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Embryology Reproduction

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EDITORIAL

Dear Colleagues and Friends,



The Italian Society of Embryology, Reproduction and Research (SIERR) and the Italian Society of Fertility, Sterility and Reproductive Medicine (SIFES-MR) have recently joined forces with the aim of highlighting the scientific activity of their members.

Initially conceived under the auspices of SIERR and later implemented through the participation of SIFES, the RIVER (Research, Innovation and Views in Embryology and Reproduction) Journal project is now a reality in the scientific literature landscape. The name chosen for this “adventure” is meant to allude to the concept of the continuously flowing river of knowledge, and also to recall how this flow is continuously fed through research, innovation and, above all, vision.

The idea of blending the expertise of our two societies in a multidisciplinary way that allows clinical experience and research to complement each other fits in neatly with the concept of a global vision.

This first issue of *RIVER Journal* takes us on a journey through topics relating to the application of assisted reproduction techniques (looking at biochemical and structural aspects, and issues linked to infection).

The issue opens with a systematic review which describes in detail the phenomena that trigger the production of reactive oxygen species in male gametes subjected to cryopreservation

(• *Mechanisms underlying human sperm cryodamage: the role of reactive oxygen species (ROS) and antioxidants*). It then continues with six concise reviews that describe different characteristics of female reproductive potential in relation to age and hormonal status (• *Meiotic errors in oocytes of young and advanced maternal age women: the U-curve of fertility* • *Follicle-stimulating hormone receptor: the impact of genetic variations and gene expression levels on fertility*) and look at possible risks associated with reproductive tract infections and contamination linked to laboratory manipulations (• *HPV infection and ART: the andrologist's point of view* • *Report on viral infections in ART* • *Cross-contamination risk management in biobanking. Lesson from the pandemic* • *Male infection: cross-contamination risk management and impact on sperm parameters*).

The issue closes with an interesting case report which addresses the problem and the possible solutions in the rare, but possible, event of contamination of the embryonic cell culture (• *Candida contamination of embryos in the ART laboratory: a case report and literature review*).

The presidents of SIERR and SIFES-MR join me, the editor in chief, in expressing our hope that this publication will arouse the interest of the scientific community dealing with reproduction (in not only the human but also the veterinary field), and that readers will feel motivated to submit papers of their own, thereby helping to drive the growth of our new scientific journal.

Lucia De Santis
Editor in chief, RIVER Journal

Mechanisms underlying human sperm cryodamage: the role of reactive oxygen species (ROS) and antioxidants

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ABSTRACT

Sperm cryopreservation is an efficient procedure for male fertility preservation, although the freeze-thaw procedure causes irreversible structural and functional changes in human spermatozoa. Indeed, the procedure is responsible for harmful changes that may affect sperm biology. In mammalian cells, cryopreservation induces a shift of redox homeostasis towards increasing generation of reactive oxygen species (ROS). The characteristics of ROS and the cellular outcomes depend on the cell type. Supra-physiological ROS levels during cryopreservation severely impact sperm survival, reproductive potential and DNA integrity, the latter a fundamental factor for fertilisation and transmission of paternal genetic information to offspring. The aim of this review is to summarise current knowledge of the main molecular mechanisms underlying ROS generation during sperm cryopreservation and its subsequent effects. In addition, we report current experimental approaches based on the supplementation of cryopreservation media with enzymatic and non-enzymatic antioxidants with the aim of minimising the harmful effects of ROS, and thus improving post-thaw sperm quality. Current data indicate that the potential use of antioxidants as constituents of the sperm freezing solution in clinical settings would require considerable attention.

KEYWORDS

Spermatozoa, cryopreservation, ROS, oxidative stress, antioxidants.

Introduction

Sperm cryopreservation is an effective procedure widely used for male fertility preservation in conditions likely to deteriorate sperm quality and/or testicular function, such as cytotoxic chemo- and radio-therapy, surgical treatments or conditions leading to severe oligozoospermia^[1-3]. Moreover, cryopreservation is widely employed in the *in vitro* fertilisation field, being routinely used to store donor semen or to preserve spermatozoa obtained from azoospermic patients who have undergone testicular sperm extraction^[4,5]. Although sperm cryopreservation is a widely used technique and has been improved over time, there are still unsolved technical and biological issues. During cryopreservation, the processes of cooling, freezing and thawing are responsible not only for a reduced proportion of surviving cells but also for harmful effects on sperm function, as demonstrated by changes in membrane lipid composition and acrosome status, increased DNA fragmentation and oxidation and decreased sperm motility and viability^[6]. The damage caused by cryopreservation can be due to osmotic stress, cold shock, intracellular ice crystal formation, excessive production of reactive oxygen species (ROS), alterations in antioxidant defence system, or combinations of these conditions^[4,7]. Indeed, the activation both of apoptotic pathways and of lipid peroxidation has been associated with impairment of sperm velocity, motility, viability, mitochondrial

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activity and chromatin and membrane integrity^[8-11]. Spermatozoa from ejaculates with poor semen quality are more vulnerable to cryopreservation damage; it was recently reported that the recovery of motile and viable spermatozoa after freezing and thawing procedures is reduced in the presence of even just one basal semen parameter below the 5th percentile of the World Health Organisation reference values^[6].

In the past, several attempts in different mammalian species have been made to reduce sperm freeze-thaw stress. Membrane fluidity was increased to prevent cold shock, and anti-freeze molecules were employed to reduce heterogeneous ice nucleation and ice crystallisation. In order to counteract ROS production during the semen cryopreservation process, it was considered important to reduce ROS sources such as leucocytes, dead and defective spermatozoa and semen radiation exposure. A plethora of studies are available that analyse freeze-thaw stress-activating pathways and propose ROS scavenging

through enzymatic, non-enzymatic, plant-based antioxidants or reductants [12]. However, most of them were carried out in animals of veterinary interest, and therefore data supporting the manipulation of sperm-freezing medium as a means of minimising oxidative damage are relatively less clear for humans than for other species [13]. Thus, a better understanding of the mechanisms underlying cryodamage is required in order to improve the quality of thawed samples [14,15]. To this end, in the present paper we review the role of ROS in cryopreservation damage and the antioxidant approaches tested so far to counteract ROS effects on human sperm biology.

