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CAROLINA CAPUCHA GARCIA

Impacto toxicológico de três terapêuticas farmacológicas em espermatozoides humanos

Toxicological impact of three pharmacological therapeutics on human sperm

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biomedicina Molecular, realizada sob a orientação científica da Professora Doutora Rosália Sá, Professora Auxiliar do Instituto de Ciências Biomédicas Abel Salazar (ICBAS) e coorientação do Professor Doutor Mário Sousa, Professor Catedrático do Instituto de Ciências Biomédicas Abel Salazar (ICBAS).

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Presidente
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Professor Auxiliar em Regime Laboral da Universidade de Aveiro

Vogal
Doutora Paula Cristina Paulo Videira da Silva
Professora Auxiliar do Instituto
de Ciências Biomédicas Abel Salazar - Universidade do Porto

Vogal
Doutora Rosália Maria Pereira de Oliveira e Sá
Professora Auxiliar do Instituto
de Ciências Biomédicas Abel Salazar - Universidade do Porto

palavras-chave

Hidroxicloroquina, Dexametasona, Remdesivir, toxicidade farmacológica *in vitro*, espermatozoides humanos, fragmentação DNA, vitalidade, motilidade, stress oxidativo

Resumo

A fertilidade masculina pode ser negativamente afetada por diversos fármacos, que induzem efeitos tóxicos no sistema reprodutor masculino, resultando em profundas alterações na formação e/ou função dos espermatozoides. Alguns fármacos são capazes de atravessar a barreira hemato-testicular e atuar diretamente nas células germinativas podendo vir a interferir com a capacidade de fertilização dos espermatozoides. A pandemia COVID-19 obrigou à mobilização de diversos recursos farmacológicos. Inúmeros fármacos foram repropostos como tratamento para a COVID-19, passando a ser amplamente administrados em todo o mundo, tornando-se ainda mais urgente o total conhecimento da segurança dos fármacos em questão. Assim sendo, foi desenhado este estudo *in vitro*, para avaliar os efeitos de três fármacos utilizados como terapia para pacientes COVID-19 nos espermatozoides humanos. Hidroxicloroquina (HCQ; antimalárico), dexametasona (DEX, glucocorticoide) e remdesivir (RDV; antiviral) foram os fármacos estudados, sendo que a DEX e o RDV foram testados individualmente e em combinação. Os espermatozoides foram expostos às concentrações terapêuticas dos fármacos e os seguintes parâmetros foram avaliados: vitalidade, motilidade, stress oxidativo e danos no DNA. De acordo com os resultados, apenas a HCQ diminuiu significativamente a vitalidade e motilidade dos espermatozoides. Todos os fármacos induziram fragmentação no DNA espérmico, ainda que sem alterar os níveis de stress oxidativo. Assim, concluiu-se que a HCQ é o fármaco com maior potencial tóxico para os espermatozoides, induzindo danos ao nível macro e microscópico, e que a fragmentação do DNA é induzida por mecanismos que não implicam unicamente produção de espécies reativas de oxigénio. Mais ainda, este estudo tornou evidente a necessidade de incluir uma análise ao DNA espérmico nos espermogramas realizados em clinicas de fertilidade. DEX e RDV parecem ter um efeito aditivo quando administrados em conjunto, no entanto é necessário um estudo mais aprofundado para confirmar os seus efeitos em concomitância.

Keywords

Abstract

Therapeutic drugs can negatively affect male fertility by inducing toxic effects that impair sperm production and/or function. Some drugs are able to cross the blood-testis barrier (BTB) and act directly on germ cells, interfering with sperm function. With the world pandemic of COVID-19, several drugs were repurposed as treatment options. As a result, these drugs started to be extensively administered worldwide. The urge for full knowledge on drugs' safety became even more evident, to avoid undesirable side effects. Therefore, the aim of this *in vitro* study was to evaluate the toxicological effects of three therapeutic drugs used in COVID-19, in human sperm cells. Hydroxychloroquine (HCQ; antimalarial), dexamethasone (DEX; glucocorticoid) and remdesivir (RDV; antiviral) were the drugs studied. DEX and RDV are also recommend in co-administration for some COVID-19 patients, thus, these two drugs were evaluated for individual combined effects. Sperm vitality and motility, sperm oxidative stress and DNA damages were assessed in sperm cells exposed to usual therapeutic regimen of each drug and compared to a control group. HCQ was the only drug to induce significant effects in sperm vitality and motility. All drugs induced sperm DNA fragmentation, without alter the oxidative stress levels. Therefore, this study suggests HCQ is the most toxic drug for sperm cells, and that sperm DNA fragmentation is induced by different mechanism rather than exclusively due to oxidative stress. Moreover, results outlined the importance to include sperm DNA fragmentation evaluation in routine sperm analysis at fertility clinics. DEX and RDV seem to have addictive effects when administered simultaneously, however, further investigation is required to confirm these effects.

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Introduction

1. Male Reproductive System

Male reproductive function relies on sensitive and tightly regulated interactions in organs and cells that constitute the reproductive system. Testes are the main male reproductive organ, where spermatozoa and steroid hormones are produced. Prostate gland and seminal vesicles are important accessory organs of the male reproductive system, responsible for the secretion of the fluids present in seminal fluid, in which spermatozoa are suspended during ejaculation. Glandular secretions contribute for sperm survival during fertilization with nutrients, among other chemical substances that increase sperm motility and protect it from the acidic residues in urethra and vaginal secretions.

Sperm formation and maturation initiates during embryonic development when undifferentiated cells divide to form spermatogonia; but it is not complete until puberty when major changes occur. When men reach puberty, spermatogonia divide into primary spermatocytes which suffer two sequential meiotic divisions to form spermatids, which then differentiate into mature spermatozoa (Figure 2). Sperm can be divided into three distinct regions, with distinct functions: (1) head, composed of the nucleus, where the genetic material (DNA) is stored, and the acrosome vesicle containing hydrolytic enzymes that aid in the penetration of the oocyte during fertilization; (2) midpiece, composed essentially of mitochondria that provide energy for sperm movement; (3) tail, consisting of contractile filaments that allow sperm to move (Figure 1). Spermatogenesis is the process by which haploid spermatozoa develop from germ cells in the seminiferous tubules of the testis. To the final stage of spermatogenesis is called spermiogenesis, which sees the maturation of spermatids into mature spermatozoa. This process involves several cellular and molecular transformations to reduce volume and maximize motility of spermatozoa. Mature spermatozoa transform into compact elongated cells, losing excess cytoplasm around the head and reducing nucleus size. The entire cytoplasmic machinery in the head, such as ribosomes, endoplasmic reticulum, or the Golgi apparatus, is eliminated and the genetic material is tightly condensed, as the chromatin-associated histones are replaced by smaller, highly charged proteins, the protamines. All phases take place within the seminiferous tubules of the testes. Sertoli cells are a type of cell that is present in the seminiferous tubules and plays an essential role in spermatogenesis, providing direct support and controlling the seminiferous tubules microenvironment to facilitate the transformation of primary spermatocytes into mature spermatozoa (Griswold, 1998).
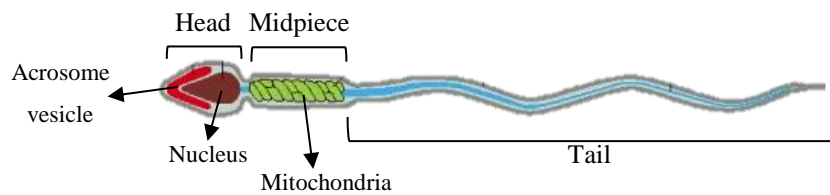
Figure 1- Human sperm structure. Adapted from Alberts et al. (2002)

Spermatogenesis is closely controlled by hormonal signalling. Testosterone is the main male sex hormone, responsible for all male characteristics, including the development of the male reproductive system and fertility. It is mainly produced and secreted in the testes, by Leydig cells; only a small percentage (approximately 5%) is obtained by the adrenal gland as part of the cortisol synthesis pathway (Mawhinney and Mariotti, 2013). In testes, testosterone production depends on hypothalamus-pituitary gonadal (HPG) axis. The signalling pathway is initiated in the hypothalamus, with the release of gonadotropin releasing hormones (GnRHs). GnRHs reach the anterior pituitary gland and stimulate the production of gonadotropins follicle-stimulating hormone (FSH) and luteinizing hormone (LH) which will affect gonadal function. FSH stimulates Sertoli cells function in spermatogenesis, while LH acts on Leydig cells inducing testosterone secretion. Testosterone, in turn, enters Sertoli cells, where it also promotes sperm formation. Therefore, gonadal function is regulated through negative feedback from the HPG. Testosterone and inhibin, a hormone produced by Sertoli cells, are major contributors to this regulation. Inhibin responds to FSH by inhibiting its secretion, whereas testosterone induces LH inhibition (Figure 3) (Mawhinney and Mariotti, 2013; Widmaier *et al.*, 2016).

Dysregulations in this system that lead to atypical gonadal steroid synthesis and secretion can have profound consequences on sexual development and function, which are then reflected in fertility issues.
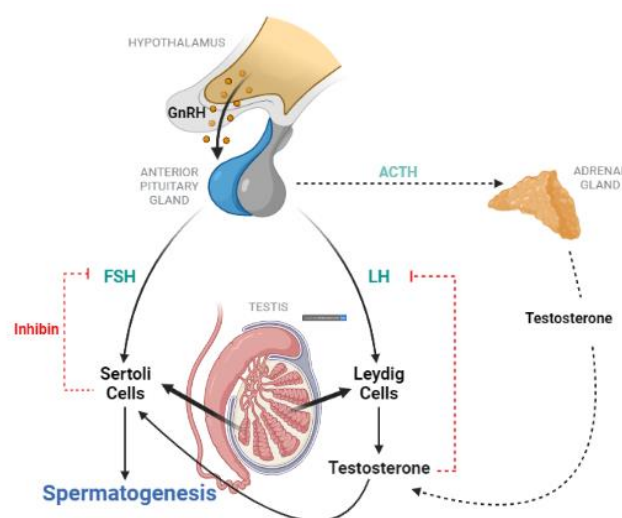
Figure 2- Hormonal controll of spermatogenesis. ACTH: Adrenocorticotropic Hormone; FSH: Follicle-Stimulating Hormone; LH: Luteneizing Hormone.

2. Male Infertility

Male infertility affects around 50% of infertile couples. Unfortunately, men who suffer from infertility of unknown cause (idiopathic infertility) still constitute a large portion of infertility cases today, which brings enormous concerns. As briefly described in the previous chapter, male reproductive function relies on several stages of developmental and complex mechanisms, which can severely impair male fertility if anyone stage is affected.

Several risk factors can influence male fertile potential, including lifestyle problems, psychological issues and sexual problems. The most common causes of male infertility include hormonal disturbances, ejaculatory dysfunction, testicular failure, or chromosomal abnormalities (Babakhanzadeh *et al.*, 2020). Usually, they are divided into pre-testicular, testicular, or post-testicular causes. Among the pre-testicular causes, hypogonadotropic hypogonadism is the most diagnosed abnormality, affecting several stages of the reproductive system. This disease is characterized by low or absent levels of gonadotropins (LH or FSH), which have serious consequences for spermatogenesis. Testicular cancer and varicocele constitute the main testicular causes of infertility. Varicocele is an enlargement of the veins in the scrotum and may affect semen parameters including total sperm count, sperm motility, and sperm morphology. Testicular cancer has direct and indirect effects on fertility. Tumour mass growth directly disrupt spermatogenesis, while the inflammatory response to the tumour may contribute indirectly. Testicular causes are associated with sperm DNA damage. Post-testicular causes mainly include defects in the vas deferens and ejaculatory ducts, which impair normal ejaculation (Parekattil *et al.*, 2020).

Drugs can be in the origin of many of these abnormalities, via side reactions that induce toxicity in the reproductive system. Disturbances in the HPG may have direct effects on testosterone activity in target tissues, interfering with testosterone receptors, or they may disrupt feedback loops in the hypothalamus or pituitary, resulting in a modification of gonadotropin release, indirectly affecting testosterone production and/or spermatogenesis. Drugs can also induce toxicity in the seminiferous tubule epithelium, including on Sertoli cells, in Leydig cells, or germ cells and affect directly sperm cells (Drobnis and Nangia, 2017).

Male reproductive potential is established through the evaluation of semen parameters, that include semen volume, total sperm count, sperm concentration, motility, vitality and morphology (spermogram), according to the low reference limit values defined by the World Health Organization (WHO) (Table 1). Low semen volume may be caused by congenital absence of the vas deferens, hypogonadism, or ejaculatory dysfunctions, which also affects sperm concentration. Sperm motility is an important indicator of the functionality of spermatozoa, while vitality identifies living sperm. In cases of idiopathic infertility, it becomes relevant to do a more detailed analysis, namely to sperm DNA (sDNA) integrity, which can be assessed by sDNA fragmentation (sDNAfrag) index or chromatin condensation status (Table 1). sDNAfrag and chromatin decondensation levels above the reference limits are indicators of male infertility. Moreover, evaluation of sperm oxidative stress also brings

important insights relative to male reproductive potential. Elevated seminal reactive oxygen species (ROS) levels have been identified in infertile men. Oxidative stress has been associated with impaired sperm physiology and function, increasing sDNAfrag and decreasing sperm motility (Parekattil *et al.*, 2020).

Awareness of human fertility issues has increased over the last decades, especially with a focus on female fertility and pregnancy; however, evidence-based knowledge regarding drugs' effects on male fertility still constitutes a major gap in clinical investigation and practice. Efforts have been made to include the assessment of male reproductive toxicity in preclinical drug development studies. Since 2011, the Food and Drug Administration (FDA) has mandated the evaluation of male reproductive toxicity as part of premarketing studies for new drugs, having released in 2015 a set of guidelines titled "Testicular Toxicity: Evaluation During Drug Development", which provide orientations for nonclinical and clinical testicular toxicity studies, in drug development research. Although this decision brings great advances in the prevention of male infertility due to the use of therapeutic drugs, most of the drugs on the market nowadays were approved long before this awareness was raised; it is important to extend these assessments to drugs already approved (Drobnis and Nangia, 2017; FDA, 2018). Common methods used in these studies include the evaluation of hormone levels (FSH, LH, testosterone, inhibin, prolactin) and semen parameters. Histopathological studies are used to assess damages in testicular tissue (Sousa *et al.*, 2017).

Table 1 Low Reference Limit values for basic sperm parameters and sDNA integrity (WHO, 2010; Agarwal et al., 2020; Hammadeh et al., 1998)

	Parameter	Low Reference Limit
SEMEN CHARACTERISTICS	Semen volume	1.5 mL
	Total sperm number	39×10^6 (per ejaculate)
	Sperm concentration	15×10^6 /mL
	Total motility	40%
	Progressive motility	32%
	Vitality	58%
	Sperm morphology	4%
sDNA INTEGRITY	sDNA fragmentation	20%
	Chromatin condensation	

3. Pharmacological Therapeutics

3.1 Hydroxychloroquine

3.1.1 Pharmacokinetic/Pharmacodynamic Properties

Hydroxychloroquine (HCQ) is a 4-aminoquinolone antimalarial and corresponds to an analogue of chloroquine (CQ), in which one of the *N*-ethyl groups of CQ is β-hydroxylated (Lim *et al.*, 2009). The introduction of the hydroxyl group reduces the drug's toxicity around 60%, which can be explained by the 2.5-fold greater tissue distribution observed in CQ. The therapeutic effects appear to be maintained (McChesney, 1983).

HCQ is administered orally, in a single or two divided doses per day, in tablets of 200 or 400 mg of the racemic sulphate salt, equivalent to 155 mg or 310 mg base, respectively (Tett *et al.*, 1988; FDA / CDER, 2017). After administration, HCQ is rapidly absorbed by the intestinal tract within 2 to 4 hours. Following a 200 mg racemic oral dose, the mean fraction of drug absorbed is estimated to be 0.74, reaching a maximum plasma concentration of 50.3 ng/ml in 3.74 hours (Tett *et al.*, 1989; FDA / CDER, 2017). As a weak amphiphilic base, HCQ is extensively captured by tissues soon after administration because of its accumulation in acidic vesicles by ion-trapping (Derendorf, 2020; Schrezenmeier and Dörner, 2020). Higher concentrations of HCQ appear in the liver, kidneys and heart (Collins *et al.*, 2018). Such tissue uptake explains the large volume of distribution (~ 4.4 x10^4 L) and the long terminal half-life (40-50 days) (Browning, 2014). Thus, it takes about 6 months for the therapeutic effects to start to be noticed (Song *et al.*, 2020). HCQ also binds to melanin with high affinity, depositing in melanin-containing tissues, such as the eyes and skin (Schrezenmeier and Dörner, 2020). However, these interactions do not appear to be a reason for the large volume of distribution. Studies in CQ have shown a similar half-life for black and white individuals (Tett *et al.*, 1990).

Upon absorption, HCQ is transported to the liver, where it is metabolized by cytochrome P450 isoforms, CYP2C8 and CYP3A4, into three metabolites. HCQ is N-dealkylated to form desethylchloroquine (DCQ) and desethylhydroxychloroquine (DHCQ). Bisdesethylchloroquine (BDCQ) appears as a secondary metabolite, resulting from the biotransformation of DCQ and DHCQ. DHCQ appears to be the main and only active metabolite (McChesney, 1983; Projean *et al.*, 2003; Collins *et al.*, 2018). HCQ and its metabolites are mainly excreted by the kidneys (40–60%), with 62% as unchanged drug, 18% as DHCQ, 16% as DCQ and 4% as BDCQ. 8–25% of the drug is excreted in the faeces, 5% is eliminated through the skin and 25–45% can remain in lean body tissue, linked to melanin (McChesney, 1983; Browning, 2014).

