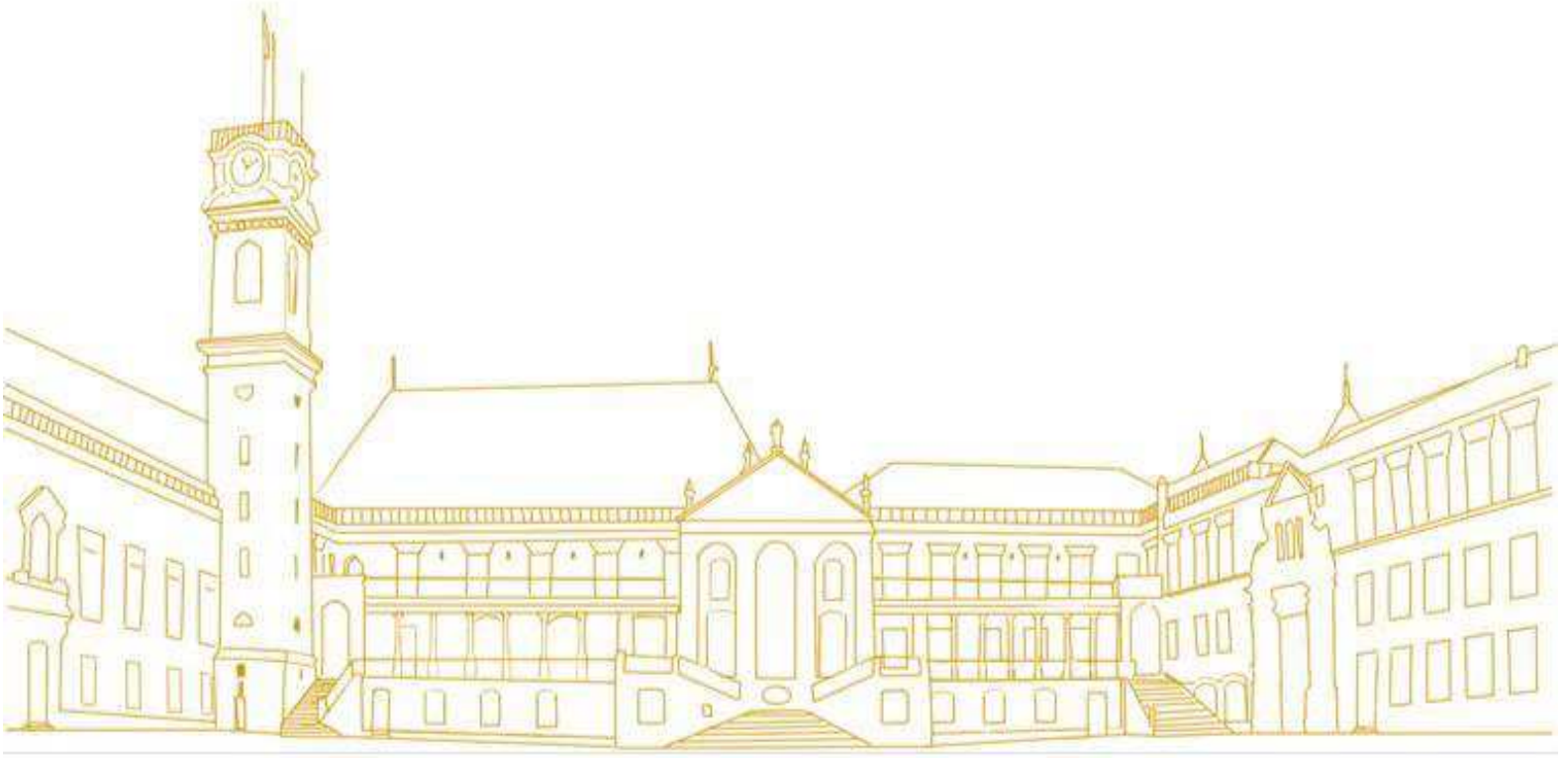
WikiPathways (https://www.wikipathways.org/) [8] database was used to explore the various pathways related to the *FMR1* gene, ovarian infertility, and DNA fragmentation.

PathVisio [85] was used to analyze, draw and editing pathways and to include the gene(s) previously unreported. Cytoscape_v3.9.1 [86] software was used to identify overlapping genes and visualize interactions of *FMR1* gene and coded FMRP with ovarian infertility and DNA damage pathways, with allows integrate them with gene expression profiles and other state data.

Biological databases like Uniprot (https://www.uniprot.org/), STRING (https://string-db.org/), and The Human Protein Atlas (https://www.proteinatlas.org/) were also used to retrieve expression profile of genes of interest.

CHAPTER 3.

RESULTS AND DISCUSSION



Chapter 3 | Results and discussion

1. Optimization of the comet assay

Optimization of the comet assay was carried out on blood samples to determine DNA damage. In presence of DNA damage, single-stranded and double-stranded damaged DNA migrate creating a tail-like structure (comet). Comets form as the broken ends of the negatively charged DNA molecule become free to migrate in the electric field towards the anode during electrophoresis. Samples with DNA damage exhibit more drag due to the migration of the breaks. Each comet is assigned a class and finally the score is calculated based on the number of comets in each class, as described in the materials and methods chapter.

1.1. Steps compromised by precoating

To further understand which downstream step depends on precoating step, the assay was performed on slides with and without a coating pretreatment using 1% normal melting point agarose (NMPA) (GRiSP), in same blood samples. Several differences were observed when agarose was placed (figure 5).

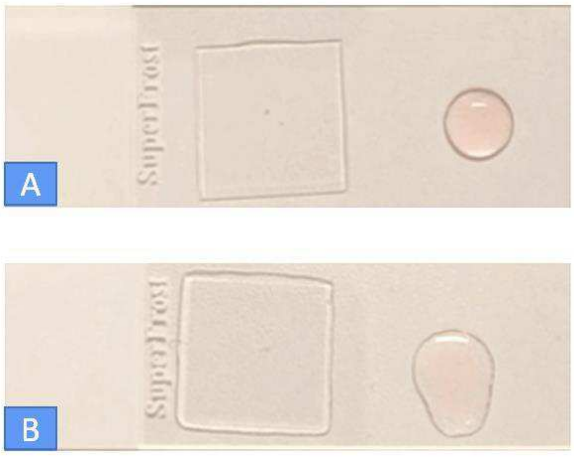


Figure 5 - Formation of the gels, sample deposition, in slide. Slides with (A) and without (B) precoating.

On the precoated slides (figure 5 – A), when the coverslips were removed after 5 min at 4ºC, the sample under agarose adhered to the slide, and the gel was kept intact throughout the following steps. In the absence of precoating (figure 5 – B) gels were correctly formed and some remained intact while others were lost. During lysis step the remain gels were compromised, which led to the conclusion that in the absence of

precoating it would not be possible to continue the assay beyond this step. Precoating with NMPA proved to be critical to ensure that the sample embedded in LMPA adheres to the slide and is not lost in the remaining steps.

1.2. Influence of the materials used

1.2.1. Type of microscope slides

Five different microscope slides were tested (table 1 - materials and methods) using the same blood sample. Under the microscope different types of impurities were observed among the five slide types (figure 6).

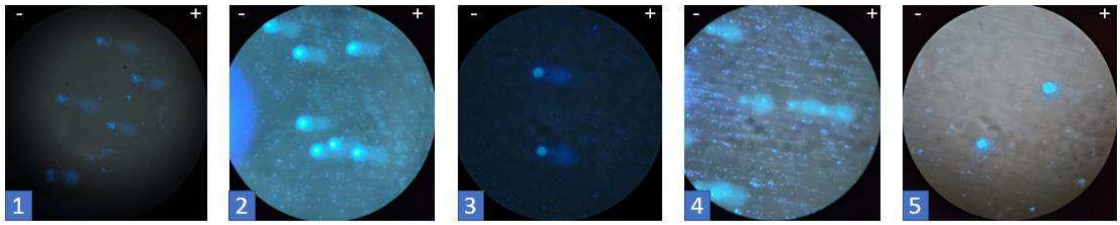


Figure 6 - Comparison of comet assay results between slide types. The number of the images corresponds to the slides described on table 1 - materials and methods.

Using slide type 1 structures other than the ones of interest were observed. In slide type 2 the background was stained hampering observation of comets. Using slide type 3 the comets were observed with no other stained structures. In type 4 comets were observed but with a background scratched with DAPI (1 µg/mL). In type 5 slides there was no background and comets were observed although faint, especially when compared to those in type 3 slides, possibly due to a "nebulous" background (figure 7).

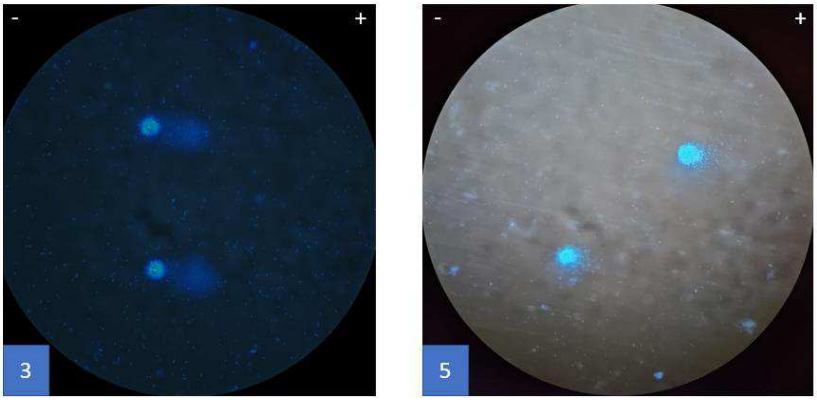


Figure 7 - Comparison between slide types 3 and 5. The number of the images correspond to the slide type described in table 1. Electrodes are represented as: (-) anode and (+) cathode. Images obtained by

OptikaISview 3.6.6 Software (OPTIKA©, Via Rigla, Ponteranica, Italy) coupled connected with the OPTIKA© 4083.B5 camera (Via Rigla, Ponteranica, Italy) coupled to the florescence microscope Eclipse E400 (Nikon, Japan, magnification ×400).

When we are viewing samples under the microscope, the field must be as clean as possible otherwise other components or structures (named impurities) may overlap with the comets or retain the DAPI (1 µg/mL) and disturb the analysis. Considering the comet and background intensities and the absence of other impurities, slide 3 showed better results.

1.2.2. Microscope coverslips adhesive proprieties

Two different glass microscope coverslips were tested: (A) Menzel Gläser SuperFrost® (Thermo Fisher Scientific, Waltham, Massachusetts, United States) and (B) HistoStar™ HistoStar™ (Vidrolab, Gandra, Paredes, Portugal) both with 22x22mm. When type (A) coverslips were removed the gel remained intact (figure 8 – A). On the other hand, type (B) showed high adherence, compromising the gel integrity after removal (figure 8 – B) and leading to gel loss in many instances (figure 8 – C). Furthermore, when using coverslips type (B) for staining, these did not detach from the gels, preventing future re-staining and visualization of those samples.

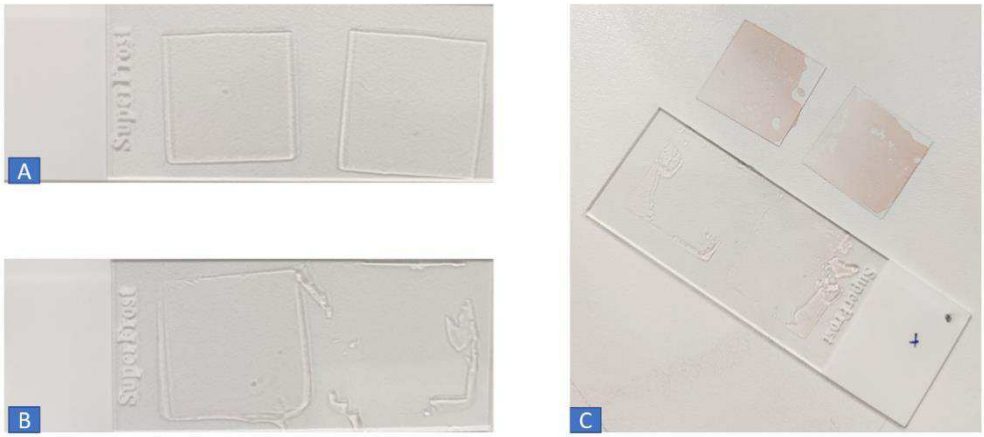


Figure 8 - Formation of gels and coverslips removal. A- Menzel Gläser SuperFrost® microscope coverslips (Thermo Fisher Scientific) showing intact gels; B- HistoStar™ (Vidrolab) coverslips compromising the integrity of the gels; C- Coverslips from HistoStar™ (Vidrolab) – examples where gels did not adhere to the slide.