ROS and ROS-scavenging systems in human sperm

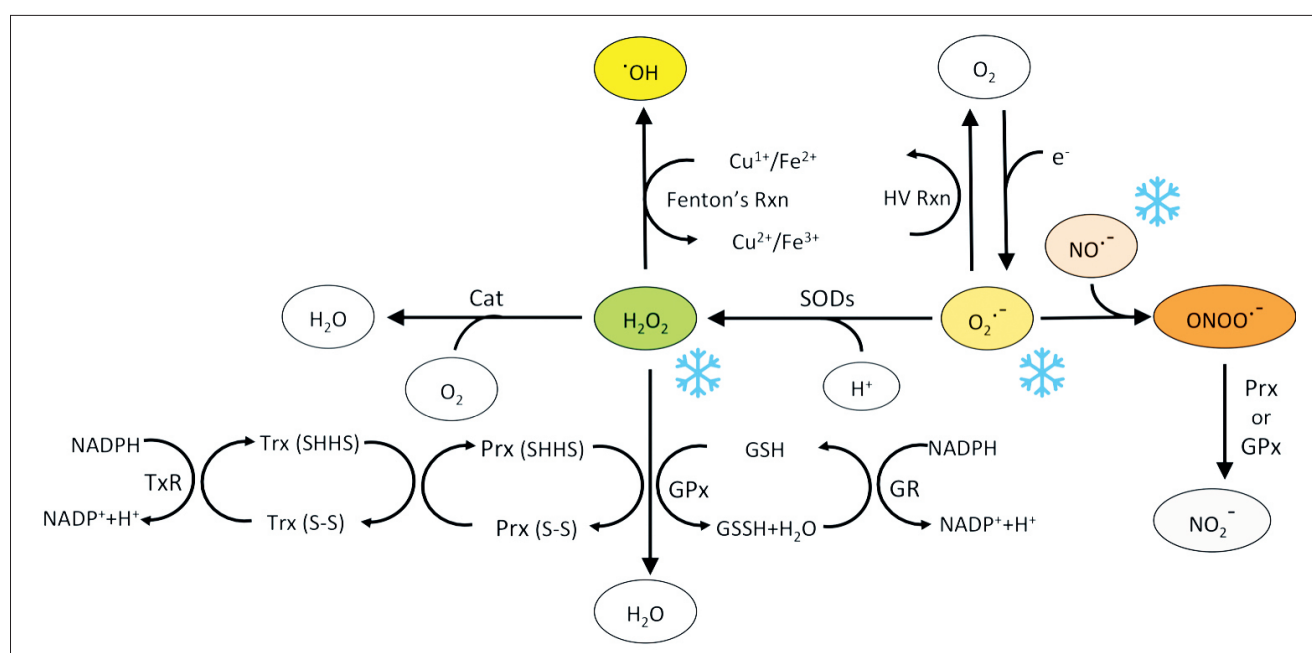
Reactive oxygen species (ROS) play a key role in sperm pathophysiology, being required for sperm capacitation, acrosome reaction, and binding to the zona pellucida [16,17]. ROS comprise both free radical and non-free radical oxygen-derived reactive molecules that are constantly generated by the mitochondrial electron transport chain, or during various enzymatic reactions. Common forms of ROS include superoxide anions ($O_2^{\cdot-}$), which are capable of dismutating, either spontaneously or enzymatically, to hydrogen peroxide (H_2O_2), singlet oxygen ($^1O_2$), and hydroxyl ($OH^{\cdot}$) radicals. The superoxide anion is extremely harmful because of its high oxidant activity and its ability to cross the biological membranes [18]. H_2O_2 reacts with only certain biomolecules and can cause the oxidation of thiol groups (SH). It is toxic at high concentrations because it can be

reduced by ferrous iron, Fe (II), into the more damaging $^{\cdot}OH$ via the Fenton reaction (Figure 1). Due to its long half-life, H_2O_2 can transduce signals at long ranges. Glutathione peroxidase-glutathione reductase and the peroxiredoxin/thioredoxin-thioredoxin reductase pathways utilise NADPH as a reducing equivalent to reduce H_2O_2 to H_2O (Figure 1).

A subset of ROS includes reactive nitrogen species such as the nitric oxide ($NO^{\cdot}$) and the peroxynitrite ($ONOO^{\cdot-}$) anions [19,20]. Under physiological conditions, spermatozoa are known to produce low amounts of ROS, mainly by mitochondrial activity [16,21]. On the contrary, excessive levels of ROS in the semen may be ascribed to the activities of immature germ cells and leukocytes, which negatively influence sperm functions and fertility potential [22,23].

Indeed, spermatozoa are particularly susceptible to oxidative injury due to their low cytoplasmic antioxidant content and the abundance of polyunsaturated fatty acids (PUFAs) in plasma membrane [16,23,24]. PUFAs provide fluidity that is necessary for sperm motility and for membrane fusion events (e.g., the acrosome reaction and sperm-egg fusion), but, unfortunately, they are also prone to free radical attack [21,25,26]. Membrane PUFA peroxidation triggers a cascade of lipid peroxidation on the sperm surface, which disrupts membrane permeability and fluidity, thus leading to irreversible loss of motility and impaired sperm-oocyte fusion [16]. To cope with excessive ROS, sperm and seminal plasma are equipped with a number of enzymatic and non-enzymatic antioxidant systems, especially at the post-testicular level [14,16]. The main antioxidant enzymes contained in seminal plasma include superoxide dismutase, catalase and glutathione peroxidase [27]. The cytosolic copper/

Figure 1 Generation and detoxification of reactive oxygen/nitrogen species. SODs, superoxide dismutases, detoxify $O_2^{\cdot-}$ by converting it to H_2O_2 (hydrogen peroxide): SOD1 is localised in both the mitochondria intermembrane space and the cytosol, SOD3 is located extracellularly, and SOD2 is found in the mitochondria matrix. Cat, catalase, reduces H_2O_2 into H_2O and is mostly located in peroxisomes. GPx, glutathione peroxidase, is found in mitochondria and cytosol. GR, glutathione reductase, is an NADPH-dependent flavoprotein that catalyses the reduction of glutathione disulfide (GSSG) to glutathione (GSH). Prx, peroxiredoxins, and Trx, thioredoxins, constitute the Prx/Trx system present in the nucleus, mitochondria, endoplasmic reticulum, peroxisome and extracellular environment. The snowflakes indicate ROS detected during cryopreservation procedures (see the manuscript for further details).



zinc superoxide dismutase (Cu/ZnSOD) and the mitochondrial Mn²⁺-dependent superoxide dismutase (MnSOD) convert superoxide anions to hydrogen peroxide. This compound is then transformed to water by catalase and glutathione S-peroxidase (GSSPx). In the peroxidase reaction, reduced glutathione (GSH) is oxidized to GSSG (oxidized glutathione). GSSG is reduced by NADPH through the action of glutathione reductase (GSSG-Rx) [119]. Seminal plasma also exhibits non-enzymatic antioxidant activity, mainly relying on ascorbic acid, alpha-tocopherol, pyruvate, GSH, L-carnitine, taurine, hypotaurine, flavonoids, alkaloids, carotenoids, urate, albumin and ubiquinol [17,27].