It is believed that the mechanisms of action of HCQ are related to its ability to influence the pH in intracytoplasmic vesicles, such as lysosomes. HCQ increases the pH within acidic intracellular compartments and interferes with processes such as protein degradation in

lysosomes and autophagy, the assembly of macromolecules in endosomes and signalling pathways involved in immune responses (Fox, 1993; Schrezenmeier and Dörner, 2020). As a chemotherapeutic agent against malaria, HCQ acts by interfering with the digestion of haemoglobin, the main food source for the erythrocytic forms of *Plasmodium* parasites. When inside human erythrocytes, absorption of HCQ increases the pH of the parasite's acidic food vacuoles, inhibiting the polymerization of heme and its subsequent clearance into the cytoplasm, where hemozoin is formed. Failure to convert heme to hemozoin causes swelling of the vacuoles due to the accumulation of free heme, which is highly toxic to the parasite, leading to its death (Stokkermans *et al.*, 2021; Krogstad and Schlesinger, 1987; Brocks and Mehvar, 2003; Kaur *et al.*, 2010).

3.1.2 Therapeutic Use

Although first described as an antimalarial, during World War II it was observed that soldiers who took antimalarials for prophylaxis and had skin rashes and manifestations of inflammatory arthritis, improved their autoimmune conditions. Since then, CQ and HCQ have also been widely adopted as an anti-rheumatic agent in multiple autoimmune disorders such as Systemic Lupus Erythematosus (SLE) and Rheumatoid Arthritis (Browning, 2014; National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK), 2021).

HCQ can exert its anti-inflammatory effects following different mechanisms. One is to interfere with antigen presentation via MHC class II (Schrezenmeier and Dörner, 2020). To present antigens via MHC II and activate CD4+ T cells, the antigen is internalized and processed through the endocytic processing pathway, involving endosomes and lysosomes. Within these compartments, the antigen is degraded into short peptides, which will eventually bind to the α and β chains of the MHC protein to form MHC class II–peptide complexes that appear on the cell surface. By accumulating inside lysosomes and increasing their pH, HCQ prevents peptides with low affinity for the α and β chains from binding and forming the antigen-MHC II complex and, consequently, the autoantigen presentation (Fox, 1993; Punt *et al.*, 2018). In addition, the immune response triggered by endosomal TLRs may be affected by HCQ. Kužnik et al. showed HCQ inhibited TLR3, 7 and 9 signalling by interacting with nucleic acids ligands. According to this study, such interaction leads to a change in the conformation of nucleic acids, which impairs the binding of these ligands to the TLR receptor binding sites (Kužnik *et al.*, 2011). Through a similar mechanism, HCQ can also act on cGAS-STING signalling, inhibiting cGAMP production by cGAS, through active-site competitive inhibition (An *et al.*, 2017). Altogether, these pathways contribute for the release of several inflammatory cytokines as part of their immune responses. Thus, in addition to antigen presentation and signalling pathways direct inhibition, HCQ indirectly reduces the production of inflammatory cytokines, such as type I interferons, TNF-α, IL-1 and IL-6 (Kužnik *et al.*, 2011; An *et al.*, 2017; Schrezenmeier and Dörner, 2020).

Over the last decade, HCQ has been studied as part of cancer therapy based on its ability to inhibit autophagy. Autophagy plays dual roles in tumor promotion and suppression. Under normal conditions, basal levels of autophagy ensure the maintenance of biological function and homeostasis, preventing tumor growth; however, it can also contribute to the development and proliferation of cancer cells by facilitating their adaptation to adverse microenvironmental conditions. Elevated levels of autophagy are found in several types of RAS-activated cancer, such as pancreatic, lung, and colon cancer (Yun and Lee, 2018). RAS-activated cancer cells rely on autophagy to maintain mitochondrial metabolic function and energy levels necessary to support tumor growth under starvation conditions. Impairing the autophagy process can promote cell death and consequently tumor regression (Guo *et al.*, 2011).

Therefore, HCQ has been studied as a primary or adjuvant treatment in these types of cancer, due to its role in directly inhibiting autophagy. By accumulating within lysosomes, HCQ blocks the fusion of autophagosomes with lysosomes and prevents the autophagy process (Lin *et al.*, 2017). Nevertheless, there are still some concerns about this therapeutic approach regarding the drug concentrations required to block autophagy within tumours. The phase II clinical trial showed that doses of 400mg and 600mg of HCQ were not sufficient to adequately inhibit autophagy (Yang *et al.*, 2013; Wolpin *et al.*, 2014).

3.1.3 Adverse Effects

Under recommended administration concentrations (5.0 mg/kg/day) (Jorge *et al.*, 2018), the most common adverse reactions due to HCQ treatment are gastrointestinal effects, including nausea, vomiting, diarrhoea and abdominal discomfort, occurring in early treatment and can often be minimized by taking medication with food. However, due to its slow terminal elimination rate, HCQ begins to accumulate when used for long periods of time and may become toxic (White *et al.*, 2020). Some case reports have described the incidence of HCQ-associated myopathy caused by lysosomal damage. After treatment discontinuation, myotoxic effects may be reversible (Abdel-Hamid *et al.*, 2008; Kwon *et al.*, 2010; Schrezenmeier and Dörner, 2020).

One of the most studied severe adverse effects of HCQ is related to vision. HCQ retinopathy is a condition that can result in permanent loss of vision (Pandya *et al.*, 2015). As previously referred, HCQ binds to melanin with a strong affinity and is deposited into melanin-containing tissues, such as the retinal pigment epithelium (RPE), in the eye. RPE cells are responsible for phagocytosis of shed photoreceptor outer segment (POS) membranes, an important component of the retinal photoreceptors (PR), specialized in detecting light and initiating the biological process of vision (Sparrrow *et al.*, 2010; Goldberg et al., 2016). The phagocytosed POS are degraded in the lysosomes of the RPE cells, although a gradual accumulation of lipofuscin occurs during the process. HCQ increases the RPE lysosomal pH and blocks the attachment of autophagosomes to lysosomes, interfering with the degradation

of POS membranes. Consequently, there is also retention of lipofuscin, which is associated with PR degeneration (Sundelin and Terman, 2002; Jorge *et al.*, 2018). The duration and dosage of treatment were accepted as the main risk factors for HCQ retinopathy. Recent studies have shown that the overall prevalence of this disease ranged from 1.6% to 8.0%, but when focusing on patients receiving treatment for more than 5 years, the prevalence was 5.2–7.5%. Efforts have been made to minimize these toxic outcomes, including new screening methods, updates on maximum daily dose recommendation or type of weight measurement (Ideal Body weight (IBW) versus Actual Body Weight (ABW)) (Jorge *et al.*, 2018). However, there is still a great challenge in early diagnosis, since most patients are asymptomatic at this stage. Symptoms only begin to be noticed after partial loss of vision and can culminate in significant deterioration of visual functions (visual acuity, peripheral vision and night vision). Discontinuing therapy usually results in disease stabilization, although vision loss may be irreversible and may continue to progress for several years after discontinuing medication (Hansen and Schuman, 2011; Ding *et al.*, 2016).

Awareness about drug toxicology has been evolving and with this it is recognized that several inter-individual factors, such as sex, age, weight or height, affect the response to drug treatment. For example, studies show that obese patients, as well as individuals with asthenic constitution, are at increased risk of developing HCQ retinopathy. A balance between IBW and ABW must be taken into consideration when HCQ dosages are defined, to avoid overdosing. Therefore, in patients where the ABW is higher than the IBW, the daily dose should be adjusted for the IBW, while if the ABW is lower than or similar to the IBW, the ABW should be taken in consideration (Marmor *et al.*, 2011; Browning *et al.*, 2014).

3.2 Dexamethasone

3.2.1 Pharmacokinetic/Pharmacodynamic Properties

Dexamethasone (DEX), also named 9α-16α-fluoromethylprednisolone, is a prednisolone analogue to which a fluorine atom on carbon 9 and a methyl radical on carbon 16 have been added. First described by Bunim and colleagues in 1958, is a synthetic analogue of the main endogenous glucocorticoid (GC), cortisol (Bunim *et al.*, 1958). GCs are a type of corticosteroid, a class of steroid hormones produced and secreted by the adrenal glands that play an important role in regulating the endocrine system, including stress management and homeostasis control. GCs are predominantly involved in metabolism and immunosuppressive/anti-inflammatory responses, while sodium and water balance are regulated by corticosteroids with mineralocorticoid activity. Changes made on the molecular structure of cortisol increased the anti-inflammatory potency of DEX and minimized mineralocorticoid effects, making this drug one of the most effective synthetic GCs and far more potent than the natural hormone. (Khan and Lee, 2008; Samuel *et al.*, 2017; Williams, 2018).

Depending on the condition being treated, DEX is administered by different routes. Oral pills are indicated for cancer treatment, such as lymphoma and multiple myeloma, in daily doses of 20 mg to 40 mg (FDA, 2019). DEX topical lotion and eye drops may also be given to treat some short-term skin and eye inflammations, respectively (Schäcke *et al.*, 2002; FDA, 2017). Most commonly, DEX is given intravenously (IV) or intramuscularly (IM) as phosphate or succinate esters prodrugs to treat inflammatory conditions. Dosing regimens range from 0.5 mg to 9 mg per day, depending on the disease being treated and its severity (FDA, 2014). Conversion of the prodrug to the active form occurs shortly after injection, with 90% of DEX being free in plasma, within 10 minutes (Hare *et al.*, 1975). After administration of a 5 mg DEX-phosphate (DP) dose, a peak plasma concentration of 51 ng/ml is reached in 0.25 hours. It shows moderate volume of distribution (1.4 L/Kg) and terminal half-life of 4.1 h (Varis *et al.*, 2000; Czock *et al.*, 2005).

As a lipophilic molecule, DEX is transported in the circulation associated with plasma proteins to reach target tissues. While endogenous GCs bind essentially to corticosteroid binding protein (CBG) (80-90%), and only 5-15% to albumin (Cidlowski, 2016), DEX does not bind to CBG and is about 75% bound to albumin (Cummings *et al.*, 1990). Given its lipophilic nature, DEX can rapidly cross plasma membranes and target cells at a systemic level. Almost all cells in the body are sensitive to GCs regulation, but inflammation precursors such as myeloid cells and lymphocytes constitute the main sites of action (Hardy *et al.*, 2020; Quatrini and Ugolini, 2021). DEX therapeutic activity is controlled intracellularly by tissue-specific 11β-hydroxysteroid dehydrogenases (11β-HSDs), 11β-HSD type 1 (11β-HSD1) and 11β-HSD type 2 (11β-HSD2). The two isoforms regulate the interconversion between the active 11-hydroxy- and the inactive 11-keto form of the drug, modulating DEX availability according to tissue-specific needs. Type 2 dehydrogenases (11β-HSD2) are found mainly in mineralocorticoid target tissues (kidney, colon, salivary glands, placenta) and are responsible for the oxidation of GCs to inactivate molecules. Unlike endogenous GCs, DEX is not metabolized by 11β-HSD2, which explains the lack of mineralcorticoid properties of this drug. 11β-HSD1 is mainly distributed in GC target tissues such as liver, adipose tissue, brain, lung, muscle and bone, with greater activity in the liver. I*n vivo*, it acts primarily as a reductase, converting 11-dehydrodexamethasone into DEX. The presence of the 11β-hydroxyl group activates DEX molecule, which becomes capable of binding to GC receptors (GRs) triggering the therapeutic mechanisms of action (Loew *et al.*, 1986; Cidlowski, 2016; Schiffer *et al.*, 2019). A downstream phase in DEX metabolism mediates the transformation of the active molecule into hydrophilic inactive metabolites, to be eliminated. Also in the liver, DEX is a substrate of the CYP3A4 enzyme. CYP3A4 catalyses the 6β-hydroxylation of DEX to 6α- and 6β-hydroxydexamethasone. As evidenced by Tomilson *et al.*, 6β-hydroxydexamethasone was identified as the major metabolite, in a 3:1 ratio compared to the 6α metabolite. In the same study, a monohydroxylated side-chain cleaved DEX (*9αF-A*) was also detected in liver microsomes (Tomlinson *et al.*, 1997). This hydroxylation step inactivates DEX and increases its polarity and water solubility (Gentile *et al.*, 1996; Schiffer *et al.*, 2019). Elimination occurs predominantly by renal excretion in

the form of metabolites, with 6β-hydroxydexamethasone being the main metabolite found in urine samples. Only a small fraction (approximately 2%) of the total dose is eliminated as unchanged drug (Minagawa *et al.*, 1986).

3.2.2 Therapeutic Use

DEX has been used to treat severe inflammatory, immune and allergic diseases, including rheumatic diseases, SLE and asthma. DEX can also be used in cancer treatment for several types of cancer, as part of the therapy regimen or to prevent secondary effects underlying conventional treatments such as anaemia and/or thrombocytopenia (NCI, 2015; Samuel *et al.*, 2017).

The therapeutic immunosuppressive effects are triggered by mechanisms that inhibit many of the initial events in an inflammatory reaction and can be distinguished into genomic or non-genomic mechanisms. Genomic effects are characterised by a slow response, involving alterations in mRNA transcription and translation, while non-genomic mechanisms do not interfere with protein synthesis and occur within minutes after DEX enters the cell. When addressing the molecular reactions underlying the anti-inflammatory effects of GCs, authors commonly refer to the classical genomic mechanisms, dependent on GC-GR binding (Stahn and Buttgereit, 2008). GRs are transcription factors (TFs), members of the nuclear receptor superfamily, which regulate the expression of several GC-responsive genes involved in metabolism and immune system. Two main isoforms of GRs are known, GRα and GRβ, which differ from each other in the C-terminal domain, responsible for steroid binding. GRα is present in most cells and contains all the domains required for GR signalling, therefore playing an important role on the GC effects. GRβ, on the other hand, has lower expression and is not able to bind to GCs, due to differences in the steroid binding domain. This heterogeneity explains the variations in genes targeted by GRs between different cell types and the consequent effects of DEX on specific genes. (Van Der Velden, 1998; Hardy *et al.*, 2020; Quatrini and Ugolini, 2021). In the absence of GC, GR is found predominantly in the cytoplasm complexed with accessory proteins in a conformation with high affinity for ligands. After being activated by the 11β-HSD1, DEX binds to GR in the cytoplasm inducing conformational changes that result in TF activation. The activated DEX-GR complex is translocated to the nucleus, where it interferes with gene expression. In the nucleus, DEX-GR can activate (transactivation) or repress (transrepression) gene transcription by different mechanisms: (1) directly, by binding to specific DNA sequences, referred as GC response elements (GREs); (2) indirectly, via interaction with other transcription factors, or interfering with the stability of specific mRNA molecules. Direct binding of GC-GR complex to GREs is mainly associated with unwanted metabolic effects via transactivation, by inducing the transcription of important proteins involved in metabolism, like hepatic gluconeogenic enzymes. However, it can also bind to negative GREs and induce repression of gene transcription (Stahn and Buttgereit, 2008; Hardy *et al.*, 2020). DEX's anti-inflammatory

activity seems to be mediated mainly by indirect transrepression of pro-inflammatory genes. This response is based on the interaction of DEX-GR complex with proteins involved in pro-inflammatory signalling pathways. Nuclear factor (NF)-kB and activating protein (AP)-1 are two main TFs responsible for the expression of several immunoregulatory genes, including adhesion molecules, cytokines, and interleukins (IL-1β, IL-2, IL-6 and IL-8) (Van Der Velden, 1998). The protein-protein binding between these TFs and the DEX-GR complex blocks the TFs function and down-regulates pro-inflammatory genes expression, either preventing TFs from binding to promoter regions or impairing its activity at sites of action, in mechanisms referred as TF sequestration and tethering, respectively. DEX-GR can also compete with TFs for gene binding sites and, consequently, inhibit the initiation of gene transcription (Van Der Velden, 1998; Scheschowitsch *et al.*, 2017; Quatrini and Ugolini, 2021). Although most of anti-inflammatory and immunosuppressive outcomes of DEX are associated with the drug's interaction with NF-kB or AP-1, these effects may also result from changes in mRNA stability and subsequent protein translation, as has been observed for some interleukins (IL-1 and IL-6). Protein synthesis depends on the stability and half-life of mRNA, which is regulated, in part, by the length of its poly(A) tail. The DEX-dependent mechanism involves transactivation of specific ribonucleases, which act on mRNA Poly(A) tails, shortening its length. Consequently, the mRNA loses its stability and is degraded (Van Der Velden, 1998; Newton *et al.*, 1999). Ultimately, through these mechanisms, DEX decreases the number and activation status of inflammatory cells, including mast cells, dendritic cells, eosinophils, and T lymphocytes at the site of infection, and regulates the expression of pro-inflammatory mediators such as TNF-a, GM-CSF, and several interleukins (IL-4, IL-5, IL-6), molecules that are overexpressed in diseases treated with this drug (Coutinho and Chapman, 2011). Asthma, for instance, consists of an inflammation in the airways, with activation of eosinophils and T cells, inducing cytokines production and release of interleukins (IL)-4 and IL-5 (Kudo *et al.,* 2013). In Rheumatoid Arthritis, the inflammatory cascade is caused by an overproducing pro-inflammatory transcription factor Tumour Necrosis Factor (TNF). TNF induces the production of many cytokines, such as interleukin 6 (IL-6), which accumulate in joints, leading to persistent inflammation and tissue destruction (Scott *et al.*, 2010).