1.2.3. Agarose Brand

Low gelling temperature agarose (LMPA) is ideal for the recovery of nucleic acid fragments after electrophoresis, as it melts at 65.5°C, near to the physiological temperature, avoiding denaturation of proteins and nucleic acids. Two brands of LMPA: (A) Bio-Rad and (B) Sigma-Aldrich® were tested in same samples. Evaluating the intensity of the comets and their structure LMPA (B) showed better results (figure 9). The use of the appropriate brand of LMPA has proven to be critical to perform the assay.

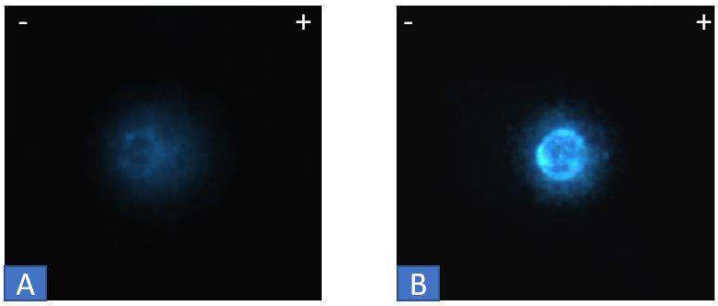


Figure 9 - Comet assay results using different brands of LMPA. Assay performed with A- Bio-Rad LMPA; B- Sigma-Aldrich® LMPA. Electrodes are represented as: (-) anode and (+) cathode. Images obtained by OptikaISview 3.6.6 Software (OPTIKA©) connected with the OPTIKA 4083.B5 camera (OPTIKA©) coupled to the florescence microscope Eclipse E400 (Nikon, magnification ×400).

1.3. Testing concentration of key reagents

Experiments were performed to ensure that proper assay conditions and reagents were used in the assay.

1.3.1. Low melting point agarose (LMPA)

The preparation of the gel is an important procedure of the comet assay as the sample is placed on the slide with the agarose to form a gel, ensuring that the sample adheres to the precoated slide. The recommended LMPA concentration ranges from 0.5-1% however the final concentration is variable depending on the type of sample [87].

Different concentrations of Sigma-Aldrich® LMPA: 0.6%, 0.8%, 1% and 1.5% were tested. Different aspects were evaluated:

- 0.6% LMPA originated very liquid gels, leading to gel disruption and electrophoretic run compromise; in cases of assay completion comets were very tenuous (figure 10 – A).

- 0.8% LMPA, the gels were intact and consolidated, allowing conclusion of the assay with intense comets easily recognizable (figure 10 – B).
- 1% LMPA showed similar results as 0.8% LMPA, however there was a slight obstruction in the migration of DNA along the gel (figure 10 – B).
- 1.5% LMPA, gels solidify faster which hampered spreading; besides affecting the electrophoretic run also hampered proper comet characterization as the comets are positioned in different optical planes (figure 10 – C).

A high percentage of LMPA prevents DNA migration, whereas a low concentration increases the fluidity of the gel and the risk of detachment. Under the established conditions the use of 0.8% agarose showed the best result.

Figure 10 – Gel flowability when applying different concentration of LMPA (Sigma-Aldrich®). A- 0.6%; B- 0.8/ 1%; C- 1.5% LMPA.

1.3.2. Triton X-100

Two different concentrations of Triton X-100 (Sigma-Aldrich®, St. Louis, Missouri, United States) were tested in the preparation of the lysis solution: 1% and 0.01%. At the lowest concentration cell nucleus became small (figure 11 – A). With 1% Triton X-100 (Sigma-Aldrich®) nucleus showed normal size and appearance and comets are visible with the predicted structure (figure 11 – B).

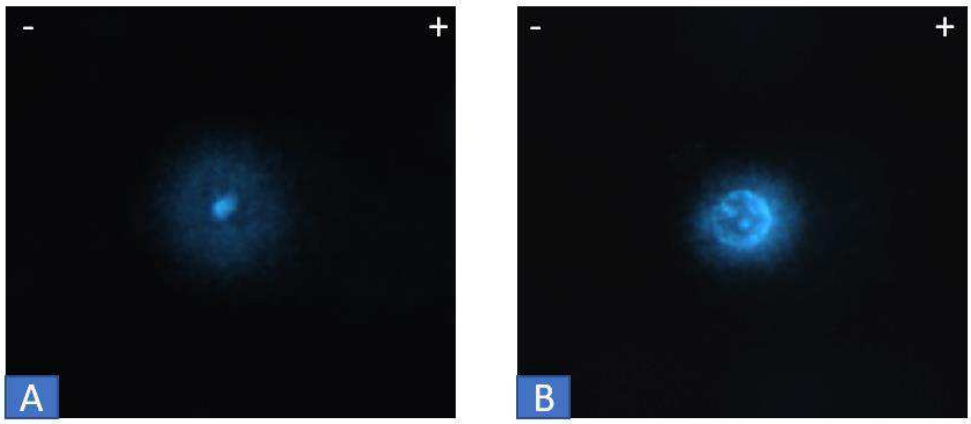


Figure 11 – Results of comet assay with different concentrations of Triton X-100 (Sigma-Aldrich®): A- 0.01%; B- 1%. Electrodes are represented as: (-) anode and (+) cathode. Images obtained by OptikaISview 3.6.6 Software (OPTIKA©) connected with the OPTIKA 4083.B5 camera (OPTIKA©) coupled to the florescence microscope Eclipse E400 (Nikon, magnification ×400).

Triton X-100 (Sigma-Aldrich®) is a detergent used to permeabilize membranes and when applied at low concentrations seems to be unable to promote lysis. Besides pH 10, Triton X-100 (Sigma-Aldrich®) concentration is fundamental to ensure the success of the lysis step allowing cell lysis to occur and the assay to run successfully.

1.4. Incubation times

1.4.1. Lysis

Comet assay was performed in the same samples, using distinct lysis incubation times: 1 and 24 h. The results showed no differences. The OECD in vivo comet assay guidelines recommend at least 1 h of lysis incubation time, although suggesting that longer lysis periods (e.g. 24 h) are also feasible. Of note, lysis conditions (including incubation time) should be kept as uniform as possible within an experiment [88] to allow a more robust comparison of results among different samples.

1.4.2. Alkaline treatment

Two different incubation times were tested: 30 and 40 min, when incubated for 30 min the comets were brighter and clearly defined, thus simplifying the classification (figure 12 – A) when compared to 40 min incubation time (figure 12 – B).

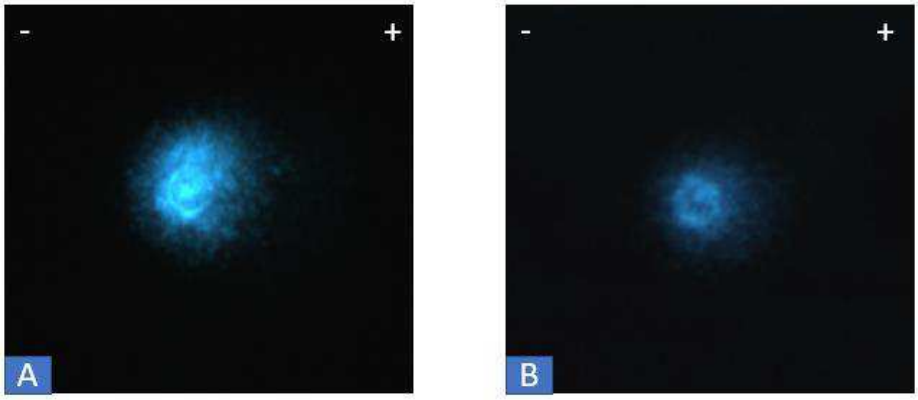


Figure 12 - Visualization of the comets stained with DAPI after different alkaline treatment incubation times: A- 30 min; B- 40min. Electrodes are represented as: (-) anode and (+) cathode. Images obtained by OptikaISview 3.6.6 Software (OPTIKA©) connected with the OPTIKA 4083.B5 camera (OPTIKA©) coupled to the florescence microscope Eclipse E400 (Nikon, magnification ×400).

These results demonstrate that a 10 min difference in alkaline treatment incubation time is not critical for the results but when a 30 min incubation time is used there is a better visualization of the comets.

1.5. Electrophoretic conditions

1.5.1. Voltage

Two voltage parameters were tested: 0.8V/cm and 1V/cm (as the distance between the electrodes is 25cm, voltages of 20V and 25V were, respectively, applied). Visually the samples subjected to 1V/cm (figure 13 – A) reveal comets with higher entrainment/migration when compared to 0.8V/cm (figure 13 – B).

Electrophoretic conditions that favor the formation of comets with very long tails are not recommended because these are prone to overlap. Running at 20V proved to be more efficient in solving this issue.

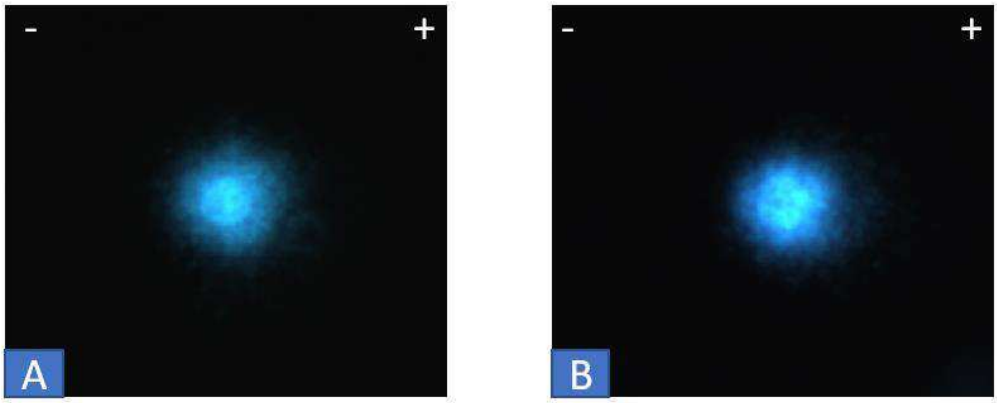


Figure 13 – Comet assay results testing two different voltages: A-20V (0.8V/cm); B-25V (1V/cm). Electrodes are represented as: (-) anode and (+) cathode. Images obtained by OptikaISview 3.6.6 Software (OPTIKA©) connected with the OPTIKA 4083.B5 camera (OPTIKA©) coupled to the florescence microscope Eclipse E400 (Nikon, magnification ×400).

This step should be controlled to increase sensitivity and importantly reproducibility. Conditions used should guarantee the possibility to sort all comet types in the sample, including those with longer tails.

1.5.2. Light and dark conditions

Electrophoresis was performed on a common electrophoresis tank exposed to light and in a tank that allows dark conditions (figure 14).

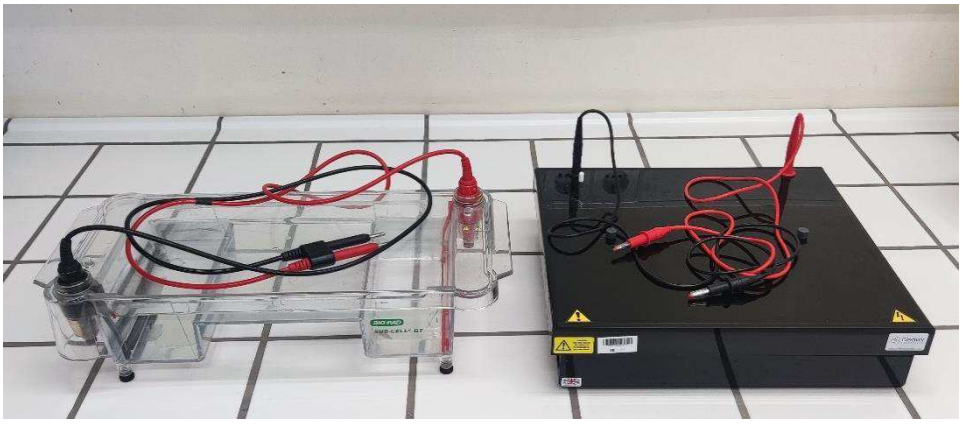


Figure 14 – Electrophoresis light and dark tanks, respectively.