A poor seminal antioxidant capacity associated with high levels of ROS has been reported in ejaculates from infertile men. Although oxidative stress is regarded as one of the main factors underlying sperm DNA damage and dysfunction in these patients, the question of whether the high ROS levels are due to increased ROS production, decreased ROS scavenging capacity or both is still under debate [17,27].

Cryodamage, ROS and sperm dysfunction

Several studies have pointed out that cryopreservation could exert detrimental effects on sperm survival by affecting sperm ultrastructural and molecular integrity [28,29]. Cold shock, osmotic stress, and ice crystal formation experienced by sperm during cryopreservation may impact sperm physiology with consequences on sperm viability and motility. Similarly, low temperatures and the presence of cryoprotectant may impair membrane cytoskeletal and membrane components, which may affect sperm surface proteins, with consequent alterations in capacitation status and sperm fertilising ability [15,30,31]. Interestingly, metabolomic and proteomic analyses have recently shown that energy pathways including the citrate cycle, glycolysis and pyruvate metabolism play an important role in human sperm cryopreservation [32-34].

Cryodamage is also involved in the downregulation of sperm mRNAs associated with fertilisation, early embryo development, capacitation and successful pregnancy. Some epigenetic-related transcripts are also affected by the cryopreservation process [35].

Oxidative stress is regarded as the main mechanism involved in cryodamage in reproductive and non-reproductive cells [13]. It may arise from osmotic stress and increased oxidative metabolism occurring during the thawing phase. Indeed, during thawing, when oxygen is reintroduced into frozen tissues, there is a burst of glucose-mediated oxidative metabolism with a consequent rise in ROS generation [36,37]. This process is mainly related to dysfunctional mitochondrial electron transport [38,39], as mitochondria represent the main source of ROS in human spermatozoa, especially after cryopreservation [40,41]. ROS in the form of superoxide (O₂^{•-}), detected in the cells of various species undergoing cryopreservation, were observed in thawing sperm from some animal species along with elevated mitochondrial H₂O₂ [13].

Thawing-related oxidative stress results in reduced sperm motility and morphology, together with increased DNA frag-

mentation [15]. Several studies, indeed, showed that ROS are the main effectors of DNA damage during the process of sperm freezing and thawing, and that DNA damage is not linked to caspase-related mechanisms [9,42,43]. Ribas-Maynou *et al.* [15] showed that cryopreservation induces a 10% increase in spermatozoa with single-stranded DNA fragmentation, whereas no effects have been observed on the amount of double-stranded DNA breaks. A significant increase in both sperm DNA fragmentation and oxidation is associated with high levels of ROS after cryostorage of semen from infertile men. Moreover, deficiencies in DNA packaging by protamines have been observed in sperm from subfertile men, making their DNA more susceptible and vulnerable to ROS attack [15]. Interestingly, the extension of sperm DNA damage seems to directly correlate with sperm dysfunction and male infertility [6].

Role of antioxidants in sperm cryopreservation

Although semen displays antioxidant systems, during cryopreservation spermatozoa are more vulnerable to oxidative stress induced by the procedure itself, since the addition of cryoprotectants during sperm preparation prior to freezing dilutes the seminal plasma, thus lowering the efficiency of its antioxidant activity. Therefore, the addition of external antioxidants could be effective in compensating for the lack of natural defences [7,44,45]. Many studies have shown that supplementation of freezing and/or thawing media with antioxidants could protect sperm from the loss of motility that occurs following cryopreservation and thawing [10]. Enzymatic and non-enzymatic antioxidant strategies have been employed, for decreasing oxygen concentration, eliminating the catalytic metal ions, and harvesting a variety of free radicals such as superoxide and hydroxyl anions and hydrogen peroxide, as well as membrane peroxidation.

Several non-enzymatic antioxidants have been shown to counteract the effects of exogenous ROS [46]. Among them, vitamin E, a natural lipid soluble antioxidant, which, in its active form (alpha-tocopherol) removes superoxide anion and hydrogen peroxide, and hydroxyl anions, and breaks peroxidation chain reactions [47,48]. Moreover, vitamin E decreases cryopreservation-related ROS generation, thus counteracting membrane lipid peroxidation [49-54]. In domestic animals, *in vitro* exposure to vitamin E improves post-thaw motility and DNA integrity, while its addition during incubation improves motility and viability of normal and abnormal spermatozoa during cryopreservation [26,44,50-54]. Recently, it was observed that gamma-tocopherol, added to cryopreservation medium, induced higher post-thaw human sperm viability and motility than alpha-tocopherol [55]. Melatonin is a powerful antioxidant secreted by the pineal gland [56]. It has been detected in seminal plasma, and sperm membrane displays a melatonin receptor [57,58]. Karimfar *et al.* [29] observed that the addition of 0.01 mM melatonin to the freezing medium improves spermatozoa viability and motility, and lowers intracellular ROS and membrane malondialdehyde (MDA). In addition, when caffeine was added to melatonin, sperm motility improvement was associated with a healthier mitochondrial status [59]. Trans-resveratrol, the

most common isomer of resveratrol, is one of the most important polyphenols of red wine; it has a powerful antioxidant activity, which it exerts by scavenging free radicals and chelating divalent cations [60]. Besides the ability to inhibit ROS generation by enzymatic and non-enzymatic systems, resveratrol also induces calcium release into cell cytoplasm and increases sperm AMP-activated protein kinase (AMPK) phosphorylation [61]. In particular, trans-resveratrol provides protection against ROS-mediated membrane lipid peroxidation and DNA damage [28], increases mitochondrial membrane potential, and decreases ROS generation [61]. Curiously, in spite of its antioxidant properties against oxidative damage to lipids, resveratrol reduced damage but did not display a protective effect on sperm motility during cryopreservation [62-64]. The addition of ascorbic acid before cryopreservation can reduce DNA damage only in infertile men [63].