Regarding non-genomic mechanisms, they only seem to be activated when high doses of treatment are administered (pulse therapy, e.g.). This type of response can be categorized into 3 different processes: nonspecific, mediated by cytosolic GR, and specific. Nonspecific mechanism is characterized by interactions between the GC and cell membranes, inducing physicochemical changes. GC are thought to intercalate into membranes and interfere with membrane-associated proteins, affecting membrane permeability. As a result, calcium and sodium cycling rapidly reduces across membranes, which contributes to immunosuppression and reduction of inflammation. The mechanism mediated by cytosolic GR requires GC-GR complex activation as in genomic mechanisms, but this response is triggered by accessory proteins that dissociate from the GR upon GC binding. These proteins, such as Heat Shock Proteins (HSP90 and HSP70) and Proto-oncogene tyrosine-protein kinase (Scr), are

involved in secondary signalling cascades that can induce the inhibition of several metabolic and inflammatory reactions. The non-genomic specific response, in turn, is initiated via interactions with membrane-bound GRs and is associated with signalling pathways mediated by G-protein-coupled receptors. Membrane-bound GRs appear to bind to endogenous GCs with high affinity, but they do not bind to most GC analogues. Thus, the non-genomic specific response does not contribute to therapeutic effects of DEX (Czock *et al.*, 2005; Stahn and Buttgereit, 2008; Timmermans *et al.*, 2019). Clinically, it is not possible to separate these effects. DEX's role in cancer treatment includes both genomic and non-genomic mechanisms. In haematological tumours, DEX is commonly used as part of therapy due to its ability to promote cancer cells apoptosis. Via transactivation, DEX up-regulates the expression of pro-apoptotic genes such as Bim, while transrepression of NF-κB and AP-1 inhibits the transcription of pro-inflammatory cytokines, such as IL-6, anti-apoptotic genes such as Bcl-xL, and important genes involved in the cell cycle. Non-genomic effects result from the interaction of DEX with mitochondrial GRs promoting the release of cytochrome C, necessary for the activation of the apoptotic pathway. As an adjuvant therapy of solid tumours such as prostatic cancer, DEX is used to diminish inflammatory reactions of chemotherapy, to reduce nausea, to decrease swelling, but also to inhibit inflammation against invasive tumour growth (Sundahl *et al.*, 2016).

3.2.3 Adverse Effects

Despite the clinical efficacy, DEX is associated with a wide range of adverse reactions, strongly correlated with dosage and duration of treatment (Hardy *et al.*, 2020). GRs are expressed in almost all cells throughout the body; thus it is not possible to fully separate the therapeutically anti-inflammatory effects from other unwanted GC-sensitive metabolic reactions. Side effects induced by short-term treatment (less than 30 days) include mood changes, weight gain and modulation of the immune system, which is translated in an increased risk of infections (Tamez-Pérez, 2015; Williams, 2018). One of the most common short-term adverse reactions is related to glucose uptake. GC-induced hyperglycaemia emerges within few days after initiating GC therapy and can progress to diabetes, with longer treatment periods. The diabetogenic action of DEX is triggered by different mechanisms, that induce both insulin resistance and glucose synthesis. By interacting with different glucose-transporters (GLUT), GLUT4 in muscle and GLUT2 in pancreatic β-cells, DEX impairs both production and function of insulin, thus supressing glucose uptake into tissues. Simultaneously, DEX stimulates glucose biosynthesis via transactivation of hepatic enzymes involved in gluconeogenesis, such as glucose-6-phosphatase (G6Pase). This enzyme catalyses the final step of gluconeogenesis, transforming the glucose-6-phpsphate into free glucose, to be released into blood. Altogether, these effects culminate in an accumulation of glucose in the bloodstream (Schäcke *et al.*, 2002; Tamez-Pérez, 2015; Paredes and Alves, 2016). High dose regimens (0.4 to 0.8 mg/kg/day DEX) and prolonged treatment periods

(more than 6 months) lead to more severe adverse reactions, affecting predominantly bone, muscle, and hypothalamic-pituitary-adrenal (HPA) axis (Tamez-Pérez, 2015; Hardy *et al.*, 2020). In bone metabolism, DEX causes a reduction in bone mineral density and bone quality, that can lead to osteoporosis. Bone homeostasis relies on the balance between bone resorption and bone formation carried out by osteoclasts and osteoblasts, respectively. This process occurs in sequential phases, that initiate with osteoclasts formation, mediated by receptor activator of nuclear factor-ligand (RANKL). RANKL, which is expressed by osteoblasts and osteocytes, binds to its receptor RANK, present in osteoclast precursors and promotes osteoclast maturation and activation. Once activated, osteoclasts secret acids (H+) and proteolytic enzymes, such as cathepsin K (CTSK), inducing bone demineralization and subsequent bone degradation. Resorption is followed by bone formation. Osteoprotegerin (OPG), an antiresorptive protein produced by osteoblasts binds to RANKL preventing the RANK/RANKL interaction and, consequently, inhibiting the osteoclastogenesis. Bone formation phase is carried out by osteoblasts, that synthetize multiple bone matrix proteins, including the main bone protein, type-I collagen, and differentiate into lining osteocytes, the most abundant cell type within the skeleton (Tanaka *et al.*, 2005; Kim *et al.*, 2020). DEX affects osteoblast function by inducing the expression of sclerostin, an inhibitor of proliferation factors like Wnt, and pro-apoptotic genes such as Bim, thus impairing osteoblast formation and differentiation into osteocytes. On the other hand, it upregulates osteoclastic formation through direct transrepression of OPG. Such effects induce a disturbance in bone homeostasis favouring bone resorption. Excessive resorption without the corresponding newformed bone contributes to bone loss and osteoporosis (Tanaka *et al.*, 2005; Hardy, Raza and Cooper, 2020; Kim *et al.*, 2020). Similarly, to what occurs in the bone, prolonged use of DEX results in loss of muscle mass and strength, leading to muscle atrophy. DEX supresses key mediators of muscle protein synthesis, including the insulin-like growth factor-1 (IGF-1) and the downstream phosphoinositide 3-kinase (PI3K)–AKT–mTOR pathway, while inducing protein degradation through proteasomal degradation. Forkhead box protein O1 (FOXO1), a negative regulator of skeletal muscle differentiation, is upregulated by DEX, which, consequently, activates E3 ubiquitin-protein ligases that mediate protein degradation in proteosomes (Glass, 2010; Hardy *et al.*, 2020). Physical exercise, by stimulating new muscle proteins formation, may contribute to prevent GC-induced muscle atrophy (Schäcke *et al.*, 2002). The most common adverse effect associated with CGs therapy is HPA axis suppression. HPA axis defines the interactions between the hypothalamus, the pituitary gland, and the adrenal gland, three main components of the neuroendocrine system responsible for the response to stress. A signalling pathway is initiated by corticotropin-releasing hormone (CRH), a hormone released by the hypothalamus upon stress signals, that stimulates the pituitary gland. Consequently, the pituitary gland secretes the adrenocorticotropic hormone (ACTH), which will bind to receptors in the adrenal gland, activating it. Finally, the adrenal gland releases GCs (cortisol) that will exert its effects on managing stress (Smith and Vale, 2006). The administration of exogenous GCs induces a negative feedback effect in the hypothalamus by inhibiting the

synthesis of CRH via transrepression, which in turn, suppresses all the downstream steps of HPA axis. Duration of treatment seems to be the main cause of adrenal insufficiency, with 100% of the patients developing this condition after 1 year of treatment. However, patients receiving DEX for less than 30 days in high doses, also experienced HPA axis suppression, which suggests the risk of developing this disease is not directly correlated to dose and duration of treatment. The effects can last weeks to months after treatment discontinuation and lead to serious conditions as Cushing's syndrome and growth retardation (Schäcke *et al.*, 2002).

GCs are commonly prescribed to treat several paediatric disorders, such as juvenile idiopathic arthritis, paediatric vasculitis and SLE, as well as asthma and other respiratory diseases (Ferrara *et al.*, 2019), but it is known children receiving long-term GC therapy experience growth failure and delayed puberty (Schäcke *et al.*, 2002). Besides managing stress, the HPA axis also controls growth by regulating the growth hormone (GH), the main hormone involved in growth process, secreted by the pituitary gland. Although it can directly stimulate all tissues, GH acts mostly in the liver inducing the production and release of IGF-1, another hormone responsible for tissue growth and maturation. The negative effects of DEX result primarily from the inhibition of GH through the stimulation of somatostatin, the inhibitor hormone of GH secretion. Additionally, IGF-1 levels are also downregulated, either as a consequence of GH inhibition, or directly by DEX (Allen *et al.*, 1998). The effects of DEX on the bone and muscle described above will also affect normal growth. Therefore, it is of extreme importance to closely monitor patients receiving GCs therapy, especially during growth age. Treatment should be limited to the minimum dosage for the shortest period of time, to minimise serious adverse reactions.

3.3 Remdesivir

3.3.1 Pharmacokinetic/Pharmacodynamic Properties

Remdesivir (RDV) or GS-5734 is a monophosphoramidate prodrug of the 1'-cyano-substituted adenine nucleoside analogue (GS-441524). It is an antiviral, first described to treat Ebola during the virus outbreak in Africa in 2016 (Warren *et al.*, 2016). Nowadays, it has been mostly associated to the treatment of the recent world pandemic disease, COVID-19. In fact, RDV was not a FDA approved drug until October 2020, when the organization approved it as the first approved drug to treat COVID-19 (FDA, 2020b).

Nucleoside analogues have been extensively explored to treat for a broad spectrum of family virus, as their active triphosphate forms have the ability to interfere with viral replication. The transformation into nucleosides triphosphates occurs inside cells, after nucleoside analogues enter through specific plasma membrane nucleoside transporters and requires three activation steps. It starts with the conversion into nucleoside monophosphate by

cellular nucleoside kinases. This first transformation is particularly difficult, and rate limiting and constitutes the main cause for the lack of efficiency of these nucleosides (Eyer *et al.*, 2018). The direct administration of the monophosphate derivative it would not be appropriate either, since it is a negatively charged molecule and cannot cross the cell membrane (Mehellou *et al.*, 2009; Siegel *et al.*, 2017). Hence, RDV was developed to overcome these limiting steps. The nucleoside monophosphate core of RDV is coated with an aryl group and an amino acid ester, resulting in a charge-neutral compound. This strategy allows RDV to cross the cellular membrane by passive diffusion, enhancing cellular uptake, while bypassing the nucleoside-kinase dependent rate limiting phosphorylation step (Eastman *et al.*, 2020; Wiemer, 2020). Once inside cells, RDV undergoes a sequence of hydrolytic transformations, involving carboxylesterases (CES1) and cathepsin A (CTSA), to form an intermediate metabolite, GS-704277. GS-704277 is cleaved by phosphoramidases, resulting in the GS-441524 nucleoside analog monophosphate. This second metabolite is further phosphorylated by cell kinases to ultimately form the pharmacologically active nucleoside triphosphate, GS-443902 (Eastman *et al.*, 2020; Humeniuk *et al.*, 2020; Yan and Muller, 2020). RDV can also be a substrate for cytochrome P450 enzymes, namely CYP2C8, CYP2D6, and CYP3A4. However, due to the immediate action of hydrolases on the prodrug, it is believed this is not the principal mechanism of metabolization (Gilead, 2020; Jorgensen *et al.*, 2020).

RDV is administered via intravenous (IV) in an initial 200 mg dose (Day 1), followed by a 100 mg daily maintenance dose, for 5-10 days (Eastman *et al.*, 2020). It shows moderate plasma protein binding, with only a free fraction of 12.1%, and a volume of distribution of approximately 93 L (Jorgensen *et al.*, 2020; Therapeutic Goods Administration, 2020). The 4.38 µg/mL mean maximum plasma concentration is reached at the end of the infusion and rapidly declines, as the prodrug distributes into tissues and blood cells through passive diffusion (Gilead, 2020; Jorgensen *et al*., 2020; Therapeutic Goods Administration, 2020). In a nonhuman primate (NHP) conducted study infected with EBOV, upon the administration of a 10 mg kg^{-1} RDV dose (~200 mg in humans) RDV exhibited a short plasma half-life ($t_{1/2}$) of 0.39 h, being rapidly diffused into cells and converted into GS-443902, within 2-4 h. Inside cells, the active form was shown to persist with a $t_{1/2}$ of 14 h and at levels capable of inhibiting over 50% of viral load in 24h. The same study demonstrated RDV and its metabolites were mainly distributed within targeted tissues of EBOV infection, such as the testis, epididymis, eyes, and brain (Warren *et al.*, 2016). In mice, RDV could be detected in blood, heart, liver, lung, kidney, testis and small intestine, with the liver and lung being the most predominant organs (Hu *et al.*, 2020). In agreement with the findings in NHP and mice, relatively high levels of RDV metabolites were also detected in human semen, when men were administered with a single or repeated dose (WHO *et al.*, 2017). More information about the distribution in humans is still required (Jorgensen *et al*., 2020).

RDV elimination is accompanied by the appearance of its metabolites, GS-704277, GS-441524 and GS-443902. Approximately 92% of the drug is recovered in urine (74%) and

faeces (18%). GS-441524 is the most abundant metabolite accounting 49% of the species detected in urine, followed by the parent drug (10%), GS-704277 (2.9%) and other metabolites. The active form GS-44309 being negatively charged, cannot cross back the cellular membrane and is only detected inside cells (Gilead, 2020; Sun, 2020).

3.3.2 Therapeutic Use

GS-443902 is an adenosine triphosphate (ATP) analogue that plays its therapeutic effect by interfering with viral genome replication, once used as substrate by viral RNA-dependent RNA polymerases (RdRp) in replacement of the natural ATP. *In vitro* and *in vivo* studies have demonstrated RDV antiviral activity against several RNA virus families, such as filoviridae (eg. EBOV), paramyxoviruses (NiV), pneumoviridae (RSV)and coronaviridae (e.g. SARS-CoV, MERS-CoV) (Gordon *et al.*, 2020). Once misintegrated, the RDV nucleoside triphosphate (GS-443902) induces premature termination of viral genome synthesis. In contrast to classic chain terminators, GS-443902 is considered a delayed chain terminator, given the fact it still possesses a reactive 3'-hydroxyl group. The presence of this hydroxyl group allows the nucleophilic attack and the integration of a new nucleotide; thus, the inhibition of RNA synthesis occurs a few residues downstream. Evidence have shown GS-443902 incorporation commonly triggers chain termination between 3 to 5 nucleotides downstream (Eastman *et al.*, 2020; Gordon *et al.*, 2020). GS-443902 is selective for viral RdRp, as proven by Tchesnokov *et al.*, in 2019. According to their repot, GS-443902 is almost as efficiently integrated by viral RdRp complexes, as the natural ATP. On the contrary, the human mitochondrial RNA polymerase (h-mtRNAP) discriminates against the inhibitor (Tchesnokov *et al.*, 2019). Warren et al., back in 2016, had already shown GS-443902 could inhibit EBOV RdRp, but not human RNA polymerases nor mitochondrial RNA polymerases (Warren *et al.*, 2016). Altogether, these findings help to explain the efficacy and low cytotoxicity of RDV. The half-maximal effective concentrations (EC$_{50}$) of RDV are in the submicromolar range (0.003 to 0.79 µM) against filo-, pneumo-, and paramyxoviruses (Lo *et al.*, 2017). The high rate of incorporation of RDV-TP likely contributes to such low EC$_{50}$ values. In a cell-based assay, the half-maximum inhibitory concentration (IC$_{50}$) for MERS-CoV was shown to be 0.025 µM and cytotoxicity was not detectable until concentrations up to 10 µM. The same effects were measured in different cell types, for both MERS-CoV and SARS-CoV, and the half-cytotoxic concentration (CC$_{50}$) was determined to be 45 µM, a concentration ~100-fold above the ones required for RDV therapeutic effect on CoV (Sheahan *et al.*, 2017). RDV *in vivo* efficacy was evaluated in the Warren *et al.* study, where the EBOV-infected NHP were administered with 3 and/or 10 mg kg−1 of RDV, in a 12- day treatment. The experiment revealed RDV treatment reduced systemic viraemia and improved survival in 33% to 66%. Moreover, it was observed that a 3 mg kg−1 dose was suboptimal and that at 10 mg kg−1 the antiviral effects were

consistently greater, suggesting this last as the optimal therapeutic concentration (Warren *et al.*, 2016).

RDV can be administered prophylactically or therapeutically, although some *in vivo* studies suggest prophylactic administration have better outcomes. Wit *et al.* used a MERS-CoV-infected NHP model and demonstrated that prophylactic-treated animals presented significant lower clinical scores (no signs of respiratory alterations and ameliorated weight loss), lower levels of MERS-CoV replication in the lung and less lung lesions, when compared to therapeutically-treated animals or vehicle control (Wit *et al.*, 2020). Sheahan *et al.* observed similar results in Ces1c$^{-/-}$ mice infected with SARS-CoV (Sheahan *et al.*, 2017). Nevertheless, in both studies therapeutically administration of RDV also exhibited clinical benefits, reducing clinical signs, viral load and lung lesions. Importantly to consider in therapeutic treatment is the timing of treatment initiation, that should be prior to the peak of viral load. After virus replication has reached its peak, RDV is still capable to reduce viral loads, yet not enough for clinical improvement (Sheahan *et al.*, 2017).