Samples subjected to light electrophoresis (figure 15 – A) showed fainter comets when compared to the dark conditions (figure 15 – B). The results reveal that electrophoresis should be preferably performed in a dark tank, to minimize exposure to light and reduce background “noise”. No differences in the DNA damage of the samples

tested were observed among the tested conditions, neither in the structure of the comets obtained. Although it influences the visualization, this step did not prove to be critical for the assay.

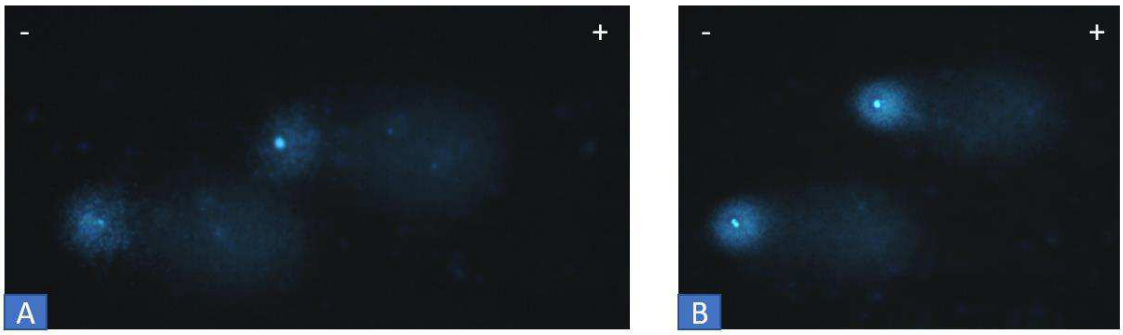


Figure 15 – Visualization of the comets stained with DAPI. Comet assay was performed under light (A) and dark (B) electrophoretic conditions. Electrodes are represented as: (-) anode and (+) cathode. Images obtained by OptikaISview 3.6.6 Software (OPTIKA©) connected with the OPTIKA 4083.B5 camera (OPTIKA©) coupled to the florescence microscope Eclipse E400 (Nikon, magnification ×400).

1.6. Positive Control (mock)

Exposing cells to a toxic agent DNA fragmentation is expected. H_2O_2 is a by-product of reactive oxygen species (ROS), which can cause DNA strand breaks through the loss of DNA bases, known as AP sites, and inhibits transcription. It is the radical most frequently responsible for oxidative damage [89]. It has been used to replicate oxidative stress *in vitro* in many different cell types and therefore widely used as a positive control in the comet assay.

Blood samples were exposed to 10% H_2O_2 [30% H_2O_2 stock solution (Pareac)] for 10 min (figure 16 – B). Negative control, without H_2O_2, is represented in figure 16 – A.

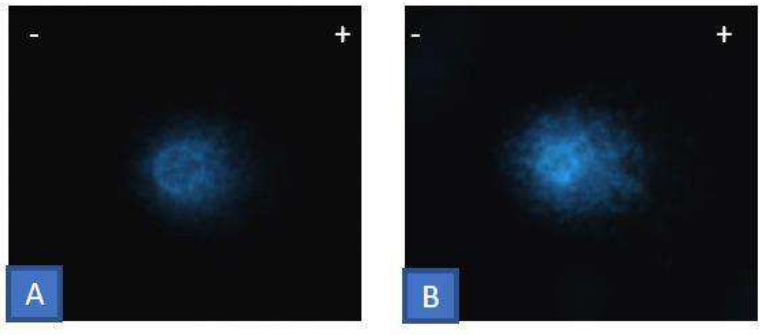


Figure 16 – Comet assay negative (A) and positive (B) control. Positive control were exposed to 10% H_2O_2 for 10 min. Electrodes are represented as: (-) anode and (+) cathode. Images obtained by OptikaISview 3.6.6 Software (OPTIKA©) connected with the OPTIKA 4083.B5 camera (OPTIKA©) coupled to the florescence microscope Eclipse E400 (Nikon, magnification ×400).

The results showed comets with some damage but without the typical fragmented structure, moreover, there was not a major difference between the positive and negative control. Therefore, it was important to understand if there could be an imbalance between the exposure time and the concentration applied.

Three different concentrations of H_2O_2 and four different exposure times were tested using 30% stock solution (Pareac). After the formation of the gels, the slides were submitted to 10%, 15%, and 20% of H_2O_2 during an incubation time of 5, 10, 15, and 20 min (figure 17).

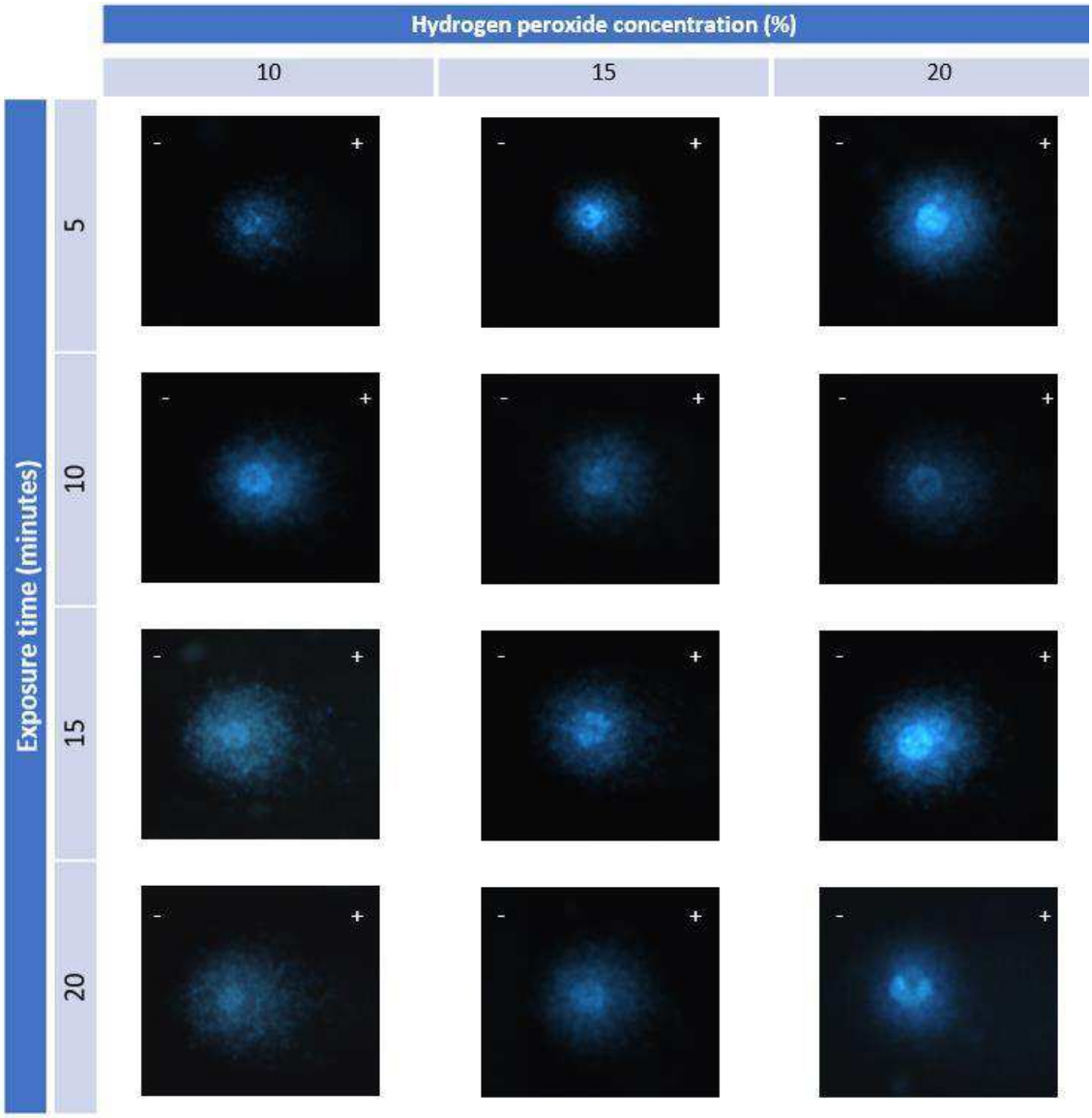


Figure 17 – Comet assay results after exposure to different incubation times (5, 10, 15, 20 min) and H_2O_2 concentrations (10, 15, 20%). Electrodes are represented as: (-) anode and (+) cathode. Images obtained by OptikaISview 3.6.6 Software (OPTIKA©) connected with the OPTIKA 4083.B5 camera (OPTIKA©) coupled to the florescence microscope Eclipse E400 (Nikon, magnification ×400).

Considering the fragmentation observed, the 10% concentration and 20 min exposure time was more suitable. However, the results did not show uniformity, as the majority of the cells did not reveal damage and higher concentrations and exposure times revealed less damage. This unexpected result suggests a recovery from damage or a low DNA toxicity of the used H_2O_2.

Brand new stock solution of 0.9M H_2O_2 (Alvita) was tested exposing the slides to 100µM H_2O_2 for 5 min. The results showed comets with different levels of fragmentation (figure 18), as well as an increase in the number of cells with damage. Furthermore, the expected halo-like structures were also observed (figure 18 – C). These results allow using this concentration and exposure time to validate the comet assay. Hydrogen peroxide (H_2O_2) exposure was used to induce breaks in the DNA, to have a (mock) positive control that allows the confirmation of a successful experiment (namely electrophoretic separation) [89].

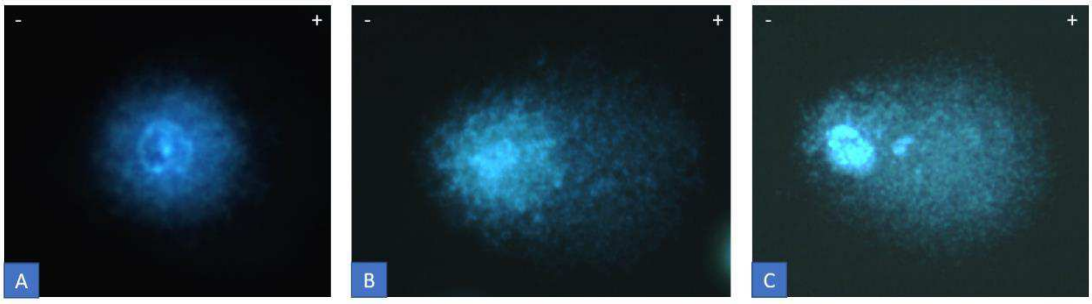


Figure 18 – Comparison of DNA migration between negative and positive control. A- Negative control; B and C – Positive control with 100µM H_2O_2 for 5 min. Electrodes are represented as: (-) anode and (+) cathode. Images obtained by OptikaISview 3.6.6 Software (OPTIKA©) connected with the OPTIKA 4083.B5 camera (OPTIKA©) coupled to the florescence microscope Eclipse E400 (Nikon, magnification ×400).

Addition of H_2O_2 directly to the gel was also tested, however this led to gel corruption, absence of uniform spread, hindering DAPI (1 µg/mL) staining and consequently compromising the analysis of the results (figure 19).