Meamar *et al.* [30] observed that the freezing medium supplemented with extract of *Opuntia ficus-indica*, which contains antioxidants and flavanoids, including resveratrol, significantly reduced sperm DNA fragmentation without improving sperm motility or viability. Another polyphenol, quercetin, has been demonstrated to ameliorate sperm motility and viability, and to reduce DNA damage as well as MDA levels [43,65]. In other systems, it has been proposed, but not yet definitively confirmed, that both resveratrol and quercetin may act by activating the NAD-dependent histone deacetylase sirtuin 1 (SIRT1), which triggers deacetylation of liver kinase B1 (LKB1) and phosphorylation of AMPK [66,67]. An interesting compound employed as a cryopreservation extender is the brain-derived neurotrophic factor (BDNF), which is a polypeptide of the neurotrophin family that is mainly expressed in the central nervous system and plays a key role in the differentiation, maturation, survival and regeneration of neuronal cells [68]. BDNF is also expressed in Leydig and Sertoli cells of the human testis, and its receptors (TrkB) have been found in spermatogonia and mature spermatozoa, suggesting a role in the paracrine regulation of spermatogenesis [69,70]. BDNF probably exerts its antioxidant activity by scavenging free radical ions, modulating the activity of antioxidant enzymes and enhancing the expression of sestrin 2, a stress-responsive gene involved in the cellular defence against oxidative damage [71,72]. This factor improves sperm motility and viability and reduces the level of intracellular H_2O_2 and caspase-3 activity [10], therefore, supplementation of sperm freezing and/or thawing media with BDNF could protect sperm cells against ROS- and apoptosis-mediated damage occurring during cryopreservation.

The supplementation of cryopreservation medium with GSH, ascorbate, myo-inositol or the mitochondria-targeted antioxidant MitoTEMPO increases sperm motility and mitochondrial functionality, and potentiates the antioxidant enzymes superoxide dismutase, catalase and GSH-peroxidase in spermatozoa, resulting in decreased activation of oxidative stress markers and a low degree of DNA fragmentation [73-76]. Elamipretide, a novel small mitochondrial targeting peptide, has recently been demonstrated to improve post-thaw motility and viability, and stability of the plasma membrane, mitochondria and chromosomes. Oxidation and acrosome dysfunction were also significantly reduced [77]. Surprisingly, a mitochondriotrop-

ic molecule such as L-carnitine, does not protect against DNA oxidation, although it enhances human sperm motility and vitality after cryopreservation [78]. Among the plethora of compounds with antioxidant properties, the efficacy of brown algae *Sargassum* has been tested in human spermatozoa [79]. Brown Algae *Sargassum* contains polyphenols, which are known to chelate heavy metals, leading to elimination of free radicals. The addition of *Sargassum* extract to the cryopreserved medium improves both total and forward sperm motility [79]. Gangliosides are sialic acid-containing glycosphingolipids expressed in the outer leaflet of the plasma membranes of all mammalian cells, including spermatozoa, and they are particularly abundant in neuronal cell membranes.

The protective effect of ganglioside micelles against ROS-induced changes is believed to stem from their ability to scavenge free radicals and prevent their harmful effects [45]. As expected, enrichment of cryopreservation media with gangliosides protected human sperm DNA integrity during the freeze-thaw process, reducing sperm susceptibility to DNA fragmentation. Moreover, gangliosides modulated the generation of superoxide anion, inhibited iron-catalysed hydroxyl radical formation and prevented the hydrogen peroxide diffusion across the sperm membrane [45]. Finally, addition of the enzymatic antioxidant catalase to cryoprotective media reduced the amount of ROS in frozen-thawed spermatozoa and greatly improved sperm motility, viability and mitochondrial function, and inhibited both DNA damage and early apoptotic events. The effects of antioxidants on cryopreserved human sperm have been summarised in Table 1.

Unfortunately, none of approaches reported above was able to abolish the cryodamage. This may be ascribed to i) reduced ability of sperm to use the added antioxidants, ii) the low amount of added antioxidant in comparison with the ROS produced, iii) loss of efficacy of antioxidant compounds during cryopreservation. Recent studies have proposed that the application of stressing conditions prior to cryopreservation would stimulate intrinsic sperm responses against cryodamage. In this context, different stressors such as hydrostatic pressure, osmotic pressure and oxidative agents have been applied at sub-lethal levels and shown to enhance sperm tolerance against stress induced by cryopreservation [80-83].

Conclusions

In this review, we summarised current knowledge about oxidative stress occurring during cryopreservation and the potential for supplementation of cryopreservation extenders with antioxidants as a means of minimising the harmful effects of ROS and improving the post-thaw quality of human sperm.

Cryopreservation leads to high generation of ROS, reducing the fertilising potential of human spermatozoa, which are particularly susceptible to ROS damage. In particular, to overcome oxidative reactions, spermatozoa are dependent on extracellular antioxidant systems contained in seminal plasma, which, however, is separated and discarded during the sperm processing. Therefore, there is need for a source of antioxidants to protect sperm cells during the cryopreservation pro-

cess. Most studies have demonstrated beneficial effects of *in vitro* antioxidant supplements in protecting spermatozoa from exogenous oxidants as well as freezing/thawing procedures. Nevertheless, further studies are warranted to establish the efficacy, suitability and safety of most of these antioxidants as supplements for cryopreservation extenders in clinical practice.

Table 1 Effects of antioxidants on cryopreserved human sperm.