3.3.3 Adverse Effects

Considering the promising antiviral activity on both *in vitro* and *in vivo* studies, the efficacy and safety of RDV was evaluated in humans. The first randomized, controlled clinical trial was developed in 2019, on the sequence of the EBOV outbreak in in the Democratic Republic of Congo. In this study, only one serious event was identified on RDV treatment arm, a hypotension event during the loading dose, followed by cardiac arrest that led to patient's death. However, an interim midstudy analysis recommended the premature termination of RDV intervention, due to the high mortality rates (>50%) in contrast with other treatment's arms (Mulangu *et al.*, 2019; Jorgensen *et al.*, 2020). Besides hypotension, other infusion-related hypersensitivity and anaphylactic reactions have been observed including hypertension, tachycardia, bradycardia, hypoxia, fever, dyspnea, wheezing, angioedema, rash, nausea, vomiting, diaphoresis, and shivering, as described in the RDV summary of product characteristics. Slower infusion rates may contribute to prevent these reactions (EMA, 2019). Studies in healthy volunteers and in patients infected with SARS-CoV2 revealed mild-to-moderate elevations in Aspartate Aminotransferase (AST) and Alanine Aminotransferase (ALT) serum levels (less than 5 times the upper limit of normal-ULN) (Gilead, 2020; Montastruc *et al.*, 2020; Zampino *et al.*, 2020). Elevations in these liver transaminases reflects hepatocellular injury (Vasimahmed *et al.*, 2020). The extent of liver injury caused by this antiviral is still not clear, but it is known that other nucleoside analogues can cause liver injury by affecting mitochondria. The nucleosides are incorporated by the mitochondrial RNA polymerase and inhibits mitochondrial RNA synthesis leading to a depletion of mitochondria or decrease in their function ('Remdesivir', 2020). This can result in accumulation of lactic acidosis, microvesicular steatosis and hepatic synthetic failure (LASH) (LiverTox, 2012). However, according to Zapino *et al.*, and despite the small

sampling, no case of RDV discontinuation was observed because of liver injury (Zampino *et al.*, 2020). Thus, ALT and AST elevations may occur during RDV therapy but are generally asymptomatic and reversible. Hepatotoxicity may become more evident when RDV is used widespread and for longer periods ('Remdesivir', 2020).

To date, safety and efficacy of RDV in pediatric individuals have not yet been assessed. Therefore, a physiologically based pharmacokinetics (PBPK) model of pharmacokinetic data from healthy adults was used to obtain pediatric doses. The resulting recommended dosage is a 5 mg/kg single loading dose on Day 1, followed by a 2.5 mg/kg dose once daily from Day 2 until the end of treatment (up to 10 days). This weight-based dosage regimen is expected to maintain comparable drug exposure as observed in healthy adults (WHO *et al.*, 2017; FDA, 2020a).

4. Impact of Pharmacological Therapeutics in Male Infertility

As referred in the previous section, drugs used in this study are associated with several adverse reactions that affect several systems of the human body. However, the adverse reactions in the male reproductive system are not described for any of these drugs.

DEX and HCQ are well established drugs used to treat several conditions that affect men in their reproductive age, thus it is surprising the lack of studies regarding the effects of these drugs in male reproduction. Most of the studies are carried out in animal models, while clinical studies use patients with active disease, which does not allow a direct association between alterations in seminal parameters and the drug, as it also may be due to the inherent pathology. RDV, on the other hand, is a recent drug, therefore adverse reactions of this drug needs to be further investigated. In this sense, it is important to note that RDV and its metabolites can be found in the testes, which makes studies on male reproductive system of extreme importance and urgency.

In this section is made a review of relevant studies about the effects of each drug in the male reproductive system.

4.1 Hydroxychloroquine

Male and female reproductive systems are extremely sensitive to drug exposure, and the resultant drug effects raise concerns regarding fertility, pregnancy outcomes and neonatal health. Dose regimens prescribed and treatment duration, can also influence the drug response and the extent of the consequences.

Surprisingly, data concerning the safety of HCQ in men trying to conceive is very limited (Bermas, 2020). A systematic review form 2019 identified only 1 case report, dated from 1987, describing a possible effect of CQ on male's fertility during an antimalarial treatment

(Mouyis *et al*., 2019). In this case report, a 33-years-old man was indicated to sperm analysis, after 4 malaria infections and subsequent treatments, which included amodiaquine-HCI, chloroquine phosphate, chloroquine sulphate, proquanil-HCI and pyrimethamine. The first analysis showed low sperm concentration and motility, that revealed to be worsened three weeks later. Even though, a 2-year follow-up allowed to conclude sperm damage was reversible, since sperm counts gradually increased over time, until reached normal parameters. Whether those changes were exclusively due to CQ or a consequence of the disease itself remained unclear (Singer *et al.*, 1987). Tiseo *et al.* performed a complete urological evaluation on SLE patients under different treatments, such as HCQ, comparing to a control group without disease. The determined parameters included hormone levels (follicle-stimulating hormone (FSH), luteinizing hormone (LH), testosterone and estradiol), a urological evaluation (testicular volume and varicocele state), conventional semen analysis, and sperm DNA fragmentation (sDNAfrag) determinations. Conventional sperm parameters were similar in both groups, but significant higher DNA fragmentation index values were detected within SLE patients. It was not found, however, any relation between this increase in sDNAfrag and HCQ treatment (Tiseo *et al.*, 2019).

Due to the lack of evidence on the safety of HCQ for male fertility or the of paternal-mediated teratogenicity, orientations about the administration of HCQ in men are based on the information available for women (Flint *et al.*, 2016; Bermas, 2020).

4.2 Dexamethasone

DEX has long been used to treat disorders that affect male reproductive tract and fertility, such as antisperm antibodies (ASAs). Antisperm antibodies (ASAs) are formed naturally upon exposure of antigenic sperm proteins to the immune system. In healthy men, sperm is physically separated from blood and immune cells through blood-testis barrier (BTB). However, infections and inflammations in the reproductive tract may lead to the disruption of the BTB, allowing the immune system to contact with sperm and create antibodies against sperm proteins. ASAs can be directed against sperm head, midpiece, or tail, affecting sperm function in multiple forms, from motility to its fertilizing capacity. Both men and women can test positive for ASAs without presenting fertility problems, but increased levels of ASAs are associated with infertility, so called immunological infertility (Vickram *et al.*, 2019; Parekattil *et al*., 2020). Studies have shown DEX therapy improve sperm parameters and conception outcomes, by reducing ASAs. In 1977, De Almeida and Soufir published a case report of an infertile couple, where the male partner was positive for ASAs. The man was treated with 2 mg DEX/ day, for 3 months, followed by decreasing doses another 3 months. Along treatment, sperm agglutination and sperm toxicity significantly reduced in both serum and seminal plasma, which was maintained after treatment cessation. Regarding sperm parameters, concentration and vitality were greatly increased with treatment, while vitality remained unchanged. Moreover, pregnancy was achieved during treatment (Almeida

and Soufir, 1977). Years later, Almeida and Jouannet, (1981) studied 14 infertile men with ASAs, who were assigned to receive 2 mg DEX/ day, for 13 weeks or 3 mg DEX/ day, for 9 weeks, respectively. Sperm agglutination and semen parameters were determined to evaluate the effects of DEX. According to the results, both serum and seminal plasma sperm agglutination decreased under treatment. Semen characteristics were also positively influenced by DEX, with a significant increase in progressive motile sperm observed in all participants, as well as in sperm count in oligozoospermic men. Sperm concentrations were maintained in men with normozoospermia (Almeida and Jouannet, 1981).

Congenital Adrenal Hyperplasia (CAH) is a genetic disease characterized by a deficiency on one of the enzymes required for the biosynthesis of hormones in the adrenal gland. The most common cause of CAH is a deficiency in 21-hydroxylase (21-OH), responsible for converting 17-hydroxyprogesterone (17-OHP) into 11-deoxycortisol, to be converted in cortisol. As a result, there is a lack of cortisol in circulation, which, consequently, increase ACTH expression by the pituitary gland. ACTH stimulate the adrenal glands, resulting in overexpression of testosterone. Testosterone plays essential roles during embryonic sex differentiation and spermatogenesis, being produced and secreted in testes by the Leydig cells, upon stimulation of HPG axis. The signalling pathway is initiated in the hypothalamus, that stimulates the pituitary gland to release gonadotropins, hormones that promote gonadal function. LL acts on Leydig cells, inducing testosterone secretion. In turn, testosterone enters Sertoli cells, also present in testes, where it stimulates sperm cells differentiation and maturation (Widmaier *et al*., 2016). Dysregulation of the HPG axis can affect the production of gonadotropins (LH and FSH) and, therefore, impair testicular function and spermatogenesis. Moreover, men with this disease tend to develop testicular adrenal rest tumours (TARTs), benign tumours formed in testis that can cause an obstruction in the seminiferous tubules and lead to mechanical oligospermia or azoospermia. In fact, TARTs are considered the main cause infertility in men with CAH. GCs are the main treatment option, with DEX being mainly used among adult patients, due to its adverse effects on children growth (Shaw, 2010; Lekarev *et al*., 2015). Multiple case reports have been described, on the effects of DEX in infertile men diagnosed with CAH. Collet and Pralong (2010) reported the case of a 26-year-old man with CAH, who presented elevated levels of ACTH and 17-OH, low levels of LH and FSH, TARTs and azoospermia. A complete disappearing of TARTs was observed after treatment (0.5 mg DEX/ twice daily), along with a decrease in the ACTH and 17-OH and an increase in gonadotropins. DEX's effects were notable, as the couple had a successful pregnancy after one-year therapy (Collet and Pralong, 2010). Mouritsen et al. (2010) described the effects of a combined treatment of 10 mg hydrocortisone, 3 times a day, plus 0.1 mg DEX/ day in a 30-year-old man. The patient was prescribed DEX due to the lack of efficacy of the former hydrocortisone regimen in controlling fertility. This therapy adjustment resulted in the regression of TARTs, as well as a significant increase in sperm parameters (concentration, motility, and morphology), which culminated in a successful pregnancy after 6 months (Mouritsen *et al*., 2010). Similar outcomes were presented by Sumida et al. (2011), who described two cases of male CAH

treated with DEX, following a dose regimen of 0.5 mg DEX/ day, for 12 months. In both cases DEX reduced ACTH and testosterone levels and testicular tumours, resulting in improved sperm count and motility (Sumida *et al.*, 2011).

A direct effect in male reproductive tract seems to occur at the hormonal level, on testosterone production. DEX induces negative feedback in the hypothalamus, inhibiting the signalling pathways, thus reducing circulating testosterone levels. This causes profound alterations in sperm cells, which will have in men trying to conceive.

Small trials in healthy men have been carried out to evaluate this effect of DEX, even though with different outcomes. Schaison *et al.* (1978), for instance, assessed the role of GCs on testosterone synthesis in eight men at reproductive age (27 to 59 years old), administered with 8 mg DEX/day, for 3 days. Results showed a significant decrease on hormone levels (approximately 50%) at the end of treatment. Similar outcomes had been reached before, first by Rosner and Conte (1966) and later by Doerr and Pirke (1976). In the first study, a decrease by 35% in excreted testosterone was detected after treatment (0.5 mg DEX every 6 h, for 2 days), comparing to the basal levels (Rosner and Conte, 1966). Doerr and Pirke (1976) verified a complete suppression of testosterone levels after DEX treatment, in 36 volunteers. The authors pointed the high-dose regimen used (6 mg DEX oral dose, followed by 3 mg every 6 h, for 24 h) as the main reason for the observed. In 1992, Veldhuis *et al.* investigated how testosterone levels varied during short-term DEX therapy. Of the 5 men included in this study, each received 1.5 mg DEX/ twice daily or the equivalent placebo, for 8 days. Hormonal levels were measured over the treatment period, being detected a significant decrease in testosterone at the last day. Interestingly, LH concentrations did not change (Veldhuis *et al.*, 1992). Such results corroborate with the suggestion made by Sapolsky, in an animal study using wild baboons. According to the author, DEX inhibits testosterone release at a testicular level by interfering with testicular response to LH stimulation, rather than impairing LH expression by HPG axis (Sapolsky, 1985).

Conversely, there is also a number of studies demonstrating testosterone is not affected by DEX. Faiman and Winter (1971), and Judd *et al.* (1992), both evaluated the effect of this GC in the circadian rhythm of testosterone, and found no effects. Were assigned 8 and 4 men to the studies, respectively, to receive the following doses: 1 mg oral DEX, followed by 0.5 mg every 6h, for 24 h (Faiman and Winter, 1971); 2 mg DEX before sleep (Veldhuis *et al.*, 1992). In 1999, Lac and colleagues developed a randomized study to investigate the reactions of adrenocorticosteroids and sex steroids to different stimuli, one of them being the administration of DEX. In this trial, 24 healthy men were randomized into three groups and assigned to receive oral placebo, DEX at low dose (0.5 mg), or DEX at high dose (1.5 mg), respectively, for 4.5 days. Testosterone levels did not vary in any of the treatment groups, comparing with placebo (Lac *et al.*, 1999), which counters what had been suggested by Doerr and Pirke.

Altogether, this review shows the conflicting results regarding the impact of DEX therapy in male fertility. Further investigation is necessary, as this drug continues to be a treatment of reference for several diseases, nowadays. Moreover, there is a clear gap in this

investigational field, regarding the effect of this GC in spermatogenesis. Only few animal studies have been developed in this matter. Yazawa et al. (2000) used a rat model to evaluate the effect of high doses of DEX (7 mg/ kg) on spermatogonia. Results showed increased DNA fragmentation in these cells after treatment, compared with controls. Orazizadeh et al. (2010) found similar effects in a male NMRI mouse model. In this study, mice assigned to experimental groups received either 4 mg/kg, 7 mg/ kg or 10 mg/ kg of DEX, for 7 days. Interestingly, the group receiving smaller dose did not show significant testicular damages. On the hand, both 7 mg/ kg and 10 mg/kg groups, demonstrated significant alterations, including decreased diameters and height, low testicular sperm count and increased spermatogonia apoptosis (DNA fragmentation). Damages were more severe at the higher the dose of treatment (Orazizadeh *et al.*, 2010). These results bring very relevant considerations on the dose-dependent effect of DEX in spermatogenesis and enlightens the urgent need for further investigation in humans.

4.3 Remdesivir

RDV is a recent drug, having been mostly studied in the context of COVID-19, during the last year. Thus, there are no known experiments regarding the effect of RDV in human fertility (EMA, 2019). As stated above, RDV can be found in animal testis and in human semen (Warren *et al.*, 2016; WHO *et al.*, 2017; Hu *et al.*, 2020); however, the impact it may have on male fertility is not yet well understood and further investigation is needed. Previous studies had shown some nucleoside analogues can have toxic effects in gametogenesis and gonad function. Ribavirin, a guanosine analogue with antiviral activity against several RNA and DNA viruses, was shown to induce cytotoxicity in germ cells and subsequently affect sperm count and morphology (Narayana *et al.*, 2002). Similar effects were observed in an animal study treated with Acyclovir, another guanosine nucleoside analogue used against herpesviruses (Narayana, 2008). In the study with rats administrated with 10 mg/kg RDV, male rats did not experience any adverse event, suggesting RDV has no toxic effects on male fertility (WHO *et al.*, 2017; EMA, 2019; Gilead, 2020).

A recent clinical trial investigated whether RDV could safely reduce the viral RNA still present in the semen of male Ebola survivors. A total of 38 participants were randomized to receive either 100mg/day RDV or matching placebo, on a 5-day treatment course. Analysis of semen samples collected during treatment and a 5-month follow-up demonstrated RDV effectively decreased viral load. Although this effect was visible in both sampling periods, statistically significant differences were only detected on the follow-up phase. Moreover, there were not registered any serious adverse event among individuals assigned to RDV group, suggesting RDV was well tolerated at the dose regimen administrated. The safety of RDV was evaluated by measuring common adverse reactions previously associated with RDV, such as nausea, vomiting, headache, and elevation of transaminases (Higgs *et al.*,

2021). Including tests on sperm cells and reproductive organs would have allowed a better understanding about the safety of RDV in men, and their fertility.

5. COVID-19 Outbreak

In December of 2019, the city of Wuhan, in China, became the centre of a pneumonia outbreak caused by the novel coronavirus, severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). This virus enters the human body through the Angiotensin-I Converting Enzyme 2 (ACE2) receptors, which can be found in various organs such as the heart, lungs, kidneys and gastrointestinal tract, and lead to a wide range of clinical outcomes, from asymptomatic to severe respiratory disorders and death (Astuti and Ysrafil, 2020; Zhou *et al.*, 2020). The wide spread of coronavirus disease (COVID-19) across the world led WHO to characterize it as a Pandemic disease, in March 2020. Since then, there were registered more than 100 million COVID-19 cases worldwide, and there is an ongoing intense search for effective drug therapies to contain this pandemic (Khuroo, 2020; WHO, 2020, 2021). Meanwhile, several unapproved or repurposed drugs have been used as treatment options for this disease.

6. Pharmacological Therapeutics in COVID-19 Treatment

6.1 Hydroxychloroquine

In the absence of specific drugs, HCQ is one of the several drugs already available that has been adopted against SARS-CoV-2 infection. Different studies, mostly *in vitro*, have suggested that HCQ can play antiviral effects in several steps of the virus replication cycle (Vincent *et al.*, 2005; Liu *et al.*, 2020). By increasing endosomal pH, HCQ impairs the activity of acidic enzymes that are responsible for disrupting the viral particle and release the infectious nucleic acid into the cytosol. Thus, the release of the viral genome into the cytosol for replication is inhibited. Additionally, HCQ can affect the interaction between the virus and the ACE2 receptors. As membrane receptors, ACE2 need to be glycosylated to turn into its active form and be able to recognize the viral S glycoprotein. This glycosylation step is inhibited by HCQ, preventing SARS-CoV2 from binding to the receptor and entry into host cells.