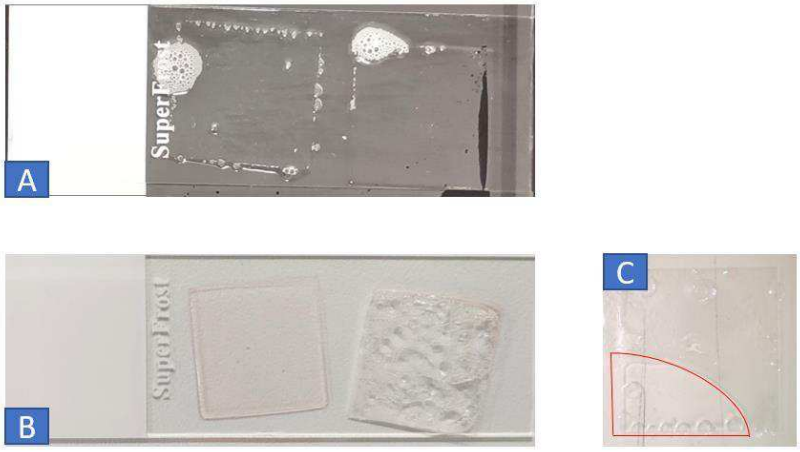


Figure 19 – Gels after slides were submitted to 100μM H_2O_2 for 5 min. A- using a staining jar - corruption is observed at the gel edges; B- applied directly on the gel - without and with H_2O_2, respectively; C- gel on the right (B) stained with DAPI (1 μg/mL), only the region highlighted in red showed staining uniformity.

Beyond the concentration used, it is important to ensure that the method of positive control exposure is also the most appropriate. The application of H_2O_2 when preparing the positive control proved to be critical for the analysis of the results.

1.7. Fixation

Comet assay was performed with and without the fixation step, using 96% ethanol. As expected, the absence of fixation did not change the results. Nevertheless, when fixated the slides can be stored for a long period of time allowing re-staining and visualization. This step did not prove to be critical for the assay results but instead for the analysis at “long term” as fixation maintains the integrity of the samples for future analyses, at any time after the experiment is finished.

1.8. Adjustment of the counting system

Comets can be classified by image analysis software or by visual analysis. Percentage of DNA in the tail is recommend to describe DNA breakage frequencies [90]. Herein, the classification of the assays was performed manually. After assay completion the slides were stained, observed under the microscope, and randomly around 100 comets per gel are classified as described in the materials and methods section.

A comet classification pattern was established in blood (figure 20) and CC (figure 21) samples to guarantee the uniformity of the results.

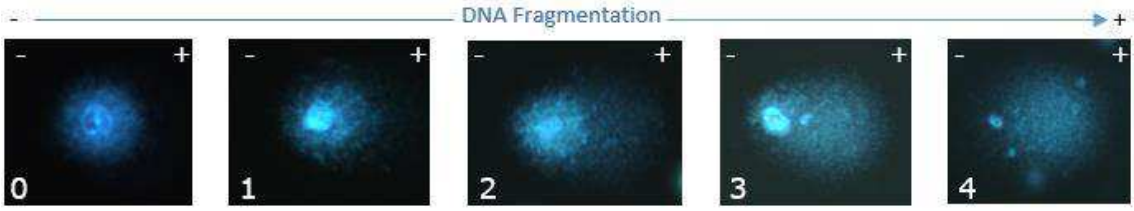


Figure 20 – Standardization of the comet assay classification in blood samples, classes 0 to 4. Electrodes are represented as: (-) anode and (+) cathode. Images obtained by OptikaISview 3.6.6 Software (OPTIKA©) connected with the OPTIKA 4083.B5 camera (OPTIKA©) coupled to the florescence microscope Eclipse E400 (Nikon, magnification ×400).

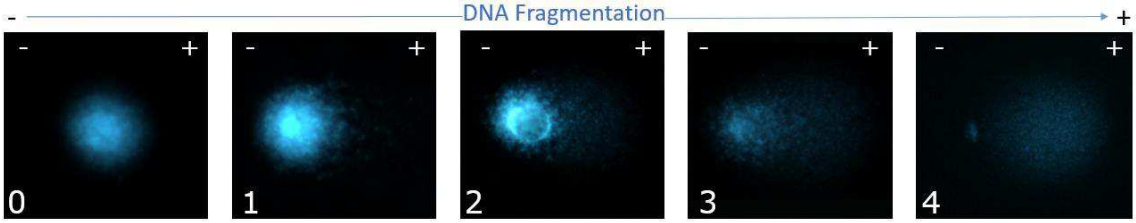


Figure 21 – Standardization of the comet assay classification performed on CC, classes 0 to 4. Electrodes are represented as: (-) anode and (+) cathode. Images obtained by OptikaISview 3.6.6 Software (OPTIKA©) connected with the OPTIKA 4083.B5 camera (OPTIKA©) coupled to the florescence microscope Eclipse E400 (Nikon, magnification ×400).

2. Assessment of DNA damage after a freezing cycle

Comet assay was performed in ten whole blood samples frozen at -70ºC, half of the samples were collected without anticoagulant, and the remaining five with, ethylenediamine tetraacetic acid (EDTA). EDTA is a polyprotic acid which contains four carboxylic acid groups and two amine groups with lone-pairs electrons that chelate calcium and many other metal ions. Calcium is required for several enzymes involved in reactions of the coagulation cascade. Thus, blood collection with anticoagulant prevents blood clotting within the collection tube, allowing the best preservation of cell components and blood cell morphology [91].

On the day of the experiment samples were placed in an ice bucket at 4°C, after 2 h the comet assay was performed. Frozen blood samples collected with anticoagulant were consistent with those obtained in pre-tested fresh samples. Frozen blood samples without anticoagulant exhibited significantly ($p<0.001$) more DNA fragmentation when compared with those containing EDTA (table 2). Furthermore, the absence of anticoagulant led to clot formation in some samples hindering the experiment. Frozen blood samples with EDTA remained intact without significant increased damage due to freezing/ thawing procedures. Storage of blood in tubes with this anticoagulant reveal to

be crucial to maintain cell's structure and the uniformity of the results. These results validate the assessment of DNA damage in whole blood samples after a freezing cycle, when collected with anticoagulant, ensuring that the results are real and not due to artifacts of freezing.

Table 2 - Average score of the ten frozen samples collected in the presence (+) and absence (-) of anticoagulant (EDTA). P<0.05 was considered to indicate a statistically significant difference.

Anticoagulant	Average score in blood cells (AU)	P value
+	3 ± 1.9	5.08×10^{-8}
-	88 ± 10.5	

3. Implementation of comet assay in cumulus cells

3.1. Fresh samples

The comet assay was performed on fresh blood and CC samples from three infertile women mean age 31.7 ± 1.2 years (range 30-33) (table 3). An increase of the DNA damage in CC was observed when compared with the corresponding blood samples which led us to suggest that different tissues might reveal different DNA damage patterns. On a first analysis of the table 3 seems to be an inverse correlation between DNA damage and the number of CC. It is tempting to think that the higher DNA fragmentation may be associated with a faster apoptosis process. However, it is not accurate to establish this connection since the number of cells counted is very low, close to the limit (only 21 cells counted in infertile 3) decreasing the specificity of the result.

Table 3 – Comet assay scores obtained on fresh blood and CC.

Infertile	Score in blood cells (AU)	Score in cumulus cells (AU)
1	36 [36]	17 [36]
2	64 [64]	115 [29]
3	105 [60]	219 [21]

*[Number of cells scored per gel]

3.2. Samples cryopreserved in liquid nitrogen

Comet assay was performed in duplicate in twelve cryopreserved samples: of these six belong to oocyte donors mean age 25.2 ± 4.4 years (range 20-32) (table 4 –

samples A to F) and the remaining six to females with infertility problems mean age 33.7 ± 3.4 (range 28-37 years) (table 4 – G to L).

Table 4 - Score obtained on blood and cryopreserved CC samples.

Sample	Score in blood (AU)	Score in CC (AU)
A	18 [100]	52 [100]
B	15 [100]	20 [100]
C	1 [100]	36 [20]
D	50 [100]	118 [100]
E	1 [100]	18 [100]
F	234 [100]	228 [100]
G	2 [100]	83 [100]
H	16 [100]	12 [100]
I	5 [100]	75 [100]
J	9 [100]	119 [100]
K	15 [100]	127 [100]
L	5 [100]	18 [100]

*[Number of cells scored per gel]

Overall, 8.33% of the samples presented DNA damage in blood and 33.33% in CC (score >100 AU). Considering the oocyte donor samples, there were no significant differences between the scores obtained in the blood and CC (Mann-Witney test p=0.180). In infertile samples there were significant differences between the scores obtained in the blood and CC (t-test p=0.005). Between oocyte donors and infertile females, no significant differences between the scores obtained in the blood (Mann-Witney test p=0.485) and in CC (t-test p=0.437) were identified.

Sample F, surprisingly reveal DNA damage in blood and CC which could be caused by exposure to external genotoxic agents or as the result of cellular pathologies. The presence in both tissues demonstrates that this damage is non-specific and unrelated with infertility, as was observed in an oocyte donor. Whereas infertile women with damage in CC did not present damage in the blood (samples J and K).

Sample D also presented DNA damage in CC and shows the highest score in the blood (after sample F) which suggests an intermediate state that eventually, over years, the fragmentation tends to increase, and may even present fertility problems.

4. Exploring linkages between female infertility, *FMR1* gene, and DNA damage

WikiPathways integrates information from different databases allowing the establishment of connections between different pathways ensuring that they are always up to date. This database was used as the basis for studying the link between *FMR1* gene and its interactions with female infertility and DNA integrity.

At first, manuscripts were explored to understand genes associated with FXS might be missing at WikiPathways and the microtubule-associated protein 1B (MAPB1) was added (annex 1 – figure 24). In this case, the PathVisio software (version 3.0), a pathway analysis and drawing software that allows analyzing, drawing, and editing biological pathways [85], were used to include the gene(s) previously unreported.

Cytoscape_v3.9.1 software [92] were then used to visualize molecular interaction networks between FXS and ovarian infertility pathways. The genes *MAPK1*, *AKT1, CREB1*, *PIP2*, *PLCG1*, *PRKCA*, *GRB2*, *SOS1*, *RAF1*, *MAP2K2*, *MAP2K1,* and *PIP3* shown to be common with the FXS, across several pathways (table 5).

Table 5 - Homo sapiens ovarian infertility pathways compared with *FMR1* gene pathway. The FXS pathway (annex 1) was crossed with pathways available at WikiPathways, using Cytoscape. Share genes are indicated. (-) Indicates absence of shared genes.

Pathway	**Genes in common**
Apoptosis-related network due to altered *NOTCH3* in ovarian cancer	*APP*; *RPS6KB1*; *MAPK1*; *AKT1*
FABP4 in ovarian cancer	-
Kisspeptin/kisspeptin receptor system in the ovary	*MMP9*; *PLCB1*; *PRKCA*; *PIP2*; *MAP2K2*; *MAPK1*; *KRAS*; *MAP2K1*; *RAF1*; *PIK3CB*; *PDK1*; *AKT1*; *PIP3*
Mammalian disorder of sexual development	-
MFAP5-mediated ovarian cancer cell motility and invasiveness	*CREB1*; *PIP2*; *PLCG1*
MFAP5 effect on permeability and motility of endothelial cells via cytoskeleton rearrangement	*CREB1*; *ITPR1*; *PLCG1*; *MAPK1*

miR-509-3p alteration of YAP 1/ECM axis	-
Model for regulation of MSMP expression in cancer cells and its proangiogenic role in ovarian tumors	-
Ovarian infertility	-
PDGFR-beta pathway	*PRKCA*; *SHC1*; *GRB2*; *PLCG1*; *SOS1*; *RAF1*
Peroxiredoxin 2 induced ovarian failure	-
TCA cycle nutrients use and invasiveness of ovarian cancer	-
RAC1/*PAK1*/p38/*MMP2* pathway	-
Endometrial cancer	*PIP3*; *GRB2*; *SOS1*; *KRAS*, *ARAF*; *BRAF*; *RAF1*; *MAP2K1*; *MAP2K2*; *MAPK1*; *PTEN*; *PIK3CB*; *AKT1*

In addition, FXS and miRNAs involved in DNA damage response pathways were compared and *CREB1* gene was retrieved as being in common. This gene was also present in ovarian infertility pathways, allowing to establish a relationship between the three study groups.

Cyclic AMP response element-binding protein 1 (*CREB1*) is known to be involved in follicular growth, ovulation, and ovarian disease [93]. This gene is expressed in various tissues, of particular interest for female fertility: vagina, ovary, fallopian tube, endometrium, cervix, placenta, and breast (figure 22).