ANTIOXIDANT	POSITIVE EFFECTS
Ascorbic acid ^[63,73]	ROS (↓) Viability and weak motility (↑) Apoptotic cells (↓) DNA damage (↓)
BDNF ^[72]	Sperm motility and vitality (↑) H2O2 (↓) Caspase activity (↓)
Brown algae sargassum extracts ^[79]	ROS (↓) Sperm motility (↑)
Catalase ^[73]	ROS (↓) Sperm viability and weak motility (↑) MMP (↑) Apoptotic cells (↓) DNA damage (↓)
L-carnitine ^[78]	Sperm viability and motility (↑)
Elamipretide ^[77]	Sperm motility and viability (↑) Plasma membrane stability (↑) MMP (↑) Acrosome dysfunction (↓)
Glutathione ^[74]	DNA damage (↓) MDA (↓) ROS (↓)
Melatonin ^[29,59,64]	Total antioxidant capacity (↑) GSH concentration (↑) Mitochondrial membrane integrity (↑) MMP (↑) SOD, catalase and GPx activity (↑) BCL-2, SOD2, GSTM1, NRF2, HSP90AA1 (↑) Lipid peroxidation and ROS levels (↓) NADPH oxidase activity (↓) Nox5 and Bax expression (↓) Sperm viability and motility (↑)
MitoTEMPO ^[75]	Sperm motility and vitality (↑) Sperm membrane integrity vitality (↑) MMP (↑) SOD activity, catalase activity, GPx activity (↑) MDA levels (↓)
Myo-inositol ^[76]	Sperm progressive motility (↑) MDA (↓) Total antioxidant capacity (↑) DNA damage (↓)
Resveratrol ^[28,61-63]	DNA damage (↓) MDA levels (↓) SOD activity (↑)
Alpha-tocopherol, gamma-tocopherol ^[55]	Sperm viability and motility (↑)
Opuntia ficus indica extracts ^[30]	DNA damage (↓)
Quercetin ^[43,65]	Sperm motility and vitality (↑) DNA damage (↓) MDA levels (↓)
BDNF, brain-derived neurotrophic factor; MMP, mitochondrial membrane potential; MDA, malondialdehyde. See the manuscript for further details.	

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Meiotic errors in oocytes of young and advanced maternal age women: the U-curve of fertility

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ABSTRACT

Italy is currently one of the countries most affected by declining birth rates, a negative trend mainly determined by several socio-economic factors that lead women to postpone pregnancy. The use of in vitro fertilization techniques cannot counteract the natural decline in fertility that accompanies advancing maternal age. This decline is associated with an increase in chromosomal aneuploidy in oocytes that, if fertilized, could result in aneuploid embryos that are often miscarried. Here, we discuss the “molecular clock hypothesis” proposed by Hoffmann’s lab, which suggests a U-shaped trend of female fertility. The high rates of chromosomal aneuploidy observed at the two “ends of the curve” may represent an evolutionary mechanism of protection that exists to balance the risks associated with pregnancy. This hypothesis lays the foundations for debating the idea that very young women are free from the risk of aneuploidy, and also suggests the existence of a safety mechanism that “protects” against possibly complicated pregnancies both after the age of 40 years and in very young women. The U-shape hypothesis described in humans will be discussed in a comparison with the fertility trends of other mammalian species.

KEYWORDS

Fertility, aneuploidy, advanced maternal age, IVF.

Chromosomal mis-segregation as a cause of reduced fertility

Italy is currently one of the countries most affected by declining birth rates ^[1], a negative trend mainly determined by socio-economic factors that lead women to postpone pregnancy pending greater economic and social stability ^[2]. The main biological cause of this fertility decline is the increase in meiotic chromosome segregation errors, and therefore in aneuploid oocytes, that accompanies maternal aging ^[3]. Chromosome segregation occurs during both the first and the second meiotic division (MI and MII) and it is a finely regulated process prone to mistakes that may result in the formation of oocytes containing two copies of a chromosome (disomic gamete) or lacking a chromosome following a non-disjunction event (nullisomic gamete) ^[4]. These chromosomal abnormalities affect 30–80% of oocytes, depending on the woman’s age ^[5], and have a substantial impact on preimplantation embryos ^[6,7].

In the period between fetal development and menarche, oocytes are arrested at the dictyate stage in the prophase of MI ^[8]. A prolonged period of quiescence, as observed in women of advanced maternal age (AMA, ≥ 35 years), may compromise the genetic stability of oocytes and increase the risk of mis-segregation ^[3]; in fact, AMA is associated with a wide range of adverse pregnancy outcomes, including a higher incidence of

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implantation failures, chromosomal abnormalities, and miscarriages ^[9,10]. The incidence of aneuploidy-related pregnancy losses is lower in women aged <40 years than in those >40 years of age (65% vs. 82%) ^[11].

Evidence suggests that multiple factors, including cohesin dysfunction, spindle instability, and alterations in chromosome telomere length, may increase the likelihood of age-dependent mis-segregation ^[12]. Alterations in the activity of cohesins (i.e., proteins involved in the separation of homologous chromosomes during MI and sister chromatids in MII) can cause segregation errors during oogenesis. In this regard, mouse model studies highlighted an association between alterations in chromosome cohesion and oocyte aging ^[13,14]. Furthermore, an imbalance in the process of chromosome cohesion often results in a high incidence of premature separation of sister chromatids (PSSC) in AMA women ^[15].

Accurate chromosome segregation also requires specific

spindle configuration and dynamics [116]. Recent studies indicate a decreasing number of proteins involved in spindle assembly in both mouse and human aged oocytes [17-21].

In addition, progressive shortening of telomeres (i.e., structures consisting of repeated nucleotide sequences which protect the ends of chromosomes) occurs at each cell division and may lead to meiotic dysfunction, morphological alterations of the spindle, as well as subsequent embryo fragmentation [13]. This hallmark of aging has been observed in both human and mouse AMA oocytes [22,23]. In particular, short telomeres have been found to be characteristic of oocytes from patients who have had recurrent in vitro fertilization (IVF) failures or fragmented or aneuploid embryos [24].

Alongside these events, associated with nuclear maturation and cell division, the reduction of women's reproductive potential with aging is also due to other cytological aspects [13].

Cytoplasmic maturation, involving organelle reorganization and storage of transcripts and proteins, is essential to support the entire oocyte maturation process, fertilization, and early embryogenesis [25,26]. In this regard, it has been found that mitochondria with structural alterations or impaired metabolic activity characterize “elderly” oocytes [27,28], and that poor-quality mitochondria may lead to early arrest of embryonic development in AMA women [29]. In particular, high mitochondrial production of reactive oxygen species in mammalian oocytes correlates with premature loss of chromosomal cohesion, segregation defects and aneuploidy [30-33]. Some studies indicate that long-term supplementation with antioxidants reduces aneuploidy in oocytes from older mice [34] and humans [30].