Moreover, HCQ can also be used as treatment for more severe cases of COVID-19, to stop the so called "cytokine storm" observed in some patients. It has been verified a subgroup of patients admitted on the ICU suffer from an uncontrolled over-production of cytokines such as IL-2, IL-6, IL-10 and TNF-α. This results in an exacerbation of the immune system, that can lead to acute respiratory distress syndrome (ARDS) and, ultimately, to multiple organ

failure and death. With its known immunomodulatory effects, HCQ is administered in these patients to suppress the release of these cytokines and reduce inflammation (Chen *et al.*, 2020; Coperchini *et al.*, 2020; Pahan, 2020). Although there are proofs of the positive effect of HCQ in COVID-19, these evidences are based mostly on *in vitro* studies and there is not enough clinical data to support the safety of HCQ in these patients (Khuroo, 2020).

Early in March 2020, an open-label nonrandomized trial, enrolling only 36 COVID-19 patients in France, revealed promising data on the use of HCQ to treat COVID-19. According to this report, HCQ significantly reduced SARS-CoV2 viral load within 3-6 days of treatment, and that this effect was significantly higher in patients with symptoms of upper respiratory tract infection (URTI) and lower respiratory tract infections (LRTI), as compared to asymptomatic patients. Even greater outcomes were observed when HCQ was co-administered Azithromycin, an antibiotic commonly used to treat pneumonia (Gautret *et al.*, 2020). Prior to this study, small clinical trials carried out in China also had shown that CQ could successfully inhibit exacerbation of pneumonia, reduce viral load, and shorten the disease course (Gao *et al*., 2020). Based on this information, on March 28th 2020 the U.S FDA authorized the emergency use of HCQ for treatment of hospitalized COVID-19 patients. Aside with the USA, several other countries started to use HCQ to treat COVID-19 from that moment (EMA, 2020d; FDA, 2020). However, randomized-controlled trials (RCTs) and systematic reviews concerning HCQ efficacy and safety on COVID-19 patients started to emerge, suggesting the use of HCQ had no clinical benefits. On May 22nd 2020, it was published the first study providing evidences of the ineffective effect of HCQ in treating hospitalized COVID-19 patients (Mehra *et al.*, 2020). Few days later, WHO announced the discontinuation of the HCQ arm in the ongoing clinical trial carried out by the WHO Trial Consortium. Even though, the WHO Solidarity Trial report released in December included the results on the HCQ efficacy already obtained, showing HCQ had no effect on patient's mortality, need for mechanical ventilation, or hospitalization duration patients (WHO Trial Consortium, 2020). Findings that were also outlined on the systematic review by Siemieniuk et al. (Siemieniuk *et al.*, 2020). A multicentre, randomized, open-label, controlled trial involving hospitalized patients with mild-to-moderate Covid-19 (receiving either no supplemental oxygen or a maximum of 4 L/min of supplemental oxygen) evaluated the efficacy of HCQ alone and in co-administration with azithromycin, on a 7-day treatment course (standard care plus 400 mg HCQ twice daily vs. standard care plus 400 mg HCQ twice daily plus 500 mg azithromycin once daily). Similarly, to what had been reported, HCQ treatment, either alone or with azithromycin, showed no significant effects on patients' clinical improvement. Additionally, this study evaluated possible adverse effects, and more events were registered in patients receiving HCQ alone or in combination with azithromycin, such as nausea, anaemia, elevation of liver-enzyme levels. Prolongation of the QT interval was also find to be more common in patients receiving HCQ plus azithromycin or HCQ alone (Cavalcanti *et al.*, 2020). This last event is, in fact, one of the greatest concerns regarding the use of HCQ on COVID-19 patients, and can have serious cardiotoxic effects (Khuroo, 2020). HCQ can bind to common drug-binding site in potassium channel pores

and block these channels. In the heart, the blockage of potassium channels affects the repolarization of action potential, which is translated by a prolongation of the electrocardiograph QT interval. Heterogeneous prolongation of QT interval predisposes to intraventricular circuits of depolarisation that can lead to potentially lethal ventricular tachyarrhythmia (White, 2007; Kamp *et al.*, 2020). Reported cases identify overdose as the main cause of these events, which given the high-doses administrated during COVID-19 treatment, explains the major concern for these patients (White, 2007). Moreover, it is known HCQ and azithromycin have a synergistic effect on the prolongation of QT intervals, meaning the co-administration of these drugs increase the risk of ventricular tachyarrhythmia, which can be lethal (Lane *et al.*, 2020). The WHO living guidelines published on December 2020, regarding therapeutics used against COVID-19. In this document, the use of HCQ to COVID-19 patients with any disease severity is strongly discouraged. This recommendation was based on evidence from the trials of no clinical improvement, and with possible harm associated, as nausea/vomiting and diarrhoea (WHO, 2020d).

Therefore, as of June 15th the use of HCQ for the treatment of COVID-19 was forbidden on the USA, by the FDA (FDA, 2020a). Before that, WHO had already announced the discontinuation of HCQ from the "Solidarity" clinical trial, and shortly after the INFARMED, in Portugal, also ceased the use of this drug, in agreement with the WHO decision (Infarmed, 2020a). More recently, updated guidelines brought recommendations regarding HCQ as a prophylactic drug against SARS-CoV2 infection. The anti-viral mechanism of HCQ led some investigators to propose this drug as prophylactic therapy, since it would reduce the chances of SARS-CoV2 infection (Pahan, 2020). As of September 2020, only two randomized COVID-19 prophylaxis trials were known. In June 2020, Boulware *et al*. published the report of a randomized-controlled trial testing HCQ as post-exposure prophylaxis, where participants were exposed to SARS-CoV2 infection and randomly assigned 4 days' post-exposure. The treatment group was submitted to an 800 mg HCQ once, plus 600 mg HCQ in 6 to 8 hours on day 1, followed by 600 mg daily dose for 4 additional days. In this study it was evidenced high doses o HCQ had no effect on the incidence of new SARAS-CoV2 infections, when compared to the control. Side effects were more frequent within HCQ group, including nausea, diarrhea, and abdominal discomfort. Cardiac arrythmias were not detected (Boulware *et al.*, 2020). Similar findings were outlined on the later pre-exposure prophylaxis randomized trial by Abella et al. Here, the enrolled participants could not have any history of SARS-CoV-2 infection, and were submitted to a 600 mg HCQ daily dose, for eight weeks (Abella *et al.*, 2020). According to the updated WHO guidelines, on March 2021, the recommendations are against the use of HCQ prophylaxis to individuals who do not have COVID-19. Used prophylactically, HCQ has a small or no effect on death and hospital admission, and small or no effect on laboratory-confirmed SARS-CoV2 infection (WHO, 2021a).

6.2 Dexamethasone

GCs were previously used to treat other human coronaviruses (hCoVs) infections, such as SARS and MERS, in patients experiencing lung injury and multi-organ damage, to reduce systemic inflammation. Similar manifestations have been observed in some cases of SARS-CoV-2 infection, thus DEX is being suggested as treatment in these patients (Lee *et al.*, 2020). Upon viral infection, SARS-CoV-2 starts to replicate and spread through the respiratory system, lodging in the lungs. As the virus continuous to replicate, macrophages and other antigen presenting cells (APCs) are activated and release several pro-inflammatory cytokines and chemokines (IL-1, IL-6, macrophage inflammatory proteins (MIP1α, MIP1β), IFN-γ inducible protein (IP-10)), initiating an immune response. Secretion of these molecules attracts more immune cells, including monocytes, macrophages, and T cells, which in its turn produce more cytokines that bring more cells to the infected site. In most of the cases (asymptomatic and mild-to-moderate disease), the first recruited cells are capable to control infection and neutralize the virus, leading to patient's recovery with minimal lung damage. However, in some patients, the immune response triggered cannot efficiently eliminate viral burden, leading to an uncontrolled viral replication accompanied by an hyperresponsiveness of the immune system to infection. Immune cells start to accumulate in the lungs, inducing an overproduction of cytokines and chemokines, such as IL-6, IL-1, IL-1, IP-10, IFN-γ and TNF, which leads to a cytokine storm. This phenomenon is responsible for the most acute manifestations of COVID-19, since exacerbated levels of pro-inflammatory proteins cause lung injury that can evolve to acute respiratory distress syndrome (ARDS), as well as enter circulation and damage other organs, leading to multiple organ failure and culminate in death. As a potent anti-inflammatory agent, DEX mitigates the systemic inflammation, through its genomic mechanism of action, as previously described in this review. The activated DEX-GR complex inhibits the production of several pro-inflammatory cytokines involved in the COVID-19- associated cytokine storm (IL-6, IL-1, TNF, IFN-γ), by blocking the transcription of the genes encoding these proteins (transrepression) (Ahmed and Hassan, 2020; Fajgenbaum and June, 2020; Tay *et al.*, 2020). Although corticosteroids have been repurposed as treatment for COVID-19 from the beginning of the outbreak (Huang *et al.*, 2020), concerns were rapidly raised given the wide range of adverse effects associated to corticosteroid therapy. Russel *et al.* summarised the available evidence on the use of corticosteroids in patients with severe respiratory virus infections, such as previous hCoVs (MERS and SARS), and found no beneficial effects in this therapy, rather than several adverse reactions including delayed viral clearance, diabetes and avascular necrosis (Russell *et al.*, 2020). Based on the same evidence, the WHO published interim guidelines on the clinical management of SARS-CoV-2 infections, where corticosteroids were discouraged based on the potential toxicity of these agents and the lack of evidence on its effectiveness in the context of the pandemic (WHO, 2020a). However, most of these data were obtained from observational studies, thus more sustained evidence was necessary for better-founded therapeutic recommendations. In March 2020, it was

launched the first and largest clinical trial evaluating the efficacy of DEX in the treatment of hospitalized COVID-19 patients, the Randomised Evaluation of COVID-19 (RECOVERY) trial. Developed by the University of Oxford, this controlled, open-label trial included more than 6000 hospitalized patients, that were randomly assigned to receive either standard of care (SOC) treatment alone, or in combination with 6 mg/day DEX, up to 10 days, administered orally or intravenously. All-cause mortality within 28-days and time to recovery/discharge were the main outcomes evaluated. Overall, both parameters were significantly lower in the DEX group, comparing to the SOC group. In a subgroup analysis based on the level of respiratory support, greatest mortality outcomes were observed within patients requiring invasive mechanical ventilation, that received DEX treatment. On the contrary, DEX did not show statistically significant effects among patients that did not require any oxygen support. With such findings, the RECOVERY trial provided robust evidence on the benefits of DEX in hospitalized COVID-19 patients requiring respiratory support (RECOVERY Collaborative Group, 2021). As the preliminary results became public in June 2020, the Medicines and Healthcare Products Regulatory Agency (MHRA), United Kingdom, issued a COVID-19 Therapeutic Alert granting authorization for the use of DEX in critically ill patients, following the dose regimens used during the trial (6 mg/day DEX, up to 10 days) (MHRA, 2020). In Portugal, this drug was already being given under a special use authorization and the national medicines authority, Infarmed, declared new recommendations would follow the European Medicines Agency (EMA) decisions, upon a review of the results (EMA, 2020e; Infarmed, 2020b). The promising findings also brought WHO to reconsider the initial orientation regarding corticosteroids therapy in COVID-19 (WHO, 2020f). Meanwhile, a living systematic review was published comparing the effects of several drugs used to treat COVID-19, as part of the BMJ Rapid Recommendations project. Were compared drugs as the antiviral RDV, corticosteroids, HCQ, azithromycin and co-administration of lopinavir/ritonavir, in COVID-19 hospitalized patients. Similar to what had been observed in the RECOVERY Trial, in this review corticosteroids also showed to be the most promising treatment for COVID-19. Data demonstrates corticosteroids have greater effects on reducing mortality, the need for mechanical ventilation, and ICU length of stay, as well as in increasing ventilator-free days in patients with severe covid-19, when compared to SOC and the other drugs evaluated (Siemieniuk *et al.*, 2020).

In September 2020, WHO published the results of a meta-analysis on the efficacy of systemic corticosteroids in the context of COVID-19, developed by WHO Rapid Evidence Appraisal for COVID-19 Therapies (REACT) Working Group. DEX, hydrocortisone and methylprednisolone were the corticosteroids evaluated in this meta-analysis that included 7 trials, among which the RECOVERY Trial. All-cause mortality and serious adverse event were the main outcomes studied. According to the results, all trials demonstrated an association between the use of corticosteroids and a lower risk of mortality within critically ill patients. Among all the corticosteroids evaluated, DEX was the drug with the lower association to mortality rates. Regarding serious adverse reactions, the more commonly observed included secondary infections, hyperglycaemia, and gastrointestinal damages

(bleeding and perforation); however, it was not possible to establish clear association between these events and the use of corticosteroids, since the definition of "serious adverse event", as well as the methods used to assess these events varied between trials (Sterne *et al.*, 2020).

Based on all the evidence, WHO finally reissued the initial recommendation against the use of corticosteroids and released a living guidance on the use of corticosteroids for COVID-19. These guidelines rely on evidence from the most relevant studies, including the living meta-analysis by Siemieniuk et al. and the REACT meta-analysis. In this document, it is recommended the use of corticosteroids in patients with severe and critical COVID-19, while a second recommendation discourages the use of these drugs to treat patients with non-severe disease, regardless their hospitalization status. Corticosteroids must be administered orally or intravenously, in single doses of 6 mg DEX daily (or equivalent), during 7 to 10 days, and treatment should be initiated only if patients meet the criteria of severe disease, independently of symptom onset (WHO, 2020b). Followed by the WHO Living Guidance, also EMA issued a statement endorsing the use of DEX in COVID-19, a decision supported by an extensive review of the available data, together with the REACT meta-analysis outcomes. Similarly, DEX was authorized only for adults and paediatric patients (12 years old or older and weighing at least 40 kg), who required any supplemental oxygen therapy (mechanical or non-mechanical), in doses of 6 mg DEX/day, up to 10 days, orally or intravenously (EMA, 2020b). In Portugal, COVID-19-related decisions are taken alongside with EMA, therefore after EMA's authorization being released, Infarmed reported the new orientations to be followed on the use of DEX in COVID-19 patients to the medical community (Infarmed, 2020c).

As the pandemic keeps ongoing, researchers continue to investigate for a better understanding of this virus, the effect it has on the global population, and effective treatments to ameliorate patient's clinical outcomes. A recent publication by Zhang et al. brought new insights on the role of DEX in the treatment of COVID-19, suggesting that DEX has antiviral effects that can contribute to the treatment of these patients, alongside with the already know anti-inflammatory actions. In this *in vitro* study, 7 GCs were evaluated for possible interactions with ACE2, the receptor used by SARS-CoV-2 to infect human cells. The results demonstrated DEX bind with greater affinity to the active sties of both ACE2, in its bioactive state. Furthermore, the effect of each GC in inhibiting viral infection was assessed in ACE2 high expressing-cell culture (ACE2h cells) incubated with SARS-CoV-2 spike pseudotyped virus. Once again, only DEX showed relevant results, by significantly reducing the number of infected cells. Such findings suggest DEX may have, in fact, an antiviral effect by impairing viral entrance into cells thorough interaction with the host receptor ACE2. Although the ground breaking results, the authors recognize further investigation is needed to support these conclusions (Zhang *et al.*, 2021).

Nowadays, DEX is still used in COVID-19 therapy due to its anti-inflammatory effects, following the recommendations summarized in the NIH COVID-19 treatment guidelines and WHO Corticosteroids Living Guidance (6 mg/day, up to 10 days, for patients requiring

supplemental oxygen support, mechanical or non-mechanical) (NIH, 2020; W. H. O. WHO, 2020b). Moreover, the NIH COVID-19 treatments guidelines also include instructions for the co-administration of DEX and the antiviral RDV. It is known COVID-19 disease is characterized by an exponential viral replication during the first days of infection (3 to 7 days) as the virus starts to spread and lodging in the lungs, which can be followed by an uncontrolled immune response, in more severe cases of disease (Ahmed and Hassan, 2020). Even though there are still lack of evidence regarding this combined therapy, both RDV and DEX separately have been shown to significantly improve patients' outcomes; therefore, co-administration of these two drugs is being recommended for patients who require elevated amounts of supplemental oxygen, or oxygen delivery through a high-flow device or non-invasive ventilation (NIH, 2020).

Despite the clear benefits of DEX in reducing COVID-19 severity and mortality, the wide range of side effects associated to DEX therapy are well known and should be taken into consideration when prescribing this drug. So far, no severe adverse reaction has yet been directly associated to the use of DEX; however, patients receiving this drug should be closely monitored for possible side effects including hyperglycemia, hypertension, gastrointestinal damages, and mood alterations. Moreover, follow-up consults should be maintained for long term reactions, in musculoskeletal and endocrine systems, for instance (Chen *et al.*, 2021).

6.3 Remdesivir

RDV antiviral activity against a broad spectrum of coronaviruses, including SARS-CoV and MERS-CoV had already been proven in several in vitro and animal models (Warren *et al.*, 2016; Sheahan *et al.*, 2017); thus, it was one the first antivirals to be proposed for the treatment of SARS-CoV-2 infection (Jorgensen *et al.*, 2020). SARS-CoV-2 binds to host cell ACE2 receptors and enters cells via endocytosis. Once inside, the viral genomic material (positive mRNA) is released in the cytoplasm and genes encoding for the viral replicase-transcriptase complex (RTC) are directly translated by host cell's machinery. This protein complex, composed by RdRp and helicase-containing subunits, is essential for the virus replication cycle, particularly the RdRp, without which the virus cannot replicate the progeny genome, nor transcribe genes encoding for structural proteins. Consequently, there is no formation of new viral particles, capable of infecting new cells (Astuti and Ysrafil, 2020). The antiviral activity of RDV falls precisely on RdRp activity. The triphosphate form of the drug is misintegrated in the viral genome by RdRp, during replication, leading to a premature termination of genome synthesis. An *in vitro* study revealed that SARS-CoV-2 RdRp, along with the other coronavirus (MERS and SARS-CoV-1) RdRp complexes, specifically stopped replication 3 nucleotides after GS-443902 integration. The study suggested the mechanism of action was based on the proximity between the 1'-cyano substituent of RDV and the RdRp, upon the incorporation of the 3rd nucleotide. This interaction causes significant distortion of the RNA positioning and prevents the correct

incorporation of the 4th nucleotide (Gordon *et al.*, 2020). Thereby, RDV inhibits the virus replication cycle and as a result, prevents viral transmission.