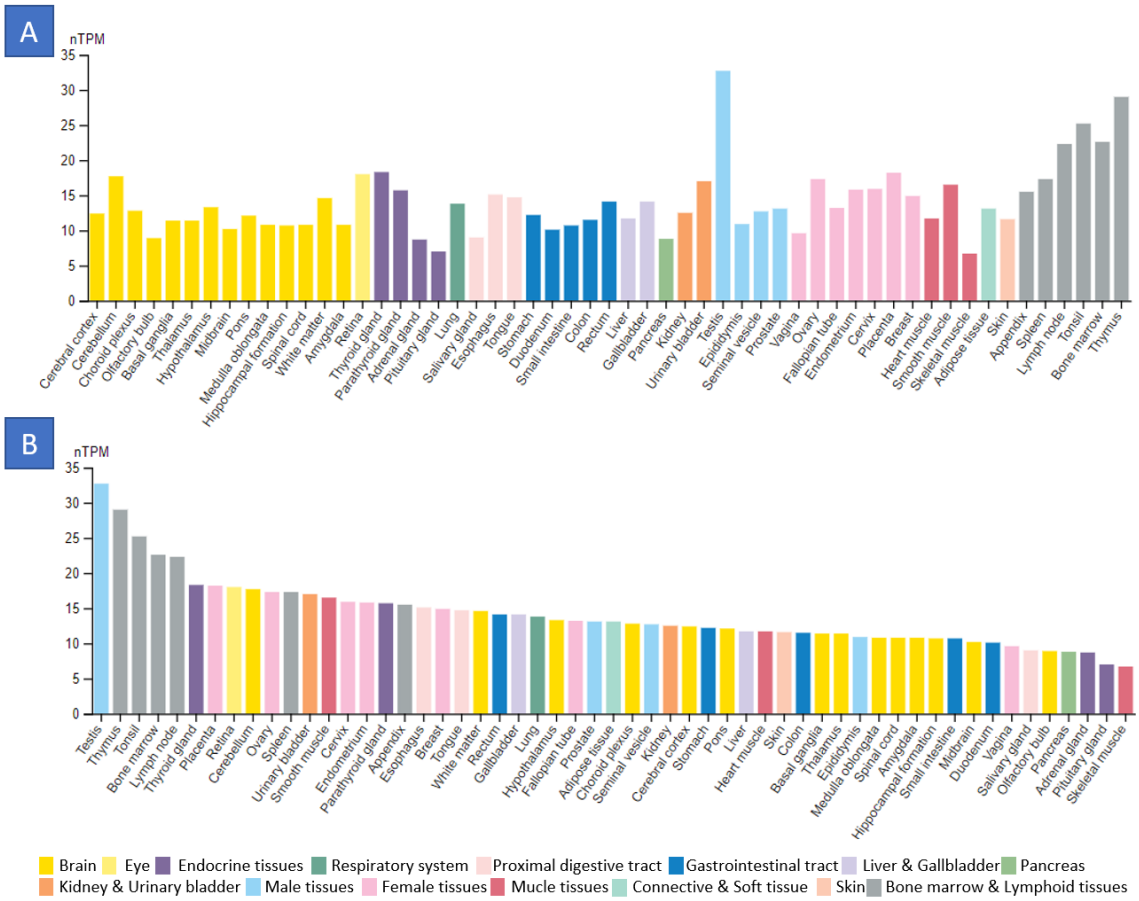
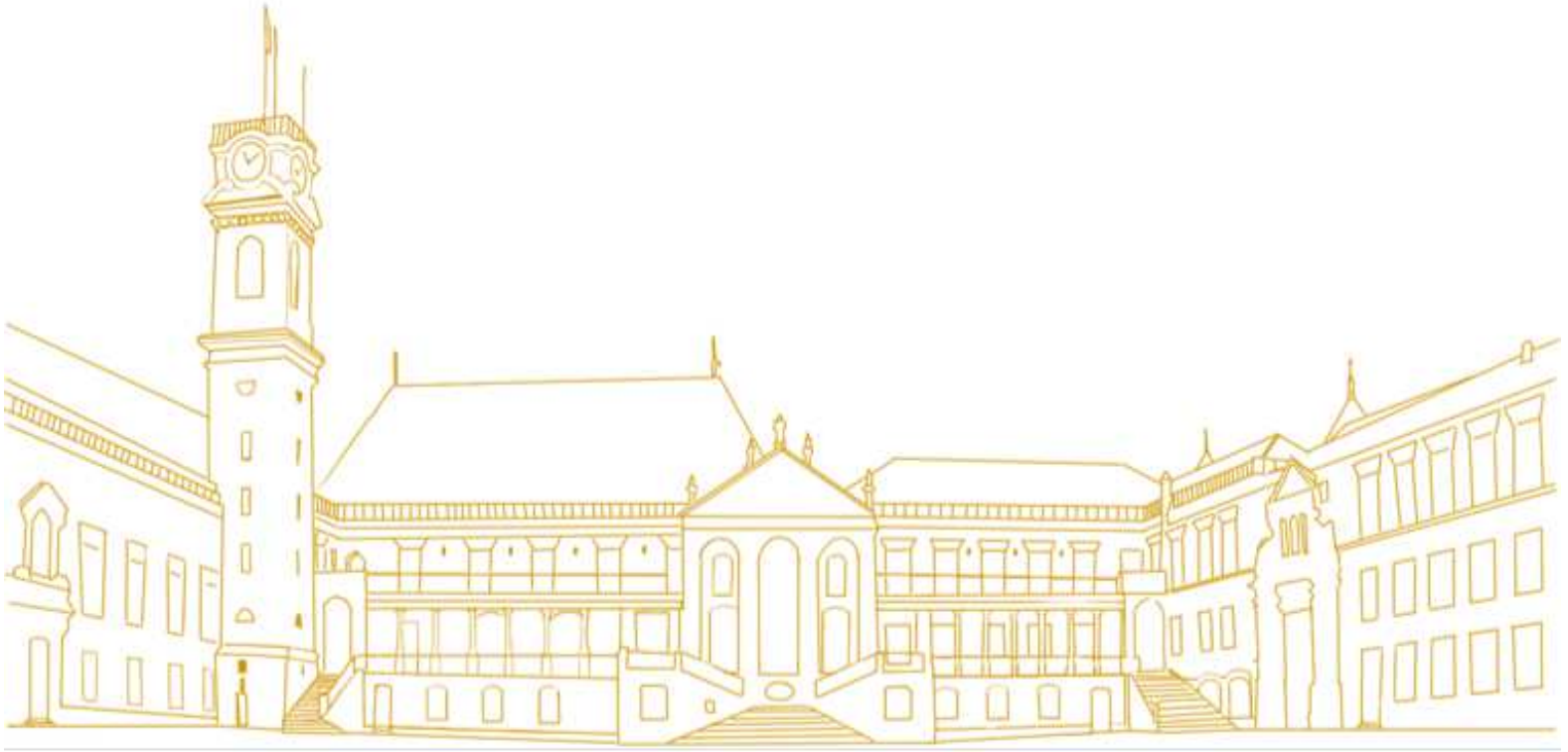


Figure 22 - *CREB1* RNA expression summary based on normalized transcript expression values (nTPM) and color-coding with is based on tissue groups. A- organized by tissue groups; B- organized by expression levels. Adapted from The Human Protein Atlas (https://www.proteinatlas.org/). Accessed on July 8, 2022.

CHAPTER 4.

CONCLUSIONS AND FUTURE PRESPECTIVES



Chapter 4 | Conclusions and Future perspectives

1. DNA damage in cumulus cells: comet assay

Since its first application more than 30 years ago [14], the comet assay has been widely used. The low cost and versatility make it the gold-standard test used worldwide to analyze DNA damage [99]. Consequently, there have been some variations in the protocol over time. Intra- and inter-laboratory variability is a disadvantage hampering the direct comparison of results and conclusions particularly between distinct laboratories [99].

Comet assay needs protocol optimization and standardization to enhance the uniformity and reproducibility of the results [99]. Therefore, significant effort was required to identify crucial parameters affecting the performance of the comet assay from sample preparation/ manipulation to the result analysis. Among these, precoating, concentration of low melting point agarose (LMPA), coverslips adhesive properties, positive control preparation and its application, concentration of Triton X-100, voltage set at the power supply and the counting system, proved to be critical steps and optimal conditions were established. The pH of lysis and electrophoresis solutions as well as the amount of buffer used in the electrophoresis and during the alkaline treatment were also important to the results of comet assay. Sample dilution, LMPA brand, lysis solution incubation time and running electrophoresis in light and dark conditions were also tested and should be considered but were not critical for the overall performance of the comet assay.

A positive control is always necessary to allow validation of the different steps of the assay towards comets classification. The key is to ensure that the toxic agent concentration used, and the exposure time are adequate to cause DNA damage allowing fragmentation to be separated during electrophoresis and visualized. Optimization of the positive control validated the assay and ensured uniformity of results.

The success in optimizing the comet assay in fresh whole blood samples has enabled the implementation of the assay in frozen samples. Frozen blood samples collected without anticoagulant (some of which were clotted), reveal a significant increase in DNA fragmentation when compared with those with EDTA. The presence of clot(s) made these samples difficult to use, as cellular constituents were retained. Despite the low number of samples tested, our results suggest that blood collection in tubes with EDTA are crucial to maintain cell's structure and the uniformity of the results. EDTA acts as a chelating agent on calcium preventing the coagulation cascade reaction and maintaining DNA integrity. To ensure the viability of the results comet assay should

be performed only on frozen blood samples collected with anticoagulant. Otherwise, lead to artefactual formation of DNA damage due to the freezing process and therefore the results should not be considered. However, for comparative studies, in which controls have been stored in the same conditions, this may not represent a significant issue.

Once optimized in blood samples the protocol was successfully implemented in cumulus cells (CC) where key points were established:

1. The dilution of CC samples in PBS is not comparable with blood samples as the number of CC will be much lower.
2. Samples should be well homogenized before start, to minimize clumping and overlapping comets.
3. Cumulus cells samples contaminated with blood should be performed one or two extra washes with PBS.
4. The comet assay protocol should be carried out rigorously as described herein.

Fresh blood and CC samples obtained dissimilar scores, suggesting that DNA damage occurs differently between these two tissues. Besides, it was tempting to assume that there was an inverse correlation between DNA damage and the number of CC, with could be associated with a faster apoptosis process. The apoptotic levels of CC are of particular interest to determine if there is a relationship between apoptosis and oocyte maturity with impact in the selection of oocytes for the infertility treatment [82]. However, it is not accurate to establish this connection since the number of cells counted is very low. Decreased number of cells may be associated with reduced CC volumes and may lead to an apparent increase in average damage levels, since scoring is not strictly limited to the center of the gel. As the number of cells per gel decreases, the operator is forced to score cells further away from the center of the gel. Thus, we propose that the increase in damage observed results from the increased contribution of "edge effects" (regions with damage accumulation).

Samples from oocyte donors and infertile female also presented different scores in blood and in the CC. Still there is no consensus about the embryonic origin of CC. In Sawyer et al. [94] studied they hypothesize that in sheep most granulosa cells develop from mesothelium cells, which are mesodermal origin, as well as blood cells. Our results point to differences between tissues of equal embryonic origin, will be interesting to study

a different embryonic origin and understand how the DNA fragmentation pattern behaves.

Analyzing the two tissues in samples from oocyte donors and infertile women we proposed that when the damage is in both tissues it is non-specific and therefore should not be correlated with infertility. In this case the damage may result from exposure to external factors (e.g. tobacco, medication) or even cellular pathology in contrast with when it is observed solely in CC. In addition, some infertile females did not present DNA damage in CC, with proves specificity of the assay and corroborates the use of comet assay. Despite the need to increase the number of samples tested this research highlights the importance of studying these two tissues and supports the hypothesis of using CC to target the oocyte status as a biomarker with clinical impact in female infertility treatments.

2. DNA damage in female fertility: *FMR1* and ovarian infertility common pathways

Primary ovarian insufficiency is one of the main causes of women infertility [6], affecting approximately 20% of women with an *FMR1* gene permutation [55<CGG<200] [95] (FXPOI; OMIM #311360) [70]. To increase knowledge on the impact of the *FMR1* premutation in infertility, *FMR1* gene and ovarian infertility pathways, described in WikiPathways, were compared to find common genes. *MAPK1*, *AKT1*, *CREB1*, *PIP2*, *PLCG1*, *PRKCA*, *GRB2*, *SOS1*, *RAF1*, *MAP2K2*, *MAP2K1,* and *PIP3* genes were shown to be common between pathways, emphasizing their importance in female infertility. Then the FXS and miRNA response DNA damage pathways were compared and reveal a single gene in common, Cyclic AMP response element-binding protein 1 (*CREB1*). Curiously, this gene has also coincident with the ovarian infertility pathways.