Comparison of aneuploidy frequencies among mammalian species

In several mammals besides humans, AMA is associated with an increase in aneuploidy incidence, with most of the regulatory cellular processes conserved across species [35]. However, only a few studies have been performed in *in vivo* ovulated MII oocytes. In the mouse, the frequency of aneuploidy in MII oocytes is 3-10% in young animals (1-3 months) [36-41], rising to 12.5% in 9-month-old adults [37], and to 30-37.5% in 12-month-old mice [37,38,40]; instead, very-old (15-19 months) mouse oocytes display a 25% rate of aneuploidy [39,41]. In the monkey, two studies report an aneuploidy incidence of 0-5% in *in vivo* ovulated oocytes from young females aged 4-15 years versus 14.3% in oldest monkeys aged 16-26 years [42,43]. Additionally, *in vitro* matured MII mare oocytes showed a frequency ranging from 15.6% in young (<14 years old) to 55.6% in old (>16 years old) individuals [44]. In cattle, the overall rate of aneuploidy was found to be between 3% and 7% [45,46].

Overall, these studies show an increase in aneuploidy rate with AMA, similar to that found in women. On the contrary, a couple of studies in *in vitro* matured MII porcine oocytes indicated no increase in the prevalence of aneuploidy, which ranged from 11-12% [47,48] in young animals aged <15 months to 8.7% in old animals (7-8 years) [47]. Overall, due to differences between the various techniques used for scoring oocyte aneuploidy, the reported frequencies are sometimes highly

heterogeneous [49-51]. Importantly, however, the use of these mammalian model organisms has facilitated the identification of key mechanisms associated with the development of aneuploidy which are also conserved in human oocytes. Nevertheless, some specific spindle dynamics predispose the human oocyte to the occurrence of chromosomal segregation errors [52]. In contrast to the mouse, which shows an acentriolar microtubule-organizing center (aMTOC)-mediated spindle assembly, human oocytes lack aMTOCs and instead show Ras-related nuclear protein-guanosine triphosphate (RAN-GTP)-driven microtubule formation. A mutation in the RAN gene prevents spindle assembly in human oocytes [52,53]. In addition, unlike oocytes of other mammals, human oocytes are deficient in the microtubule motor protein kinesin superfamily protein C1 (KIFC1), which stabilizes the spindle [54-57]. Supplementation with exogenous KIFC1 was proposed as a promising strategy to reduce spindle instability and the risk of aneuploidy in human oocytes [56]. Considering the large difference in lifespan between humans and other mammals, it is plausible that human oocytes are more susceptible to error-causing factors which, accumulated throughout the reproductive years, contribute to aneuploidy [6].

The U-shaped curve of fertility

In recent years, the incidence of meiotic aneuploidy in human oocytes has been carefully investigated.

A better understanding of the maternal aging-related mechanisms leading to aneuploidy is essential for the development of intervention strategies that may reduce its occurrence and detrimental effects, thus preventing abnormal reproductive outcomes. Human females are among the few mammals that experience menopause [58]. According to the most supported hypothesis, named the “molecular clock hypothesis” [59], the occurrence of aneuploidy may be a protective mechanism for restricting fertility, and possible gestations, to a specific period in a woman's life. Indeed, it is thought to be an evolutionary adaptation whose purpose is to minimize risky pregnancies in very young and, especially, in AMA women. Indeed, on examining the chromosome arrangement of oocytes from very young, adult and AMA women, chromosomal aneuploidy is seen show a U-shaped trend, with a high incidence from puberty to <20 years, a decrease at the ideal reproductive age (20-34 years), and a rapid increase from 35 years to menopause [59]. As oocyte aneuploidy is one of the main causes of infertility, this phenomenon is reflected in the fertility curve, which also follows a U-shaped trend, albeit an inverted one, given that fertility is higher in adult life, when the occurrence of chromosomal errors during meiosis is lower [59] (Figure 1A).

Recent evidence indicates that the U-shaped trend shown by the occurrence of chromosomal errors is associated with different segregation error mechanisms. In oocytes during the GV-to-MII transition taken from young (ideal reproductive age) and AMA women, a single nucleotide polymorphism analysis of the pericentromeric chromosomal region (closely linked to segregation events during meiosis) revealed that three different main types of errors, occurring in a temporal sequence

during aging [59,60], underlie the U-curve of aneuploidy [4]: (i) non-disjunction of homologous chromosomes during MI (MI NDJ), when the gain or loss of an entire chromosome occurs; (ii) PSSC, in which the sister chromatids of a homologous chromosome separate during MI; and (iii) reverse segregation (RS), in which the sister chromatids of both homologous chromosomes already separate during MI. Looking at these meiotic anomalies individually, MI NDJ is the most frequent alteration in women under 20 years of age and progressively decreases with increasing maternal age; PSSC, instead, increases with age; while RS is significantly higher in AMA patients (Figure 1B).

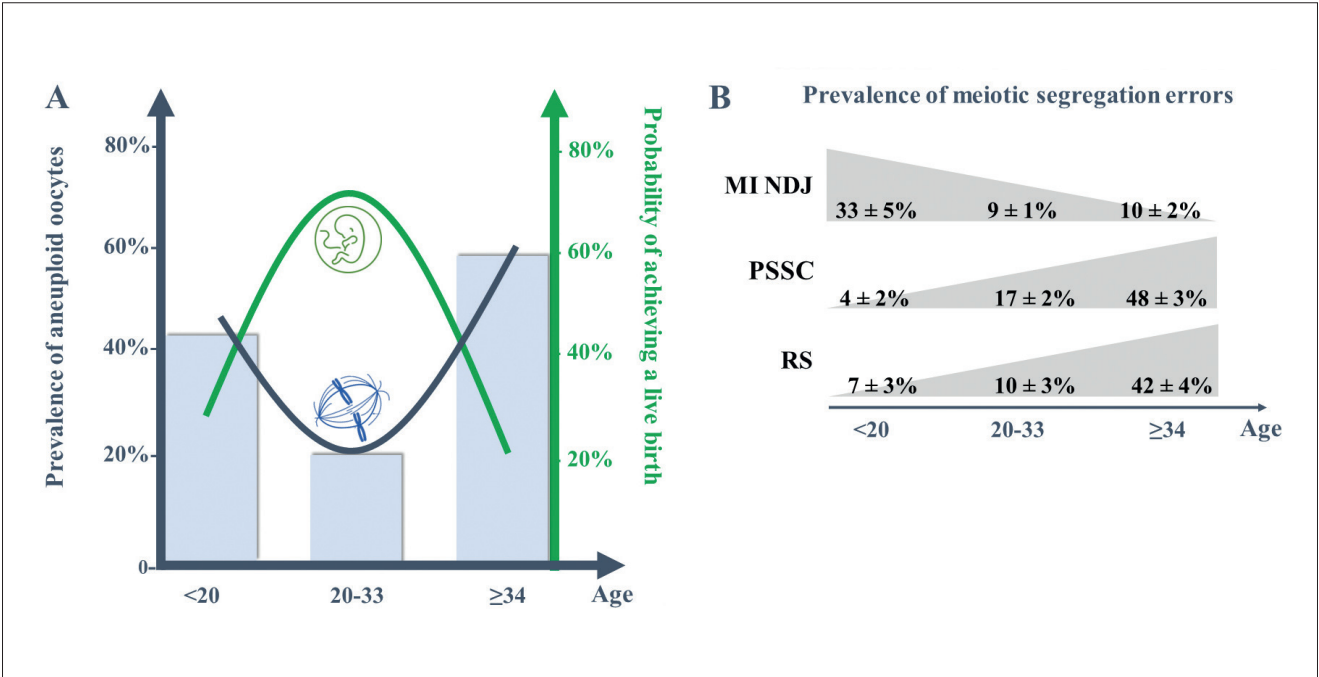
Whilst the mechanism explaining the higher trend of MI NDJ events in adolescents is still unclear, there is emerging evidence of a weakening of centromeric cohesion between two homologous chromosomes as the molecular mechanism determining aneuploidy in AMA women [61]. In the presence of an extensive loss of centromeric cohesion, which determines the loss of a single functional unit of the homologous chromosomes during MI, alterations such as a complete reversal or deterioration of bivalents may correlate with the marked increase in RS in the AMA group. Therefore, weakening centromeric cohesion may play a role as a “molecular clock” that limits the reproductive capacity of women by predisposing certain chromosomes to specific errors. Taken together these data indicate that the U-curve of aneuploidy is the result of specific timing of the incidence of different segregation errors, and that this phenomenon shapes the U-trend of fertility. They also show how the reproductive phase is finely regulated by the appearance of the different types of aneuploidy.