Based on *in vitro* evidence, Wang *et al.* developed the first clinical trial to evaluate the efficacy and safety of RDV in COVID-19. Hospitalized patients with severe COVID-19 were randomly assigned to receive either a dose regimen of 200 mg RDV (day 1) followed by 100 mg RDV (days 2–10) in single daily infusions, or the same amount in placebo infusions. Clinical improvement, mortality, the need for mechanical ventilation and viral clearance, were the main parameters evaluated in this trial. According to the results, RDV did not show significant differences in any of the clinical outcomes studied, when compared to the placebo group. Adverse events observed throughout the trial, including hypoalbuminaemia hypokalaemia, and anaemia, occurred in similar proportions in both groups. Altogether, these findings suggest that the dose regimen of RDV used was well tolerated but it was not sufficient to induce significant beneficial clinical outcomes, at least in patients with severe disease (Wang *et al.*, 2020). Conversely, preliminary results from the Adaptive Covid-19 Treatment Trial (ACTT-1), a multinational clinical trial involving USA and several countries in Europe, demonstrated RDV was superior to placebo. This study included hospitalized patients with mild-to-moderate or severe disease, that were randomly assigned to the same dose regimen administrated in the Wang *et al.* trial. Patients who received RDV had a significantly shorter time to improvement and recovery, as well as low mortality rates. Moreover, RDV appeared to be more efficient when administrated earlier in the disease and may prevent the progression to more severe states, as evidenced by recovery rates in patients randomized within the first 10 days of symptoms, comparing with patients assigned later than 10 days after de onset of symptoms, and the lower proportion of patients needing respiratory support throughout the study (Beigel *et al.*, 2020).

Given these results, on May 1st, 2020, the FDA issued an Emergency Use Authorization for emergency use of RDV in the treatment of hospitalized patients with severe COVID-19 (FDA, 2020b). By the end of June, also the EMA granted a conditional marketing authorisation for the use RDV in hospitalized adults and pediatric patients (12 years of age and older and weighing at least 40 kg) requiring supplemental oxygen. With this, RDV became the first drug against COVID-19 to be recommended for authorization in the European Union (EMA, 2020b). Infarmed, the National Medicines Authority in Portugal, decided in agreement with EMA. According to Infarmed's announcement this drug was already available in Portugal to treat particular cases, under exceptional authorizations (Infarmed, 2020e). RDV was recommended in a dose regimen of a single 200 mg loading dose on Day 1 followed by 100 mg once-daily doses maintained for a minimum of 4 days and no more than 9 days (EMA, 2020b; FDA, 2020c).

Concurrent with the findings on severe cases of disease, Spinner *et al.* looked for the efficacy and safety of RDV in hospitalized patients with moderate COVID-19. Patients were enrolled into three different groups, corresponding to a 10-day RDV treatment course, a 5-day RDV treatment course, and continuing with standard care. RDV was administrated in accordance with the doses already authorized. At the end of both treatment courses (day 11), results

indicated a significant improvement in the clinical status of the patients enrolled on the 5-day RDV treatment, when comparing with standard care. In the 10-day RDV treatment group, clinical improvements only showed to be statistically significant on day 14. The number of patients experiencing adverse reactions was similar among the 5-day RDV group and standard care, but when comparing standard care with the 10-day RDV percentages showed to be significantly different. Additional endpoints, such as duration of oxygen support, hospitalization and all-cause mortality rates, were also evaluated and no significant differences were observed between the RDV and standard care groups (Spinner *et al.*, 2020). Based on these results, the FDA reissued the initial emergency authorization to include patients with moderate disease. Given the continuous emerging of new trials and updating outcomes, on October 22nd, 2020, FDA reconsidered once again the emergency authorization and finally approved RDV as therapy for COVID-19 patients requiring hospitalization. RDV was the first FDA approved drug for the treatment of adults and paediatric (12 years of age and older and weighing at least 40 kg) COVID-19 patients. Because this approval did not include all paediatric subpopulation, Emergency Use Authorization continues to be valid for the emergency use in hospitalized paediatric COVID-19 patients weighing at least 3.5 kg to less than 40 kg (FDA, 2020b, 2020d).

On November 20th, 2020, WHO released a conditional recommendation against the use of RDV in hospitalized patients, irrespective disease severity (WHO, 2020e). The decision was founded on an evidence review from 4 international randomised trials, including the ACTT and the WHO Solidarity Trial, one of the largest international randomized trials, that evaluated the effect of 4 drugs, including RDV, on important COVID-19 clinical outcomes, such as mortality, need for assisted ventilation and duration of hospitalization (BMJ, 2020; WHO, 2020c). Interim results from this study revealed no significant effects on any of the outcomes studied, in any of the drugs' arms (WHO Trial Consortium, 2020). Given these results, specialists argued that there was no strong evidence proving the efficacy of RDV in survival or other clinical improvements (BMJ, 2020). Despite WHO recommendation, FDA and EMA each issued a statement informing RDV would maintain its conditions of approval, already in force. As explained in both authorities' declarations, although WHO Solidarity Trial had provided relevant results, they are not directly comparable nor contradictory with the ACTT findings, the main support for the authorizations. Therefore, the decision was to maintain RDV available until new information emerges, from data review (EMA, 2020c; Infarmed, 2020d; FDA, 2021). In December, Buckland and his colleagues published a case report that brought new insights regarding RDV effects on COVID-19, in a specific group of patients. The article described the use of RDV in a COVID-19 patient suffering from X-linked agammaglobulinaemia (XLA), a genetic antibody deficiency characterized by severe hypergammaglobulinemia and the absence of mature B cells in the peripheral blood. XLA is caused by mutations in the gene encoding Bruton's tyrosine kinase (BTK), an essential protein for the maturation of B cells. As a result, B cell development is impaired and, consequently, patients present reduced levels (<1 %) of circulating B lymphocytes and immunoglobulins (Suri *et al.*, 2016; Buckland *et al.*, 2020). It is known immune response

has a great role in COVID-19 pathology and progression, in an independent way of viral replication (NIH, 2020). Thus, patient's immunodeficient condition allowed to evaluate the antiviral activity of RDV in COVID-19, without the contribution of the immune system to disease progression. According to the report, this patient experienced clinical improvements within 36h after RDV administration, which were accompanied by a decrease in the viral load. These findings show that RDV has a strong effect on clinical and virological response in COVID-19 (Buckland *et al.*, 2020). Moreover, it suggests the results observed in the RCTs may not be due to the lack of antiviral activity of RDV, rather the immune response action on disease progression.

Nowadays, RDV is recommend for hospitalized patients who require supplemental oxygen or oxygen delivery through a high flow device or non-invasive ventilation, in the dose regimen approved by FDA: 200 mg on Day 1 followed by once-daily maintenance doses of 100 mg. Treatment duration may vary from 5 to 10 days, according to the severity of disease at baseline or if patients do not demonstrate clinical improvement at day 5. RDV is not recommend for patients requiring mechanical ventilation, given the lack of evidence showing drug's benefit at such advanced stages of the disease. As previously stated, and confirmed by Buckland *et al.*, there are two main contributors for COVID-19 disease. Early stages of infection are characterized by an intense replication of the virus, spreading throughout the body, and accumulating into target tissues. More advanced states of the disease are mainly due to an exacerbated immune response, as the body tries to respond to infection. Based on this understanding, RDV has often been administrated in combination with DEX, a corticosteroid with anti-inflammatory and immunosuppressive activity used to control the immune-mediated damage of lung tissue in COVID-19 patients (Ahmed and Hassan, 2020; NIH, 2020). Although this combined therapy has not yet been studied in clinical trials, DEX has shown very promising results in improving patients' outcomes, being considered a drug of excellence for COVID-19 treatment. According to NIH COVID-19 treatment guidelines, combined therapy of RDV and DEX is prescribed for patients who require elevated amounts of supplemental oxygen and patients requiring oxygen delivery (NIH, 2020).

7. Objectives

The aim of this in-vitro study was to evaluate the toxicological effects of three therapeutic drugs used in COVID-19 on human sperm. Evaluation of drugs' effect on human sperm is an important predictor of their effects on male infertility. Hydroxychloroquine (antimalarial; HCQ), dexamethasone (glucocorticoid; DEX) and remdesivir (antiviral; RDV) were the drugs studied. Moreover, DEX and RDV were used in co-administration, thus, besides to the individual, we also evaluated the combined effects of these drugs. Sperm viability, motility, sperm oxidative stress and sperm DNA damages were assessed in human sperm cells exposed to the usual therapeutic regimen of each drug and compared to control sperm.

Sperm DNA damage can be evaluated in different ways. Our analysis included the assessment of chromatin condensation status and sDNAfrag. During the final stages of spermatogenesis, sperm nucleus undergoes significant rearrangements to become more compact. Chromatin condensation results from the replacement of histones, nucleoproteins that stabilize chromatin in somatic cells, for smaller and stronger proteins, named protamines. Protamines tightly condense sperm chromatin and protect sperm DNA. When this replacement is incomplete, some parts of sperm chromatin are not correctly condensed (immature chromatin) and become more susceptible to oxidative stress and sDNAfrag. Exogenous factors, including drugs, can interfere with protamines and induced chromatin decondensation. We assessed chromatin condensation through the aniline blue (AB) staining assay, which distinguished histone-rich chromatin (immature chromatin) from protamine-rich DNA (mature chromatin) (Sellami *et al.*, 2013). sDNAfrag was evaluated following the direct assay, TdT (terminal deoxynucleotidyl transferase)-mediated dUDP nick-end labelling (TUNEL), which measures both single and double-strand DNA breaks (American Society for Reproductive Medicine, 2008). In both techniques, only morphologically normal sperm cells were considered. We believe to be enhancing clinical value of our results, considering the assisted reproductive technology (ART) procedures.

Materials and Methods

1. Ethics

The ethical guidelines were strictly followed in this study, as requested in the National Law on Medically Assisted Procreation (Law n° 58°/2017) and in the National Council on Medically Assisted Procreation guidelines (CNPMA-2018), with regard to biological material and clinical databases of patients involved in the research. Written informed consent was obtained prior to the initiation of the work and individual anonymity was guaranteed throughout the research. As this study did not involve experiments on humans or animals, approval from the Ethics Committee and the Declaration of Helsinki, revised in Tokyo 2004, on human experimentation was not required.

2. Patient selection and semen collection

Semen samples from patients who sought sperm analysis at the fertility clinic were collected by masturbation in sterile containers after a 3-day period of sexual abstinence. After sample collection and liquefaction, semen parameters were evaluated by professional embryologists according to World Health Organization (WHO) guidelines (WHO, 2010). 40 samples were selected to enrol the study based on the following inclusion criteria: a semen volume ≥ 1.5 mL and a sperm concentration $\geq 15 \times 10^6$/mL. Samples presenting blood, leukocytes and/or microorganisms were excluded.

3. Experimental Design

3.1 Hydroxychloroquine

After the clinical semen analysis, 1mL of the remaining ejaculate was centrifuged at 1500 rpm for 10 minutes and the seminal fluid discarded. The resultant pellet was resuspended in 100 μL of pre-warmed sperm preparation medium (SPM; Origio, Jyllinge, Denmark). Considering the sperm concentration of each patient, samples were diluted in SPM to obtain a final sperm concentration of 15×10^6/mL. The diluted sample was equally distributed into 3 different experimental groups: Time 0 group (T0); control group (CT), both corresponding to sperm incubated with SPM; and HCQ group, where 50.3 ng/mL of hydroxychloroquine sulphate (H0915; Sigma-Aldrich, St. Louis, USA) was added to the sperm sample. The CT

and HCQ groups were incubated in a humidified incubator with 5% CO_2 at 37 °C for 2 h, after which the following parameters were evaluated: total progressive motility, vitality, hypo-osmotic swelling, morphology, chromatin condensation, DNA fragmentation and DNA oxidative damage. T0 group was directly evaluated for chromatin condensation, DNA fragmentation and DNA oxidative damage. This procedure was repeated for each of the 20 patients.

The HCQ concentration used in this study was that considered pharmacologically and physiologically relevant and reflects the peak serum concentration reached after the administration of the therapeutic dose in humans (Tett *et al.*, 1989; FDA / CDER, 2017).

3.2 Dexamethasone and Remdesivir

After the clinical semen analysis, 1mL of the remaining ejaculate was centrifuged at 1500 rpm for 10 minutes and the seminal fluid discarded. The resultant pellet was resuspended in 100 µL of pre-warmed sperm preparation medium (SPM; Origio, Jyllinge, Denmark). Considering the sperm concentration of each patient, sperm samples were diluted in SPM to obtain a final sperm concentration of 15×10^6/mL. The diluted sample was equally distributed into 5 different experimental groups: Time 0 group (T0); control group (CT), both corresponding to sperm incubated with SPM; DEX group, where 51 ng/mL of dexamethasone (D4902; Sigma-Aldrich, St. Louis, USA) was added to the sperm sample; RDV group, corresponding to the sperm sample incubated with 4377.9 ng/mL of remdesivir (Cayman chemical, Michigan, USA); and DEX/RDV group, which consisted of the concomitant exposure of the semen sample to both drugs. The experimental groups, except for T0, were incubated in a humidified incubator with 5% CO_2 at 37 °C for 2h and the following parameters were evaluated at the end: total progressive motility, vitality, hypo-osmotic swelling, morphology, chromatin condensation, DNA fragmentation and DNA oxidative damage. T0 group was directly evaluated for chromatin condensation, DNA fragmentation and DNA oxidative damage. This procedure was repeated for each of the 20 patients.

Both DEX and RDV concentrations used in this study were those considered pharmacologically and physiologically relevant and reflect the peak serum concentration reached after the single administration of the therapeutic doses in humans (Varis *et al.*, 2000; Therapeutic Goods Administration, 2020).

4. Sperm parameters analysis

Sperm parameters were evaluated following the WHO guidelines (WHO, 2010). For sperm motility, 10 µl of each sperm sample were placed on a glass slide and covered with a coverslip to be analysed. Sperm cells were scored as progressive motile, in situ motile or

immotile sperm. Vitality was assessed using the eosin-Y test. 10 µl of a 0.5% eosin-Y solution (Merck, Darmstadt, Germany) in NaCl were added to 10 µl of each sperm sample and 10 µl of the mix was dropped on a glass slide and covered with a coverslip for observation. Dead (stained) and vital (unstained) spermatozoa were distinguished. Finally, in the hypoosmotic swelling test (HOST), 10 µl of sperm samples were mixed with 100 µl of an hypoosmotic solution (0.375 g sodium citrate dihydrate (S-4641) plus 1.351 g D-Fructose (F-0127) in 100 ml ddH$_2$O) (Sigma Aldrich, St. Louis, USA) and incubated at 37ºC for 30-120 min. After incubation, 20-30 µl of the mix was placed on the glass slide and covered to observe the reactive (swollen tails) and non-reactive (normal tail) cells.

5. Determination of sperm chromatin condensation

Sperm chromatin condensation was determined by acidic aniline-blue staining assay (AB staining), as previously described (Rabaça *et al.*, 2020). Briefly, 10 µL of each sample was spread on glass slides treated with 3- aminopropyltriethoxysilane (APES) and left to air-dry. The samples were fixed with 3% glutaraldehyde (Merck, Darmstadt, Germany) in 0.2M phosphate buffered saline (PBS, pH 7.4) for 30 min at room temperature (RT). Then, the samples were stained with 5% aqueous aniline-blue (Sigma-Aldrich, St. Louis, USA) in 4% acetic acid (VWR, Radnor, USA) (pH3.5) for 5min, at RT and then washed in running tap water and allowed to air-dry. On each slide, a minimum of 200 morphologically normal sperm cells were evaluated under an Olympus CX21 optical microscope (Olympus Corporation, Tokyo, Japan). Sperm cells were discriminated between dark blue stained heads (AB positive) indicating immature histone-rich chromatin, and unstained heads (AB negative) mirroring protamine-rich mature chromatin. The results were expressed in percentage of dark blue stained sperm heads (AB positive) in the total of morphologically normal sperm cells counted.

6. Determination of sperm DNA fragmentation

sDNAfrag was evaluated by the terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) assay using the In-Situ Cell Death Detection Kit (Roche, Mannheim, Germany), as previously described (Sá *et al.*, 2015). 10 µL of each sample was spread on APES-treated glass slides, left to air-dry, and fixed with 4% paraformaldehyde (Merck, Damstadt, Germany) in PBS for 1 h, at RT. Slides were then washed in PBS, permeabilized with 0.1% sodium citrate in 0.1% Triton-X (5 min, 4 °C) and washed in PBS two times. The TUNEL mixture was added, and slides were incubated in a dark-moist chamber at 37 °C, for 1 h. After the incubation period, the slides were washed in PBS and finally counterstained

with mounting medium containing DAPI (Vectashield antifade medium containing 4',6-diamidino-2-phenylindole, DAPI; Vector Laboratories, Burlingame, CA, USA). On each slide, a minimum of 200 morphologically normal sperm cells were evaluated on a Nikon Eclipse E400 fluorescence microscope (Nikon, Tokyo, Japan) and distinguished as having DNA fragmentation, if exhibiting intense green fluorescence (TUNEL-positive) or being normal, if presenting DAPI staining only (TUNEL-negative). The results were recorded as a percentage of sperm with green fluorescence (TUNEL-positive) in total counted normal sperm (DAPI-stained).