CREB1 gene has been described as an important regulator of the quality and number of oocytes and reproductive decline in aging [93]. Furthermore, Zhang et al., showed that *CREB1* inhibited the *Has2*, *Ptgs2*, and *Igfbp4* mRNA levels in mouse CC, suggesting that *CREB1* may be implicated in folliculogenesis, ovulation, and luteinization by regulating the expression of these genes [93]. Besides, Zhang et al. [93] and Lee A. et al. [96] demonstrated that *CREB1* down-regulation could be a major candidate gene for downregulating WNT signaling and consequently induce oocyte apoptosis. These findings provide a basis for further exploring the physiological significance of *CREB1* in granulosa cumulus cells. *CREB1* being the only gene in common that is involved in the three groups of pathways, led us to propose an interaction between them with a cumulative or repressive effect. Yet, to the best of our knowledge, no studies relate

FMR1 and *CREB1* genes to FXPOI. Despite the *in silico* analysis predicting an interaction, no literature on this subject was identified (PubMed accessed July 8, 2022). In mouse cerebellum, increased *FMR1* gene expression promotes increased *CREB1* gene expression. Therefore, the study of *CREB1* gene expression in human CC and particularly in samples belonging to females with a *FMR1* premutation is auspicious.

The *AKT1* gene, was not identified as a common gene to the three pathways through *in silico* analysis. However, literature analysis reveals that *AKT1* is a key protein that regulates many apoptotic pathways in response to DNA damage [97]. Which emphasizes the importance of complementing literature and bioinformatic analysis. *AKT1* expression was significantly higher in females with poor ovarian reserve and POI [73]. A link between *FMR1* and *AKT1* genes has not yet been established, however, Rehnitz et al. observed an *AKT1* gene downregulation in patients with POI and *FMR1* premutations, when compared to those without a premutation [97]. This finding enabled the addition of the *AKT1* gene to the DNA damage response and ovarian infertility pathways (annex 2), as well as establishing a relationship with POI. Besides, *AKT1* expression in CC may serve as a marker for a positive pregnancy outcome [67]. Furthermore, the fact that *CREB1* and *AKT1* genes are related to DNA fragmentation suggests an interaction between these and ovarian reserve, oocyte maturation and CC fragmentation.

Overall, the use of WikiPathways database provides the grounds supporting the importance of analyzing the expression of these genes in CC, to understand how these cells reflect the oocyte status and the possible use to identify a novel biomarker to select the “best” candidate to ensure the success of the ICSI technique.

CHAPTER 5.

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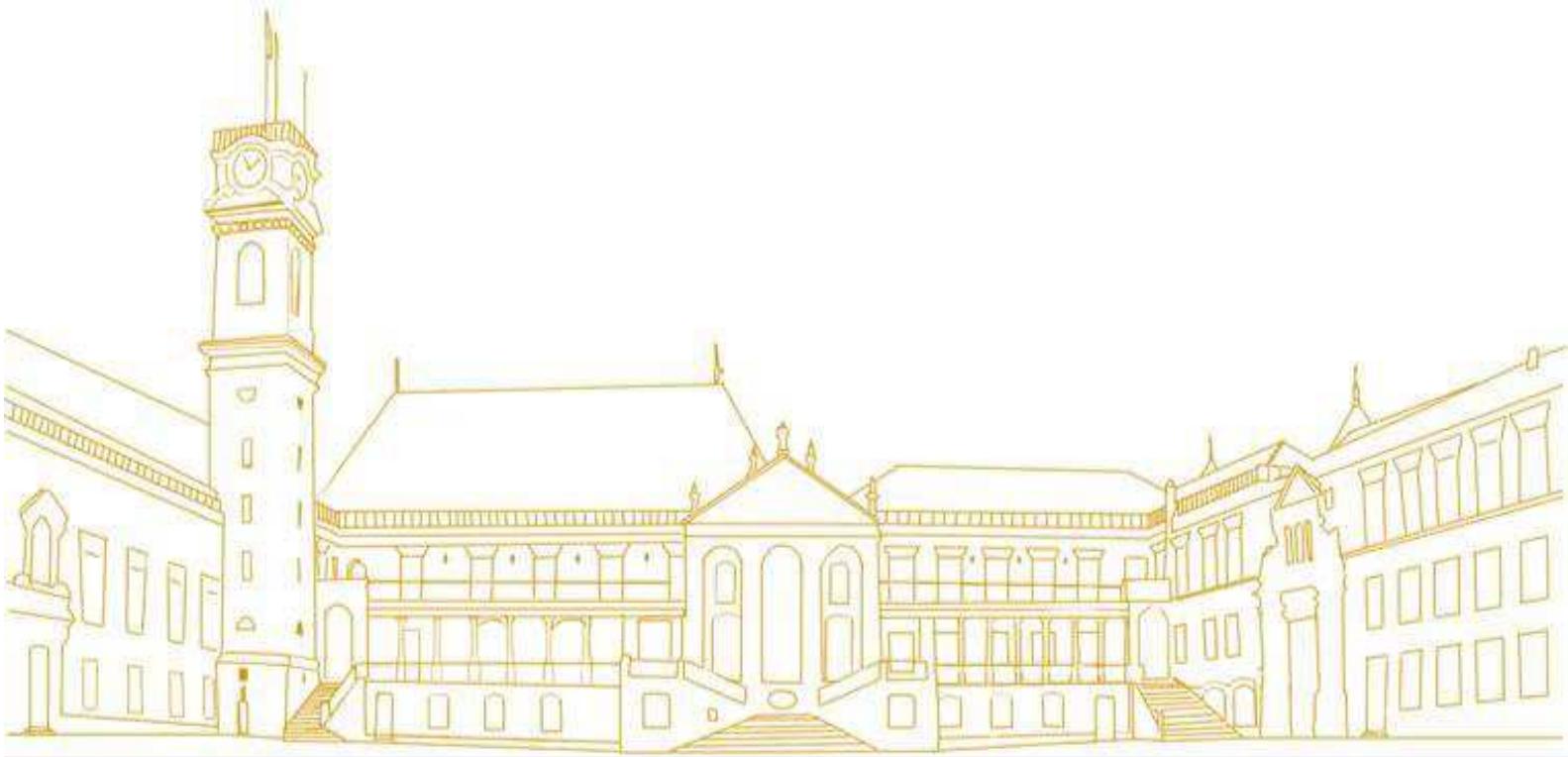

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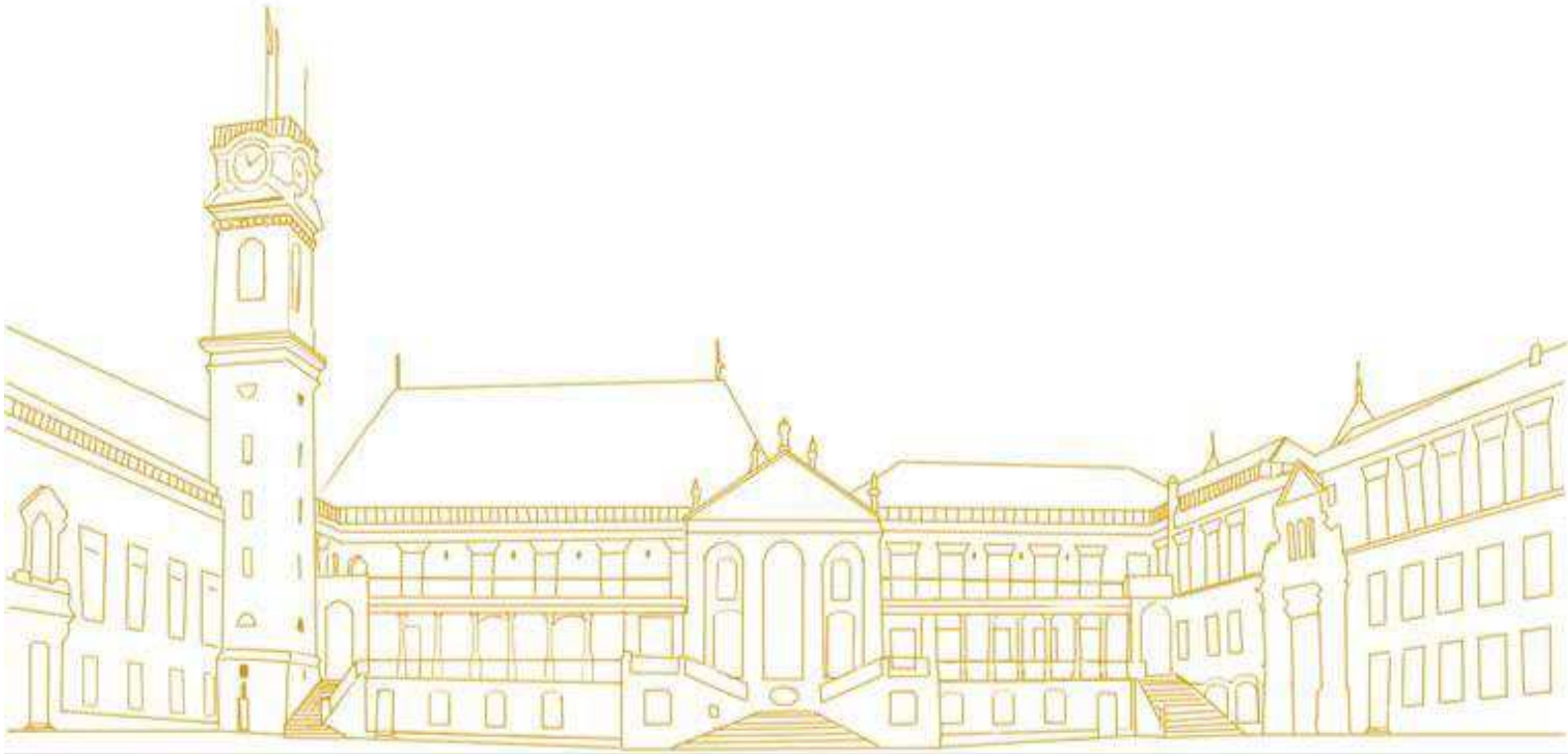
ANNEXES