Clinical and biological implications

The mechanisms underlying the U-shaped fertility curve have important implications for both clinical practice and evolutionary biology. Interestingly, recent clinical studies are focusing attention on the increased risk of aneuploidy in the oocytes of young women. Sills *et al.* [62] reported a high aneuploidy rate (53.2%) in embryos derived from donor eggs (mean $\pm$ SD age 24.0 ± 2.7 years), where 88.1% of embryo aneuploidy was of maternal origin. In line with these findings, a large retrospective review of more than 15,000 trophectoderm biopsies found the lowest risk (20–27%) of embryo aneuploidy in women of 26–30 years of age, and a higher incidence (>40%) in women aged 22 and 23 [3]. Moreover, a recent study revealed that egg donors aged 23–29 years were linked to a higher chance of recipient clinical pregnancy and live birth after the first embryo transfer compared with donors aged 18–22 years [63]. The results of these studies, contrary to the common belief that “younger is better”, suggest that a younger donor age does not necessarily correlate with a higher chance of pregnancy in egg donation cycles. Considering that most countries set a lower age limit of 18 years for egg donors [64], increasing this age limit might be an option [65].

In relation to the increase in aneuploidy in older women, several evolutionary explanations for the prolonged duration of post-reproductive life in humans have been proposed. Among these, the so-called grandmother hypothesis is attracting increasing interest. First proposed by Peter Medawar in his essay “An Unsolved Problem of Biology” [66], and subsequently elaborated on by others, the hypothesis suggests that older females

Figure 1 (A) A graphic representation of the “U” trend of female fertility. The distribution of meiotic segregation errors defines the “U-shaped” aneuploidy trend (blue curve). Showing an opposite trend, meiotic error rates throughout the entire reproductive lifespan shape the trend of natural fertility (green curve), reported as the probability of achieving a live birth. (B) Each period of a woman’s life is characterized by specific types of aneuploidy: MI NDJ is significantly higher under the age of 20 years, and progressively decreases with increasing maternal age; PSSC increases with increasing age, while RS is significantly higher in the elderly oocytes [59]. (MI NDJ, non-disjunction of homologous chromosomes during meiosis I; PSSC, premature separation of sister chromatids; RS, reverse segregation).



increase their genetic fitness, by helping to raise and support grandchildren, rather than by having other offspring of their own. In this way, natural selection, by favoring features that promote post-reproductive survival, might have driven the evolution of longer post-reproductive lifespans ^[67]. So far, study of the post-reproductive lifespan phenomenon has been limited to “natural fertility” populations that lack access to modern health care and technology, such as the Hadza hunter gatherers of Tanzania ^[68], and it has produced convincing evidence that the menopausal phenomenon is not an artefact of modern living or of today’s reproductive choices. The grandmother hypothesis has also been debated alongside the “mother hypothesis”, which suggests that menopause may have evolved in order to reduce the mortality risk associated with additional pregnancies and deliveries in older mothers with existing children, and thus to increase the latter’s chances of survival ^[69]. Overall, this evidence suggests that further understanding is essential in order to guarantee proper counseling of women who opt for social egg freezing or choose to take part in egg donation programs, so that they can be helped to choose the most appropriate time to freeze their oocytes.

Conclusions

Infertility is defined as the absence of conception following at least one year of unprotected sexual intercourse ^[70]. In patients undergoing IVF, it is advisable to investigate the cause of the infertility and define a specific treatment path. The literature reports a significant increase in meiotic errors in human oocytes with female aging ^[71]. Multiple factors, including cohesin dysfunctions, spindle instability, and alterations in chromosome telomere length, play a role in meiotic chromosomal mis-segregation ^[12]. On the basis of the emerging “U-shaped” aneuploidy trend it seems possible to predict a high incidence of chromosome alterations not only in women of AMA, but also, for the first time, in young women ^[59]. In particular, it has been suggested that whole-chromosome MI NDJs are associated with increased rates of aneuploidy in young girls, a phenomenon whose underlying mechanism is still unclear, whereas centromeric and more extensive cohesion weakening drives increases in PSSC and RS and limits fertility in AMA women. This new perspective, which reopens the discussion on the underlying molecular mechanisms, lays the foundations for a new interpretation of the natural curve of fertility. The presence of a biological mechanism that protects women from risky pregnancies, by concentrating meiotic anomalies at a very young age and at AMA, might represent a “safety system”, evolutionarily associated with improved reproduction and fitness. In clinical practice, this could be translated into a more targeted approach characterized by treatment personalization, more developed knowledge and technologies, and greater patient safety.