7. Determination of sperm DNA oxidative damage

Sperm DNA oxidative damage was determined by luminol-based chemiluminescence assay. Samples were pipetted into 2 well of a white 96-well microplate (Costar, Kennebunk, USA) corresponding to the test sample, and to the sample positive control (spC+). PBS was used for the controls: Blank, Negative control (CT-) and Positive control (CT+). To both positive controls (sCT+ and CT+), it was added hydrogen peroxide (H_2O_2) to induce oxidative stress in cells. Lastly, luminol solution 5mM in DMSO was added, except in the Blank. This solution interacts with reactive oxygen species (ROS), a strong biomarker of cellular oxidative stress. Luminescence of the samples was monitored, after gently horizontal vortex, on a Synergy H1 microplate reader (Biotek, Vermont, U.S.A) using the Endpoint reading method (0.2s detection time/well). Results were generated automatically by Gene5 Microplate Reader and Imager software.

8. Statistics Analysis

Statistical analysis was performed using SPSS statistics software (Version 27, IBM, USA). Since both control and experimental groups arise from the same semen samples, paired samples were considered. First, samples' distribution was assessed by Shapiro-Wilk test to decide which statistical test was best suited for the analysis. For samples with normal distribution, the paired-sample T test was performed, while samples that failed the normality test were evaluated by the non-parametric Wilcoxon signed-ranked test. All data are shown as median (interquartile range; IQR) and complemented with mean ± standard error of mean (SEM). A p-value < 0.05 was considered statistically significant.

Results

1. Patient characteristics

Patients enrolled in this study had their sample evaluated at the time of collection. As expected, given the inclusion criteria used in patient's selection, no significant differences were found in male ages and basic semen parameters between patients, in the groups of experiments (HCQ and DEX plus RDV). Parameters evaluated included semen volume and pH, sperm concentration, motility, normal morphology, vitality and membrane integrity (HOST). Percentages of sperm immature chromatin and sDNAfrag were also evaluated at baseline, with sDNAfrag showing to be significantly different between patients. Results are described in Table 1.

Table 2 Demographic data, basic sperm parameters, chromatin condensation and sperm DNA fragmentation values at baseline, of patients enrolled in the study

	HCQ GROUP (N=20)		DEX+RDV GROUP (N=20)		
	Median (IQR)	Mean ± SEM	Median (IQR)	Mean ± SEM	p-value
AGE (YEARS)	38.0 (10.0)	36.95 ± 5.1	36.5 (5.0)	36.8 ± 3.4	NS
VOLUME (ML)	3.1 (1.9)	3.1 ± 1.4	3.3 (2.6)	3.4 ± 1.4	NS
PH	7.9 (0.0)	7.9 ± 0.1	7.9 (0.2)	7.8 ± 0.1	NS
CONCENTRATION (NO. SZ/ML)	60.3 (60.6)	71.7 ± 47.3	74.5 (68.3)	85.7 ± 56.99	NS
MOTILITY (%)					
TM (%)	62.5 (11.0)	62.3 ± 7.9	62.5 (17.0)	62.8 ± 10.6	NS
TPM (%)	49.5 (10.8)	47.7 ± 9.0	45.5 (19.8)	46.2 ± 12.9	NS
NM (%)	3.5 (4.8)	4,5 ± 3.4	3.0 (3.0)	3.6 ± 2.2	NS
TZI (%)	1.7 (0.2)	1.7 ± 0.1	1.6 (0.2)	1.6 ± 0.2	NS
VITALITY (%)	75.5 (12.5)	75.5 ± 7.97	79.0 (7.3)	77.6 ± 7.5	NS
HOST (%)	68.0 (14.0)	66.7 ± 9.7	68.0 (16.0)	69.1 ± 9.4	NS
AB+ (%)	11.5 (7.7)	11.5 ± 5.1	14.36 (6.18)	13.6 ± 4.8	NS
TUNEL+ (%)	9.5 (11.8)	13.6 ± 8.8	20.5 (7.0)	21.0 ± 4.3	0.002

Dex = dexamethasone, HCQ = hydroxychloroquine, RDV = remdesivir.
IQR = interquartile range, SEM = standard error of mean, NS = not significant.
tm = sperm total motility, tpm = sperm total progressive motility, nm = normal morphology,
TZI = teratozoospermia index, HOST = sperm hypo-osmotic swelling test.
AB+ = positive sperm after aniline blue staining, TUNEL+ = terminal deoxynucleotidyl transferase dUTP nick end labelling.
significance at p < 0.05

2. Hydroxychloroquine

2.1 Effects on Basic sperm parameters analysis

Upon the experimental incubation period, samples from both control and HCQ groups were assessed. According to the results shown in Table 2, the HCQ group showed significant differences in the mean percentage of viable sperm ($\rho = 0.008$) and total progressive motile sperm ($\rho < 0.001$), when compared to the control group. Besides the progressive motility, total motility also had a significant decreased in the presence of HCQ ($\rho < 0.001$). On the other hand, HCQ had no significant effects on sperm cytoplasmic membrane integrity, as confirmed by the HOST.

2.2 Effects on chromatin condensation and sperm DNA fragmentation

Alterations in sperm chromatin condensation were assessed by the acid AB staining assay. Through this test, we obtained the percentage of sperm with immature chromatin (AB positive) in each sample. A significant increase in uncondensed chromatin was found in the HCQ group compared to the control (Table 2).
TUNEL assay was used to evaluate sDNAfrag, returning the percentage of sperm with DNA fragmentation (TUNEL positive). Results showed a significant increase in sDNAfrag among the HCQ group, when compared to the control (Table 2).

Table 3Effects of Hydroxychloroquine in sperm parameters, chromatin condensation and sperm DNA fragmentation

		SPERM PARAMETERS					
		VITALITY (%)	TPM (%)	TM (%)	HOST (%)	AB+ (%)	TUNEL+ (%)
	Mean ± SEM	49.87 ± 16.68	44.76 ± 16.68	51.57 ± 16.95	86.40 ± 6.4	9.58 ± 3.77	21.39 ± 11.9
	Median (IQR)	52.95 (29.14)	48.37 (26.30)	54.30 (30.06)	87.88 (7.15)	8.75 (4.08)	18.75 (9.38)
	Mean ± SEM	42.68 ± 14.46	35.99 ± 17.18	42.34 ± 16.97	85.92 ± 6.89	10.70 ± 4.43	35.83 ± 15.01
	Median (IQR)	46.38 (23.11)	31.46 (30.88)	39.16 (33.15)	87.70 (6.22)	9.75 (5.51)	32.75 (14.63)
P-VALUE		0.008	<0.001	<0.001	NS	0.028	<0.001

CT= control, HCQ = hydroxychloroquine

IQR = interquartile range, SEM = standard error of mean, NS = not significant.

TM= sperm total motility, TPM = sperm total progressive motility, HOST = sperm hypo-osmotic swelling test.

AB+ = positive sperm after aniline blue staining, TUNEL+ = positive sperm after terminal deoxynucleotidyl transferase dUTP nick end labelling

significance at $p < 0.05$

Moreover, with TUNEL assay it was possible to distinguish different sDNAfrag patterns, according to the region of the sperm head that emits fluorescence (TUNEL positive). Head (H) (Fig.3A) was considered when most sperm head was fluorescent. The post-acrosomal region (PAR) (Fig.3B) was scored when only the lower zone of the head emitted green fluorescence. Finally, the equatorial region (ER) (Fig.3C), a defined bright line surrounding the middle of sperm head, and the acrosome vesicle region (AVR) (Fig.3D), which corresponds to the upper zone of the sperm head, the acrosome region, were distinguished.
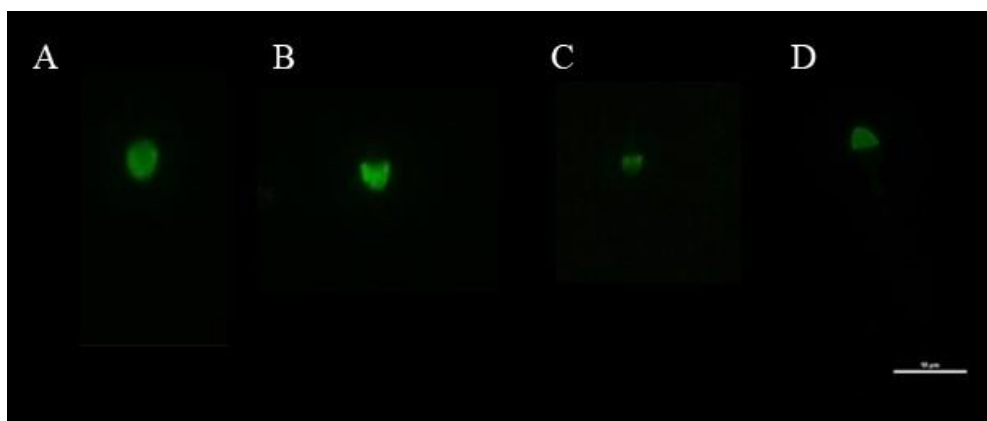
Figure 3 TUNEL staining patterns. A- Head (H); B- Post-acrosomal region (PAR); C- Equatorial region (ER); D-Acrosome-vesicle region (AVR)

Table 3 describes the mean and median percentages of each pattern, in both groups. A significant increase was observed in the H (ρ <0.001) and PAR (ρ = 0.005) patterns, in the HCQ group. The ER and the AVR did not show significant differences between groups.

In Table 4, fragmentation patterns are compared within the same group. In both groups, all the patterns differ significantly from each other.

Table 4 TUNEL patterns for Hydroxychloroquine

		H	AVR	ER	PAR
		TUNEL PATTERNS			
	Mean ± SEM	22.80 ± 15.64	1.45 ± 2.14	5.10 ± 4.25	13.45 ± 9.97
	Median (IQR)	18.50 (14)	1 (2)	4 (6)	10.50 (13)
	Mean ± SEM	40.35 ± 26.22	2 ± 2.1	6.45 ± 4.11	22.9 ± 13.68
	Median (IQR)	33 (36)	1.50 (3)	5.50 (5)	19 (18)
	P-VALUE	**<0.001**	**NS**	**NS**	**0.005**

CT= control, HCQ= hydroxychloroquine
H = head region, AVR = acrosome vesicle region, ER = equatorial region, PAR = post-acrosomal region.
IQR = interquartile range; SEM = standard error of mean; NS = not significant.
significance at p < 0.05

Table 5 Comparison between TUNEL patterns within the same group

	H vs AVR	H vs ER	H vs PAR	PAR vs AVR	AVR vs ER	ER vs PAR
	TUNEL PATTERNS					
	p-value					
CT	<0.001	<0.001	0.004	<0.001	0.004	0.002
HCQ	<0.001	<0.001	0.013	<0.001	<0.001	<0.001

CT = control, HCQ = hydroxychloroquine
H = head region, AVR = acrosome vesicle region, ER = equatorial region, PAR = post-acrosomal region.
significance aT P < 0.05

2.3 Effects on sperm oxidative stress

Sperm oxidative stress was determined by luminol-based chemiluminescence assay. A significant increase in oxidative stress was found in both sCT and HCQ groups, compared to the blank and assay negative controls. However, exposure to HCQ did not induce significant changes in sperm oxidative stress when compared to the sCT. In fact, the mean value for oxidative stress in sCT (12.45 ± 25.38) was higher than in the HCQ group (7.85 ± 6.11), although the medians were equal for both groups (Table 5). Assay blank, negative and positive controls confirm the authenticity of the results.

Table 6 Sperm oxidative stress in the Hydroxychloroquine group

| | Median (IQR) | Mean ± SD | P-VALUE | | | | |
			Blank	CT-	CT+	sCT	HCQ
BLANK	3 (2)	3.35 ± 1.53					
CT-	3 (2)	3.15 ± 1.93	NS				
CT+	364 (308)	422.85 ± 332.1	<0.001	<0.001			
SCT	5 (5)	12.45 ± 25.38	0.017	0.018	<0.001		
HCQ	5 (8)	7.85 ± 6.11	0.005	<0.001	<0.001	NS	

CT- = negative control, CT+ = positive control, SCT = control, HCQ = hydroxychloroquine
IQR = interquartile range, SEM = standard error of mean
significance at p < 0.05, NS= not significant

3. Dexamethasone and Remdesivir

The effects of DEX, RDV and both drugs in combination were analysed at the same time. Simultaneous analysis using the same group of patients allowed not only the comparison between the control and each treatment individually, but also between treatments.

3.1 Effects on basic sperm parameters

Median and mean percentages of the sperm parameters studied for each group are detailed in Table 6. Compared to the control, there were no significant differences in the basic sperm parameters, in the DEX, RDV or DEX plus RDV groups. Even though, numerical differences could be noted. A slight decrease in the percentages of live and motile sperm was detected in three experimental groups, relative to the control group. This trend was more visible in the DEX plus RDV group (57.46 ± 11.54 vs. 54.12 ± 14.13; 46.07 ± 11.37 vs. 44.20 ± 12.41; 41.06 ± 11.62 vs. 39.55 ± 12.55).

3.2 Effects on chromatin condensation and sperm DNA fragmentation

Regarding the effects on sperm chromatin condensation (AB), both RDV and DEX plus RDV groups demonstrated significant differences in the mean percentages of sperm with immature chromatin, relative to control (ρ <0.001). DEX showed no significant effects. The

sDNAfrag increased significantly in the presence of DEX and RDV (ρ <0.001), which was also verified in the presence of both drugs simultaneously (ρ = 0.001) (Table 6).

Table 7 Effects of Dexamethasone, Remdesivir and combined treatment of Dexamethasone plus Remdesivir in sperm parameters, chromatin condensation and sperm DNA fragmentation

		VITALITY (%)	TPM (%)	TM (%)	HOST (%)	AB+ (%)	TUNEL+ (%)
	SPERM PARAMETERS						
	Mean ± SEM	57.46 ± 11.54	41.06 ± 11.62	46.07 ± 11.37	87.50 ± 5.59	12.83 ± 3.95	23.47 ± 6.2
	Median (IQR)	57.89 (19.61)	40.41 (15.36)	46.72 (11.13)	89.39 (5.81)	12.5 (5.25)	22.75 (9.81)
	Mean ± SEM	56.59 ± 13.59	40.80 ± 11.69	45.85 ± 11.84	87.79 ± 5.51	14.40 ± 5.28	28.08 ± 7.33
	Median (IQR)	59.24 (21.49)	42.24 (12.93)	45.83 (17.10)	88.64 (5.5)	14.68 (7.74)	26.75 (12.40)
P-VALUE		NS	NS	NS	NS	NS	<0.001
	Mean ± SEM	57 ± 12.80	39.04 ± 10.55	45.16 ± 11.09	87.97 ± 5.01	18.03 ± 6.20	31.77 ± 8.04
	Median (IQR)	57.96 (20.47)	41.79 (15.22)	45.94 (16.95)	89.13 (6.47)	17.5 (9.13)	33 (12.36)
P-VALUE		NS	NS	NS	NS	<0.001	<0.001
	Mean ± SEM	54.12 ± 14.13	39.55 ± 12.55	44.20 ± 12.41	88.05 ± 6.97	17.49 ± 6.1	29 ± 8.82
	Median (IQR)	56.54 (23.68)	38.33 (24.56)	43.45 (25.70)	89.72 (4.40)	17.75 (5.5)	28.75 (9)
P-VALUE		NS	NS	NS	NS	<0.001	0.001

CT= control, DEX = dexamethasone, RDV = remdesivir.
IQR = interquartile range, SEM = standard error of mean, NS = not significant.
TM = sperm total motility, TPM = sperm total progressive motility, HOST = sperm hypo-osmotic swelling test.
AB+ = positive sperm after aniline blue staining, TUNEL+ = positive sperm after terminal deoxynucleotidyl transferase dutp nick end labelling
significance at P < 0.05

As in the HCQ experiment, sDNAfrag patterns were characterized by evaluating sDNAfrag. The H pattern significantly increased among the three experimental groups, when compared to the control. The DEX and RDV groups also showed a significant increase in the ER pattern (ρ=0.037; ρ=0.001). No significant changes in AVR and PAR patterns were found (Table 7).
Additionally, we compared the different patterns to each other, within the same group. Statistically significant differences were found between AVR and all the other patterns, within the four groups studied. sDNAfrag in the PAR region did not differ significantly from the ER within the DEX, RDV and DEX+RDV groups, but there were significant differences within the control group (ρ= 0.022). The opposite occurred when comparing

PAR with the H pattern, where all groups showed significant differences, except for the control group (Table 8).