ANNEX 1 | FXS pathway

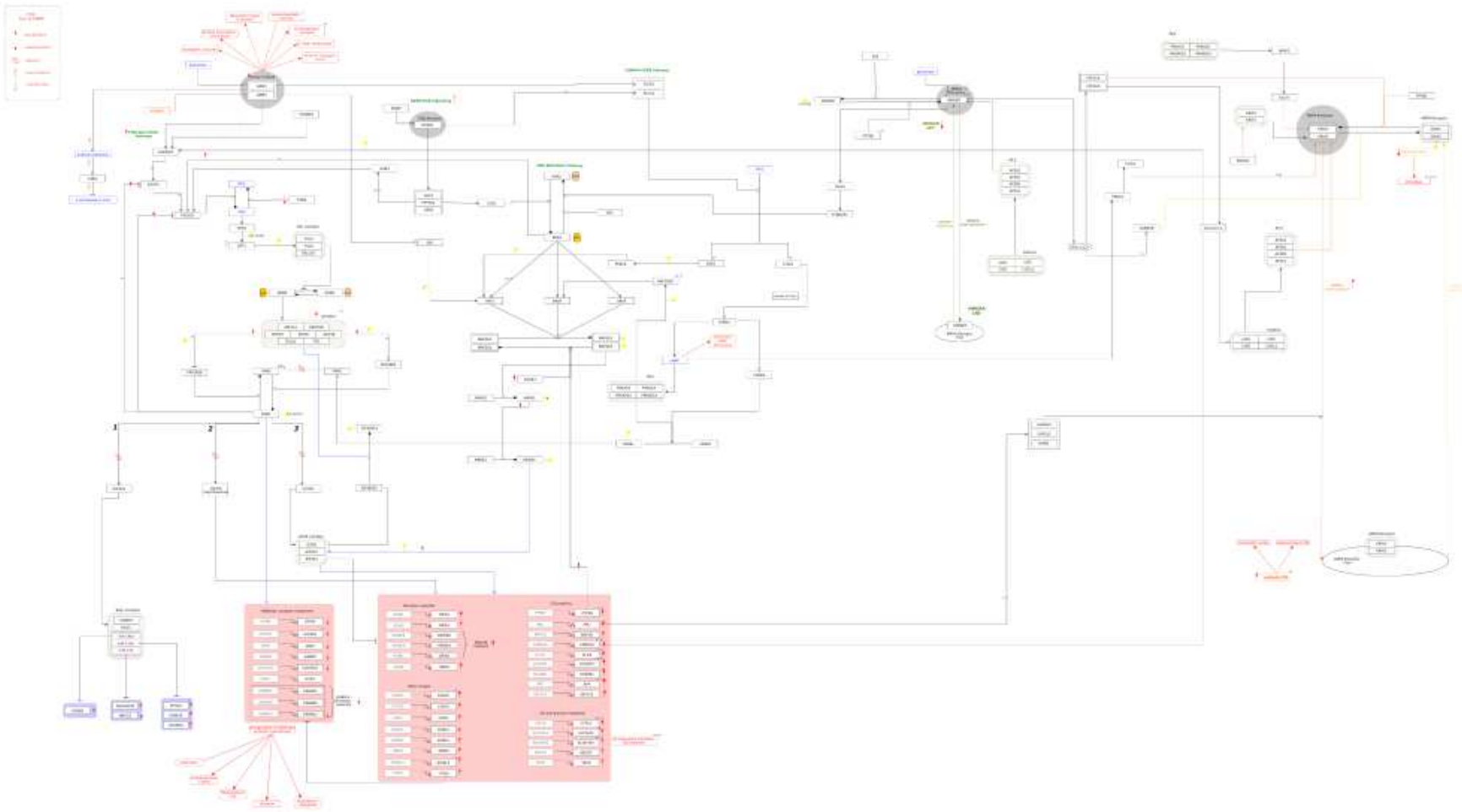


Figure 23 - FXS pathway available in WikiPathways (https://www.wikipathways.org/). Accessed in June 16, 2022.

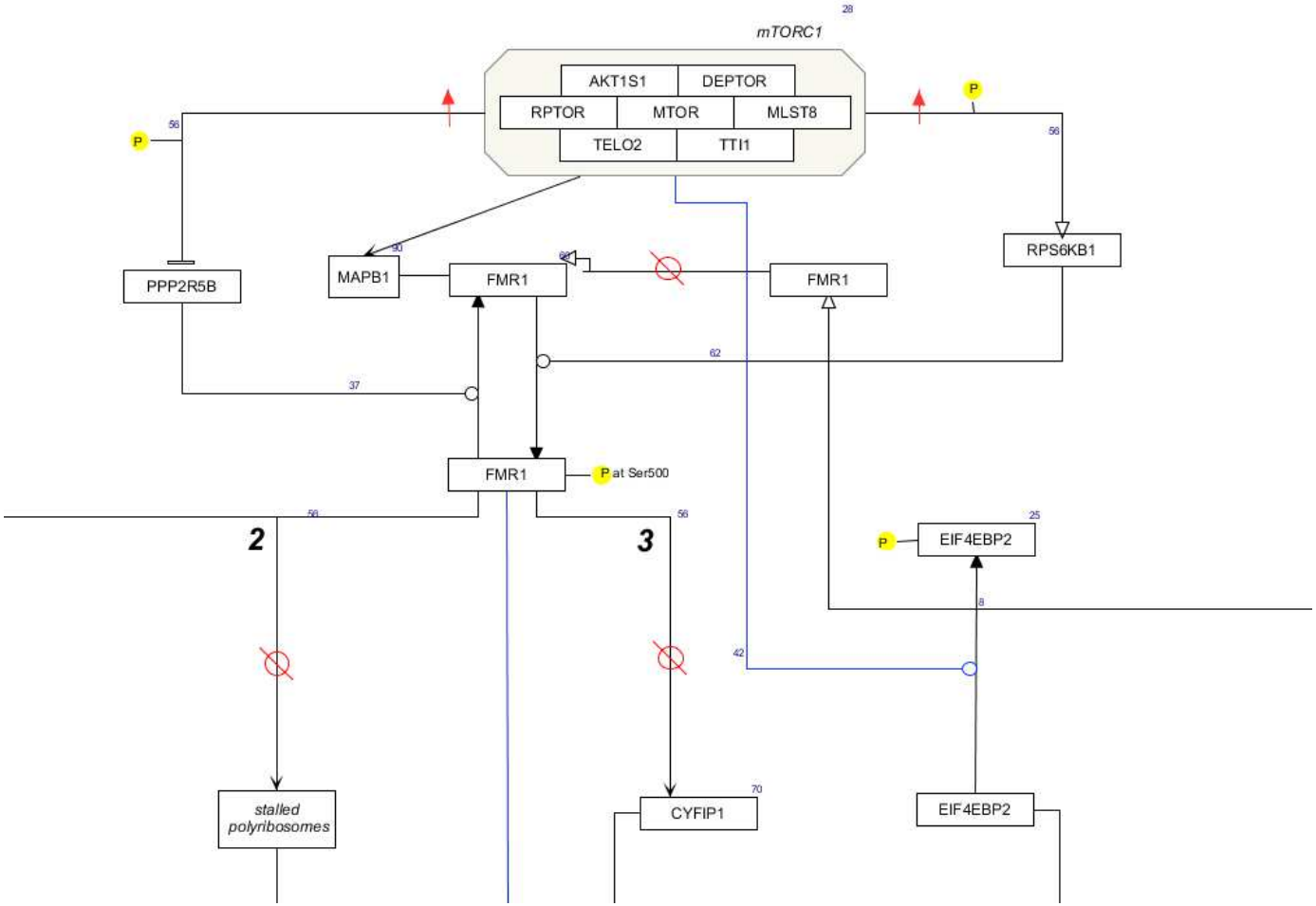


Figure 24 - FXS pathway available in WikiPathways with the addition of MAPB1 protein.

ANNEX 2 | *AKT1 gene*

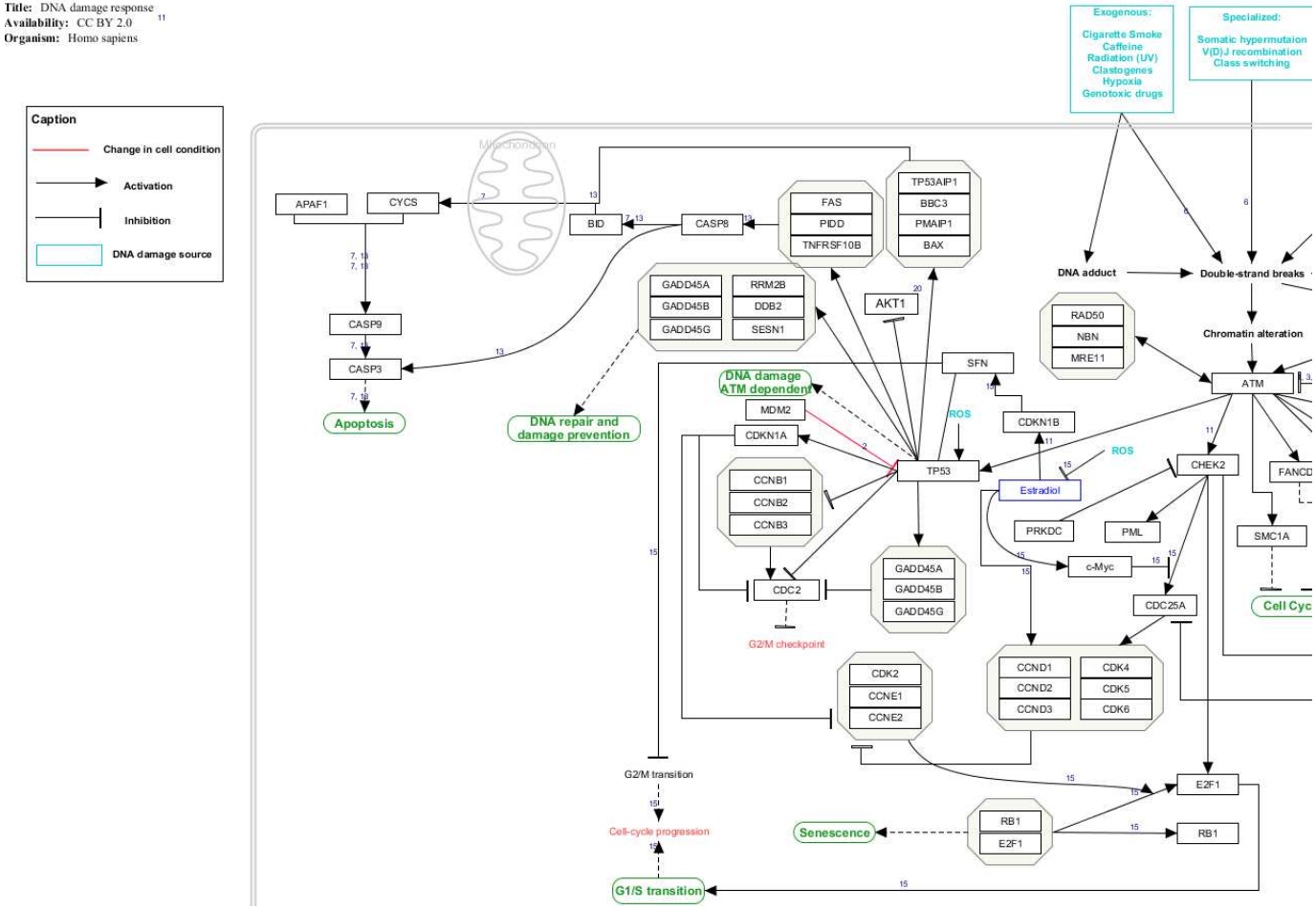


Figure 25 - DNA damage response pathway available in WikiPathways with the addition of *AKT1* gene.

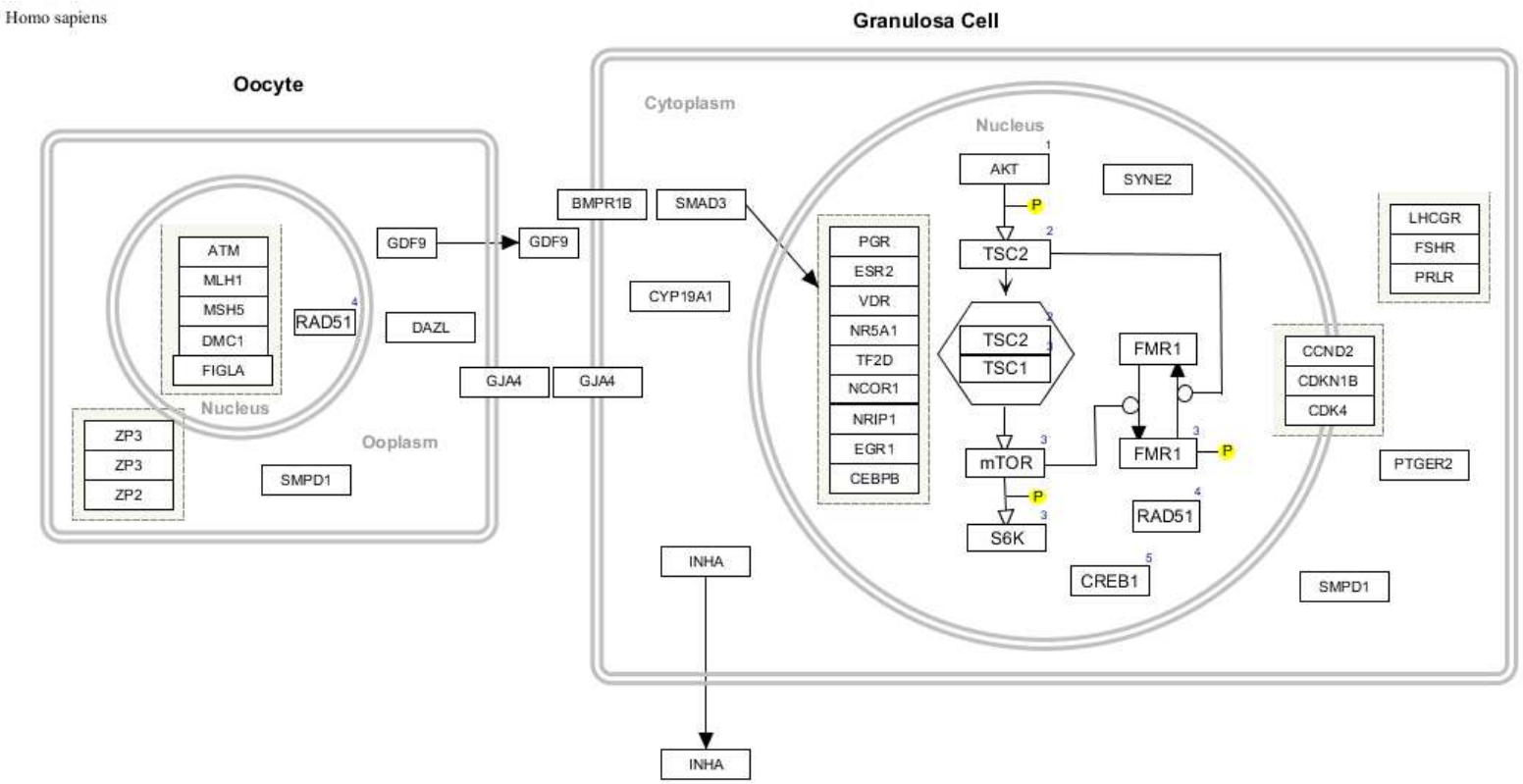


Figure 26 – Ovarian infertility pathway available in WikiPathways with the addition of *AKT, TSC1, TSC2, FMR1, RAD51, S6K* genes.