Table 8 TUNEL patterns of Dexamethasone, Remdesivir and combined treatment of Dexamethasone plus Remdesivir

		TUNEL PATTERNS			
		H	AVR	ER	PAR
	Mean ± SEM	19.43 ± 8.95	2.81 ± 2.61	9.72 ± 3.89	15.05 ± 7.44
	Median (IQR)	18 (12)	2.5 (5)	9 (4)	14 (11)
	Mean ± SEM	25.90 ± 10.80	2.45 ± 2.33	11.95 ± 4.03	15.9 ± 8.01
	Median (IQR)	25 (15)	2 (5)	12.5 (8)	16 (13)
P-VALUE		0.004	NS	0.037	NS
	Mean ± SEM	30.30 ± 13.53	3.15 ± 2.3	15.85 ± 7.4	14.5 ± 9.55
	Median (IQR)	29 (21)	3.5 (4)	14 (12)	11.5 (12)
P-VALUE		<0.001	NS	0.001	NS
	Mean ± SEM	27.20 ± 11.94	3 ± 2.81	12.4 ± 4.74	15.55 ± 10.20
	Median (IQR)	26 (21)	2.5 (3)	12 (6)	16.5 (15)
P-VALUE		0.004	NS	NS	NS

CT= control, DEX = dexamethasone, RDV = remdesivir.
IQR = interquartile range, SEM = standard error of mean, NS = not significant
H = head region, AVR = acrosome vesicle region, ER = equatorial region, PAR = post-acrosomal region.

Table 9 Comparison between TUNEL patterns within the same group

	TUNEL PATTERNS					
	H vs AVR	H vs ER	H vs PAR	PAR vs AVR	AVR vs ER	ER vs PAR
	p-value					
CT	<0.001	0.002	NS	<0.001	<0.001	0.022
DEX	<0.001	<0.001	<0.001	<0.001	<0.001	NS
RDV	<0.001	0.001	<0.001	<0.001	<0.001	NS
DEX+RDV	<0.001	<0.001	<0.001	<0.001	<0.001	NS

CT= control, DEX = dexamethasone, RDV = remdesivir.
H = head region, AVR = acrosome vesicle region, ER = equatorial region, PAR = post-acrosomal region.
significance at P < 0.05

3.3 Comparison between Dexamethasone, Remdesivir and Dexamethasone plus Remdesivir groups

Besides evaluating the effects of each drug, separately and in combination, against the control group, the DEX, RDV and DEX+RDV groups were also compared with each other. Table 9 describes the comparison between DEX and RDV groups and the DEX+RDV group, for all the sperm parameters studied, including sDNAfrag and chromatin condensation. The mean percentages obtained in the RDV group did not differ significantly from the DEX+RDV group, in any of the parameters. Comparing DEX with the DEX+RDV group, significant differences were detected only in the percentages of uncondensed chromatin. All other parameters showed no significant differences.

Table 10 Comparison between Dexamethasone and Remdesivir groups with Dexamethasone plus Remdesivir group

| | DEX + RDV | | | | | |
	VITALITY	TPM	TM	HOST	AB+	TUNEL+
VITALITY	NS					
TPM		NS				
TM			NS			
HOST				NS		
AB+					0.006	
TUNEL+						NS
VITALITY	NS					
TPM		NS				
TM			NS			
HOST				NS		
AB+					NS	
TUNEL+						NS

Dex = dexamethasone, RDV = remdesivir.
TM = sperm total motility, TPM = sperm total progressive motility, HOST = sperm hypo-osmotic swelling test.
AB+ = positive sperm after aniline blue staining, TUNEL+ = positive sperm after terminal deoxynucleotidyl transferase dutp nick end labelling
Significance at $p < 0.05$, NS= not significant

When comparing the sDNAfrag patterns, the DEX group did not differ significantly from the DEX+RDV group. The RDV showed significant differences in the ER (Table 10).

Table 11 Comparison between TUNEL patterns of Remdesivir and Dexamethasone plus Remdesivir groups

	DEX+RDV			
	H	AVR	ER	PAR
	P-VALUE			
H	NS			
AVR		NS		
ER			NS	
PAR				NS
H	NS			
AVR		NS		
ER			0.024	
PAR				NS

DEX = dexamethasone, RDV = remdesivir.
H= head region, AVR= acrosome vesicle region, ER= equatorial region, PAR= post-acrosomal region.
significance at p < 0.05

The comparison between the DEX and RDV groups showed significant differences in the percentages of uncondensed chromatin (AB+) ($\rho < 0.001$) and in sDNAfrag (TUNEL+) ($\rho = 0.012$). The percentages of viable and motile sperm had no significant changes (Table 11). The sDNAfrag patterns did not show significant differences either, as can be observed in Table 12.

Table 12 Comparison between Remdesivir and Dexamethasone groups

	DEX					
	VITALITY	TPM	TM	HOST	AB	TUNEL
VITALITY	NS					
TPM		NS				
TM			NS			
HOST				NS		
AB					<0.001	
TUNEL						0.012

Dex = dexamethasone, RDV = remdesivir.
TM = sperm total motility, TPM = sperm total progressive motility, HOST = sperm hypo-osmotic swelling test.
AB+ = positive sperm after aniline blue staining, TUNEL+ = positive sperm after terminal deoxynucleotidyl transferase dutp nick end labelling
Significance at p < 0.05, NS= not significant

Table 13 Comparison between TUNEL patterns of Dexamethasone and Remdesivir groups

	DEX			
	H	AVR	ER	PAR
	P-VALUE			
H	NS			
AVR		NS		
ER			NS	
PAR				NS

Dex = dexamethasone, RDV = remdesivir.
H= head region, AVR= acrosome vesicle region, ER= equatorial region, PAR= post-acrosomal region.
significance at p < 0.05

3.4 Effects on sperm oxidative stress

As in the HCQ experiment, no significant differences were found in oxidative stress between sCT and groups treated with DEX, RDV and DEX plus RDV, respectively. Moreover, comparisons between experimental groups (DEX *vs.* RDV, DEX *vs.* DEX+RDV and RDV *vs.* DEX+RDV) did not show significant differences either. Compared with the assay controls (blank, negative and positive controls), all groups showed significant differences, including the control group. Oxidative stress values were significantly higher compared to blank and negative controls, and significantly lower compared to the positive control. Although no statistical differences were found between the groups, numerically an increase in the value of oxidative stress was noted in the DEX group, compared to the sCT.

Table 14 Sperm oxidative stress in Dexamethasone, Remdesivir and Dexamethasone plus Remdesivir groups

	Median (IQR)	Mean ± SD	P-VALUE						
			Blank	CT-	CT+	sCT	Dex	RDV	Dex+RDV
BLANK	3 (3)	4 ± 2.38							
CT-	3 (3)	3.45 ± 1.6	NS						
CT+	1027 (1184)	1289.5 ± 705.32	<0.001	<0.001					
SCT	6.5 (12)	12.95 ± 13.8	0.002	0.001	<0.001				
DEX	9.5 (10)	16.2 ± 21.77	<0.001	<0.001	<0.001	NS			
RDV	8.5 (9)	13.15 ± 16.7	0.002	<0.001	<0.001	NS	NS		
DEX+RDV	9.5 (9)	12.25 ± 12.29	<0.001	<0.001	<0.001	NS	NS	NS	

CT- = negative control, CT+ = positive control, SCT = control gorup, DEX = dexamethasone, RDV = remdesivir
IQR = interquartile range, SEM = standard error of mean
significance at P < 0.05, NS= not significant

Discussion

HCQ and DEX are well-established drugs, used for decades to treat a wide range of diseases that affect men and women of reproductive age (Browning, 2014; Samuel, Nguyen and Choi, 2017). Therefore, it is also important to consider the possible toxic reproductive effects, aside with the better known adverse effects. In fact, awareness of the subject has been growing among the medical community and physicians who prescribe these therapeutics. However, most of the investigation is focused on women's fertility and pregnancy. Little is known about the toxic effects on male fertility. RDV is a very recent drug, only approved in 2020 as a treatment for COVID-19. Data on its toxic effects in humans remain sparse and based almost exclusively on toxicological studies carried out during drug development (WHO *et al.*, 2017).

In an attempt to increase knowledge about the impact of these drugs on male fertility, this *in vitro* study was developed. We evaluated the direct effect of the three drugs on human sperm, evaluating different sperm parameters, including sperm viability and motility, oxidative stress and alterations in sperm DNA.

According to our results, progressive and total motility as well as sperm vitality were decreased in the presence of HCQ. Similar findings have been described for CQ, an HCQ analogue (Hargreaves *et al.*, 1998). On the other hand, Tiseo et al. (2019) found no significant effects of HCQ on sperm motility in a study with SLE patients.

Curiously, no differences were found in the HOST test. Similar to the eosin-nigrosin vitality test, this test also elucidates about sperm viability, through the evaluation of sperm plasma membrane integrity. Thus, it would be expected that both assays would have similar outcomes.

Given the mechanism of action of HCQ in other cells (Lin *et al.*, 2017), it is possible that this drug is affecting sperm viability and motility by interfering with autophagy within these cells. Mitophagy, a selective form of mitochondrial-targeted autophagy, plays an important role in spermatozoa, helping to maintain functional levels of mitochondria in the midpiece, which provide the energy required for sperm to move (Aparicio *et al.*, 2016). Thereby, it is possible that HCQ impair sperm motility by inhibiting mitophagy. In fact, Aparicio *et al.* (2016) proved that CQ affected autophagy proteins in spermatozoa. Moreover, incorrect autophagy results in an accumulation of cellular metabolites inducing oxidative stress, which leads to apoptosis (Filomeni *et al.*, 2015). However, in this study no significant effects on oxidative stress upon exposure to HCQ were found.

Unlike HCQ, DEX and RDV do not seem to influence sperm viability or motility, either total and progressive, since no significant differences were found between each group and the control. It is important to note this analysis was carried out *in vitro*, meaning that physiological interactions were not taken into consideration. As far as DEX is concerned, this is particularly important. Several *in vivo* and clinical studies have demonstrated that

DEX influences male fertility, due to its effect on hormonal signalling in the male reproductive system (Drobnis and Nangia, 2017). DEX affects the HPG axis and interferes with testosterone production, which consequently affects spermatogenesis. As a result, men can have altered sperm parameters. In this sense, our results bring interesting insights into the adverse effects of DEX on male fertility, since it excludes the direct effect on sperm viability and motility.

In vivo studies evaluating other nucleoside analogues with similar therapeutic mechanisms of RDV, as Acyclovir and Ribavirin, have shown significant alterations in sperm parameters, including sperm motility and morphology. According to the authors, these drugs induce DNA mutations in germ cells, which are, then, reflected in abnormal mature sperm cells (Narayana *et al.*, 2002; Narayana, 2008). In our study, RDV was exposed to mature sperm cells *in vitro*, therefore it is not possible to establish a direct correlation. Nonetheless, based on such findings, our results outline the need for an *in vivo* investigation of the effects of RDV in male reproductive system, as the outcomes may be different. On the other hand, the effects on sDNAfrag were confirmed. RDV induced sperm DNA damage in mature sperm cells, increasing the percentages of immature chromatin and sDNAfrag.

In fact, sperm DNA damages were observed in all therapeutic groups. The mechanisms behind sperm DNA damages can have different causes and affect sperm DNA in different ways. The sperm DNA can be targeted to protamines, affecting chromatin condensation, or directly to DNA, which normally induces sperm DNA breaks. Intrinsic or extrinsic factors can be in the origin of these alterations. Intrinsically, several moments during spermatogenesis can induce sperm DNA damages, like an incorrect apoptotic process during negative selection, or inadequate replacement of histones by protamines, for instance (Sakkas and Alvarez, 2010).

Incorrect chromatin condensation makes sperm DNA more susceptible to fragmentation. Indeed, our results confirmed this phenomenon. Except for DEX, all treatment groups had significantly increased percentages of immature chromatin and sDNAfrag, compared to the controls. Furthermore, sDNAfrag showed higher levels in general, and treatment-exposed groups all exceeded the threshold value (20%) (Agarwal *et al.*, 2020) used to distinguished abnormal sDNAfrag levels. Percentages of sperm with uncondensed chromatin among the experimental groups did not exceed the threshold (20%) (Hammadeh *et al.*, 1998), despite the significant increase compared to the control. Our findings suggest HCQ, DEX and RDV may interact with protamines, but mainly induce sperm DNA damages by directly interfering with DNA.

Exogenous toxins, like drugs, normally affect sperm DNA by inducing oxidative stress in sperm cells (Agarwal *et al.*, 2020). With that in mind, we evaluated oxidative stress in our experiment to address the possible origin of sperm DNA damage. Interestingly, our results demonstrated that none of the drugs seemed to induce oxidative stress in sperm cells, as no differences were found in the reactive oxygen species levels, between the controls and

treatment groups. This suggests HCQ, DEX and RDV induce sDNAfrag by different mechanisms, rather than oxidative stress as is commonly seen with other drugs.

Our data reinforce the need to include sDNAfrag evaluation in semen analysis carried out in fertility centers, as it becomes obvious some factors affect sperm cells without inducing physiological alterations. Sperm with sDNAfrag can be physiologically normal and capable of fertilizing oocytes. However, it affects embryo development if the oocyte is unable to repair the DNA damage (Sakkas and Alvarez, 2010). Moreover, our results reflect the percentages of sDNAfrag among morphologically normal sperm cells, which allows a better approximation of the values that could be find among the sperm fraction used in ART.

Within the sDNAfrag analysis, we distinguished different sDNAfrag staining patterns, according with the head region stained. According to our results, there was a clear trend in patterns, following the order: H>PAR>ER>AVR. Statistical differences between experimental and CT groups were significant only for the two most common patterns (H and PAR), suggesting that sperm cells were more susceptible to sDNAfrag in those regions and exposure to the drug leads to sDNAfrag.

The head of mature sperm cells contains the nucleus and the acrosome vesicle, which aids in fertilization. Sperm nucleus is specifically organized with centromeres located in the centre of the nucleus, forming the chromocenter, and telomeres located in the nuclear periphery. Chromosomes in the chromocenter are distributed from the apical region, where most protein-coding genes are found, to the basal region, a poor gene region (Sá *et al.*, 2015; Wiland *et al.*, 2016). Histones and protamines are also distributed according to gene content. Protamines are found mainly in the apical and central regions, where there is greater gene density, while histones stay in the basal region. Considering the sDNAfrag staining patterns, the AVR corresponds to the apical region and the ER to the central region, while the PAR corresponds to the basal region (Sá *et al.*, 2015). Accordingly, our results showed all drugs, except RDV, induce sDNAfrag mainly in protamine-poor regions, which, as explained earlier, makes chromatin more susceptible to DNA damage. Higher percentages in the H staining pattern may indicate apoptotic sperm cells as sDNAfrag is generalized throughout sperm nucleus.

It was mentioned in the introductory chapter that DEX and RDV are also used in co-administration in some COVID-19 patients; therefore, we included combined treatment in our work and assessed whether the effects on sperm would be different from individual medications. For all evaluated sperm parameters, we compared the results of the DEX group with the RDV group, and these groups were also compared with the group exposed to both drugs simultaneously. Overall, the effects of combined treatment did not differ significantly from each drug individually. Significant differences were observed only for the AB test between the DEX and DEX plus RDV groups, where DEX plus RDV showed a significant increase in immature chromatin control percentages, compared to the control, and the DEX group did not. This difference can be attributed to the presence of RDV in the drugs-combined group, since RDV was also shown to significantly increase immature chromatin

compared to the control. When comparing the two drugs individually, significant differences were found for the percentages of chromatin condensation and sDNAfrag. RDV seems to be more toxic than DEX. Altogether, our findings suggest that DEX and RDV may have additive effects rather than synergetic or antagonistic effects, when administered toegether. However, further investigation should be held to assess in more detailed the exact contribution of each drug.

Conclusion

This *in vitro* study evaluated the effects of three distinct drugs on sperm cells. Sperm from normozoospermic patients were exposed to therapeutic doses of HCQ (antimalarial), DEX (glucocorticoid), and RDV (antiviral), individually, as well as to combined treatment of DEX plus RDV. Sperm parameters evaluated included sperm motility and viability, sperm oxidative stress, and sperm DNA damage.

All forms of treatment considered in this experiment showed to have toxic effects on sperm DNA. Except for DEX, which effects were found only in sDNAfrag, all drugs induced both sDNAfrag and chromatin decondensation. Of the identified sDNAfrag staining patterns, H was the most observed, followed by PAR, ER and finally AVR. These patterns increased significantly between treatment groups. sDNAfrag staining patterns evaluation add, therefore, relevant and detailed information about the toxic effects of the drugs studied on sperm DNA, since we were able to identify the nuclear region affected by these drugs and infer on the genes involved. This might be helpful to understand other physiological alterations in sperm cells when exposed to these drugs.

Once again, it became evident the importance to consider the evaluation of sDNAfrag in routine sperm analysis carried out at fertility clinics. DEX and RDV had no effect on basic sperm parameters., a normal spermiogram would suggest that both drugs were safe in sperm, when in fact, our sperm DNA analysis showed a significant increase in sDNAfrag in the presence of both drugs.

Surprising results were observed in the sperm oxidative stress evaluation, as no significant differences were detected between each treatment and the respective control group. Reactive oxygen species have long been identified as a major cause of sDNAfrag by investigators. However, our study showed that HCQ, DEX and RDV induced DNA damages without increasing oxidative stress. Therefore, it would be interesting and relevant to further investigate the mechanisms behind these drugs in sperm DNA.

HCQ proved to be the most dangerous drug for sperm cells, having induced toxic effects in almost all parameters evaluated, namely, sperm viability, motility and sDNA damages. Clinically, these findings are very relevant, since HCQ is an established drug used for many years in the treatment of several diseases that affect men of reproductive age. Men prescribed to take HCQ should be made aware of these effects and advised to do regular spermiograms. Regarding DEX, RDV and the combination of the two drugs, no relevant differences were found between these groups, which leads us to conclude that DEX and RDV have additive effects when combined.

Overall, we believe our study brings important new insights into how drugs of different pharmacological classes, but used in the same clinical setting, can affect sperm. We do know, however, that in vitro studies do not represent true physiological interactions. Therefore, in vivo studies should be held to confirm our results and, if relevant, initiate clinical studies.

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