ADDENDUM

To the Master's dissertation: " Assessment of DNA damage in cumulus cells of infertile women " I thought it would be useful to add the present Addendum to my dissertation describing the other practical activities developed in the scope of clinical genetics at the Molecular Genetics Unit, Centro de Genética Médica Doutor Jacinto Magalhães, Centro Hospitalar Universitário do Porto.

1. Characterization of the laboratory

The Molecular Genetics unit of the Centro Genética Médica Doutor Jacinto Magalhães (CGMJM), Centro Hospitalar Universitário do Porto, encourages multidisciplinary work, integrating research clinical and laboratory components, with the mission of providing health care and training in the field of clinical genetics. In addition, this laboratory provides a wide range of genetic tests and advanced technologies, as well as a highly competent multidisciplinary team. During this dissertation, carried out in this unit, I had the opportunity to learn and develop several techniques, used in pre and pos natal molecular genetics diagnosis of monogenetic disorders.

2. Nucleic acid separation

Genomic DNA/RNA extraction from peripheral blood, or other tissues, was performed automatically by EZI Advance XL (Qiagen, Hilden, Germany) or manually using Salting-out method [98]. Quantification was performed by spectrophotometric quantification with NanoDrop™ ND-1000 (Alfagene®, Alagoa, Carcavelos, Portugal).

2.1. gDNA extraction

Automated DNA extraction was performed with EZ1 Advanced XL (Qiagen), based on magnetic bead technology, allowing extraction and purification of the DNA in a timesaving, cost-effective way. The equipment can process up to 14 samples in each run (≈20 min in case of blood). This method requires a small amount of initial sample - around 350µL of whole blood allows a quantitatively and reproducibly recover of DNA, even from complex samples - besides being a closed system minimizing the risk of contamination. Therefore, is ideal for in-process testing of contaminants and impurities. The DNA obtained by this extraction method showed DNA concentration of around 50

to 60 ng/µL. Examples extracted with this method included blood (n[1]=10), amniotic fluid (n=1), and chorionic villus samples (n=2).

Manual extraction by salting-out [98]Miller method is based on salting of cellular proteins by dehydration and precipitation with saturated NaCl solution [98]. The DNA obtained is of high concentration (approximately 500ng/µL) purity and molecular weight particularly useful when identifying and quantifying large repetitive DNA sequences (e.g. identification a *FMR1* gene full mutation, expansion of over 200 CGG repeats).

In clinical practice, the DNA obtained by automated extraction guarantees an adequate concentration for most of the subsequent techniques. However, there are some methodologies, such as Triplet Repeat Primed PCR (TP-PCR) and Southern blot that require higher DNA concentrations, for which the use of the salting out (n= 25) method is recommended.

2.2. RNA extraction

RNA extraction is based on the use of a lysis buffer to promote membrane rupture and the use of organic solvents like phenol-chloroform to recover RNA after centrifugation. RNA extraction is particularly challenging as this single-stranded molecule is very unstable. Moreover, RNA can be easily degraded by heat, hydrolysis and RNase if not immediately processed or appropriately stored. RNases are present in the surrounding environment and degrade RNA faster, when compared to DNA, so RNA extraction and preparation and use must be in a controlled, careful, and specific local using dedicated pipets (for instance) to avoid contamination and degradation.

RNA extraction (n=2) provide quality, pure, and intact total RNA, critical to use in downstream experiments such as: RT-PCR, qRT-PCR, array analysis, Northern blots, nuclease protection assays, RNA sequencing and transcriptome analysis.

Expression studies can be performed in a single step (conversion of RNA to cDNA and then amplificated) or in two steps (reverse transcriptase step and then amplification), providing information on transcriptional activity for a certain gene. Real time PCR is generally used as it is faster, sensitive, and quantitative. The one-step extraction also reduces the risk of contamination.

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[1] number of samples tested

3. Triplet-primed polymerase chain reaction (TP-PCR)

The principle of this technique is based on the use of three primers (instead of a pair): a forward primer, marked with a fluorochrome, which binds to the region flanking the upstream region of interest (ROI, usually a trinucleotide repetitive region), a triplet repeat primer with the repeats at its 3′ end to enable random annealing and a tail primer that is complementary to the 5′ overhanging sequence of the triplet repeat primer to extend and amplify the triplet repeat primer products.

As the PCR reaction occurs, the repeat primer will randomly anneal along the repeat, amplifying fragments of different sizes. The fragments are then separated by capillary electrophoresis on the ABI PRISM® 3130*xl* Genetic Analyzer (Applied Biosystems™, Foster City, CA, United States) and the amplified fragments can be analyzed using GeneMapper® software version 4.0 (Applied Biosystems™) This technique was used in the context of the [CGG] repetitive region of the *FMR1* gene, located in the 5´UTR, to determine the AGG interspersion pattern [99]. In addition, the protocol described by Silva C. et al. [100] was optimized with the aim of providing a screening strategy to detect the number of GCC repeats in *AFF2* gene with the accuracy of homozygosity, as well as to identify the false negatives of multiplex-PCR with female samples. This methodology (n= 152) is also used in the lab to determine the number of CTGs in DMPK gene involved in Myotonic Dystrophy type 1.

4. Sanger sequencing

Sanger sequencing is intended to amplify a specific target region of template DNA using an oligonucleotide sequencing primer, which binds in the region adjacent to the sequence of interest. The synthesis of the complementary DNA strand begins at the primer-specific site and ends with the incorporation of a chain-terminating dideoxynucleotide triphosphates (ddNTP) that are randomly introduced instead of its corresponding deoxynucleotide triphosphates (dNTP). These ddNTPs have an H-terminal group (instead of OH- as in dNTPs) hampering further introduction of dNTPs in the sequence. Thus, amplified fragments will have different lengths [101].

Each ddNTP (ddATP, ddTTP, ddGTP, ddCTP) is marked with a different fluorescent label. When a ddNTP is attached to the elongating sequence, the base will show a signal based on the associated nucleotide. Conventionally, A is indicated by green fluorescence, T by red, G by black, and C by blue. The florescence intensity is detected and translated into SeqScape® software version 2.5 (Applied Biosystems™).

When a heterozygous variant occurs within a sequence, loci will be captured by two fluorescent signals of equal intensity, except in mosaics. In case of a homozygous variant is present, the expected fluorescent color is replaced completely by the new base pair(s) color [102].

Sanger sequencing (n=34) was used to analyze the 17 exons of the *TPO* gene. Pathogenic variants in this gene are associated with disorders of thyroid hormonogenesis, including congenital hypothyroidism, congenital goiter, and thyroid hormone organification defect [101].

This methodology is also applied to identify variants in other genes involved in monogenic disorders, for instance *CFTR* (Cystic fibrosis), *NOTCH3* (CADASIL), *MED12* (X-linked intellectual disability), among others.

After identification the variants need to be interpreted in terms of their role in clinical phenotype. For example gnomAD is a crucial tool for helping to identify which variants are too common to be causing a patient's specific disease. An example of variant interpretation workflow can be found in Maia et al. [103].

5. Multiplex ligation-dependent probe amplification (MLPA)

MLPA® (Multiplex Ligation-dependent Probe Amplification) (n=4) is a semi-quantitative technique that allows the study of copy number variation (CNVs). This technique consists in 6 steps: denaturation, hybridization, ligation, amplification, fragment separation and analysis.

In an initial phase, denaturation of the DNA strand takes place. Next, hybridization of the DNA with a mixture of probes occurs (each one specific to the region to be studied). Each probe contains two hemi probes, one derived from synthetic oligonucleotides and the other from bacteriophages. Ligated probes are amplified in a multiplex PCR using a single universal primer pair. Finally, the fragments are separated by capillary electrophoresis. PCR products are loaded into a capillary electrophoresis device and separated by length. Each fragment corresponds to a specific MLPA probe.

The migration pattern of the MLPA fragments is then compared with the migration pattern of the molecular marker to size each amplified fragment. Data analysis was performed in Coffalyser.Net™ (MRC Holland, Willem Schoutenstraat, Amsterdam, Netherlands).

MLPA can be used in the molecular diagnosis of several diseases related to the presence of deletions or duplications in specific genes (*SMN1* responsible for Spinal muscular atrophy, for example) and diseases characterized by abnormal DNA

methylation (Methylation-Specific-MLPA, namely Prader-willi and Angelman syndrome) [104].

6. Human androgen receptor assay (HUMARA)

Human androgen receptor assay (HUMARA) (n=75), first described in 1992 by Allen et al. [105], relies on a polymorphic trinucleotide repeat CAG located in the first exon of the androgen receptor (*AR*) gene to determine the X-chromosome inactivation pattern in female samples. *AR* gene is located close to the X-inactivation center and previous reports have shown that its methylation pattern correlates well with the methylation of the X-chromosome. X-chromosome inactivation (XCI) is an epigenetic phenomenon in which one of the X chromosomes is transcriptionally methylated during the blastocyst stage, to compensate the imbalance in the dosage of X-chromosomal genes in females [106,107]. HUMARA is a semiquantitative method [105] using methylation-sensitive endonucleases to distinguish the inactivated X chromosome.

Restriction enzymes acts as "molecular scissors", recognizing and cutting specific DNA sequences, they are highly specific: each type of enzyme recognizes and cuts only a particular nucleotide sequence, usually consisting of 4 or 6 pairs of nitrogenous bases. *Rsa*I (recognition site 5'-GTAC-3') and *Hha*I (5'-GCGC-3') are used, which will recognize the sequence and in case of *Hha*I digested only when the allele is not methylated. A female should have one inactive and one active allele that the enzyme *Hha*I will recognize and cut (active) so there will be no further amplification. The percentage of methylation of the *AR* gene is determined using the area values, normalized to the corresponding undigested allele peak area as obtained by GeneMapper® software version 4.0 (Applied Biosystems™) [105].

A set of female samples were analyzed, aiming to evaluate whether the methylation status of the fragile mental retardation protein translational regulator gene (*FMR1*) can provide an XCI pattern similar to that obtained by HUMARA.

HUMARA was performed to assess the XCI pattern of infertile women and oocyte donor samples. This pattern was then compared with the results of the *FMR1* gene methylation obtained by AmplideX® mPCR *FMR1* Kit (AmplideX® mPCR *FMR1* Kit, Asuragen®, Austin, TX, United States), ranges were established to provide the XCI pattern using the *FMR1* gene methylation pattern through mPCR [108]. Ranges were defined to establish the XCI pattern using the methylation pattern of the *FMR1* gene by mPCR for the use in HUMARA homozygous samples. Furthermore, a set of key points and a stepwise analysis towards obtaining an accurate result for the XCI pattern and to minimize the underlying pitfalls were propose.