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Follicle stimulating hormone receptor: impact of genetic variations and gene expression levels in fertility

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ABSTRACT

To date, Assisted Reproductive Technologies (ARTs) increase the probability of conception starting from the collection of more than a single oocyte produced in a regular ovulatory cycle. To achieve this aim many ovarian stimulation protocols have been proposed, some with very good performances but also with some important side effects and, more important, with not many possibilities to personalize the hormonal treatment according to patients characteristics.

Although the hormonal and clinical parameters remain the only proven factors to aid in the selection of the best possible hormone stimulation for each patient, none of the commonly used markers has an optimal predictive value if considered individually. Therefore, a complementary strategy that is emerging in recent years is pharmacogenetics. The candidate genes to date are follicle hormone (FSH) and its receptor (FSHR), in which single nucleotide polymorphisms (SNPs) are able to modulate the expression and functions of the genes.

The FSH-FSHR complex initiates a cascade of molecular events in the gonads, from the increase of cyclic AMP (cAMP) to the transduction of enzyme-encoding mRNA products, which modulate the synthesis of steroid hormones. In this way, FSH stimulates folliculogenesis and steroidogenesis in the ovary and testicular development and spermatogenesis in the testis. The administration of FSH in the treatment of infertility, in both sexes, aims to induce these activities in order to allow infertile couples to carry out the pregnancy.

Many studies on the genetic polymorphisms of FSH and its receptor identified which of these variants could be considered as a marker able to predict the individual responses of patients undergoing ovarian stimulation.

KEYWORDS

Clinical embryology, FSH, Polymorphism, Ovarian functions.

Introduction

Physiological and molecular aspects of FSH

The stimulating FSH is a glycoprotein with a fundamental role in the development of the gonads and sexual maturation during puberty in male and female.

In particular, FSH in women induces the maturation of ovarian follicles, which are egg cells surrounded by follicular cells. During the fertile period of the woman, about every 28 days, an ovarian follicle is brought to maturity. While the follicle grows releases the Inhibin that, through a negative feedback, stops the release of FSH into the adenohypophysis.

In males, FSH activate the production of androgen binding protein (ABP) in Sertoli cells, in seminiferous tubules, and it is essential for spermatogenesis.

FSH is a heterodimer composed of two glycoproteins. Its structure is similar to LH, TSH and hCG, all hormones produced by the pituitary gland. The two polypeptide units that make up the FSH dimer are called alpha and beta subunits. The alpha subunit is the same for all LH, TSH and hCG hormones,

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and contains 96 amino acids. The beta subunit, on the other hand, is specific for each hormone and in the case of FSH it contains 118 amino acids and is called FSHB. The activity of FSH is mediated by the binding of FSHB with its receptor (FSHR) located in the plasmalemma of Sertoli cells in the testes and in the cells of Granulosa in the ovaries. The FSHR protein is produced following the transcription of the FSHR gene mapped on the chromosome 2p16.3

The National Centre of Biotechnology Information database reports 731 point mutations at the FSHR gene level⁽¹⁾. Since the research for these mutations occurs by studying patients with alterations in the reproductive function, mutations

are classified as activating, inactivating or neutral, depending on the function of FSHR⁽²⁾. Point mutations in the encoding DNA of FSHR gene induce amino acid changes that modulate the receptor signalling expression and pathway with important clinical consequences.

In addition, allelic variants or SNPs have been discovered giving rise to different haplotypes that modify the effect of FSH in tissues.

Polymorphisms

Polymorphism means the presence of two or more different gene forms in the same locus with a frequency within the population of at least 1%. The polymorphisms may be due to mutations, insertion or deletion of more or less long stretches of the DNA sequence, which may induce modifications of the amino acid produced by a single codon. The frequencies of genetic polymorphisms within a population are determined by evolution and by the process of natural selection. However, these are allelic variants that can be transmitted to offspring, therefore inheritable. In fact, some analyses of these variants take advantage of the chromosome property called Linkage disequilibrium (LD) which is based on the observation that two neighbouring variants tend to be segregated together more frequently than two distant variants whose probability of recombination is greater. Allelic variation involving a single nucleotide is called SNPs (Single Nucleotide Polymorphisms).

SNPs can occur within a coding sequence of a gene, within an intronic region or an intergenic region. In any case, SNPs within a gene do not necessarily modify the encoded amino acid sequence, since the genetic code is degenerated. The presence of these variants is fundamental to define haplotypes of a population, particular combinations of alleles determined by the specific combination of SNPs in a given genomic sequence.

The studies carried out on the gene sequence coding for the FSHR have detected eight polymorphic sites at the level of the coding regions but six of these are asymptomatic while the remaining two are relevant in the variation of the receptor responses to the stimulation with FSH. One of these polymorphisms is found at the level of the gene coding for the amino acid residue at position 307 occupied by an Ala or Thr, important for interaction with the ligand. The second one is located in the coding portion for the amino acid residue 680 occupied by an Asn or a Ser, important in signal transduction⁽³⁾.

Analysis strategies for the identification of polymorphisms

The study of polymorphisms allows to make predictive investigations on the development of a disease or to provide valuable information on how the disease could develop. Indeed, the polymorphisms that are located near a disease gene can be used to find the disease gene, so they are therefore very frequently used in association study maps. The most common association studies are the ones cases-control, in which the comparison between the allele frequencies of genetic polymorphisms within the genome of subjects with pathology and healthy control subjects. Many polymorphisms are specific to each population; therefore, in 2002 the "International HapMap project" was created to guarantee to genetic research a database of useful infor-

mation to lead large-scale genome-wide association studies. In fact, the decrease in costs and the increase in the density of polymorphisms in the genotyping panels makes it possible to analyse the whole genome for the study of complex pathologies, substituting this approach to the used analysis of a candidate gene. Another useful tool for association studies is the National Centre for Biotechnology Information (NCBI) SNPs database (<http://www.ncbi.nlm.nih.gov>) where SNPs are registered with a specific nomenclature⁽⁴⁾.

The techniques used to study the receptor for FSH and its gene are different depending on whether the gene sequence and its variants are investigated, or the structure and function of the receptor protein encoded by these variants. Several research groups have developed techniques based on "Polymerase Chain Reaction" (PCR), which allows amplifying the whole FSHR gene or parts of it. Numerous studies refer to the methods originally used by Gromoll, author of the primers and protocols for 16 PCR procedures that amplify the whole FSHR gene⁽⁵⁾.

Most of the primers used in these studies have exon 10 as a target sequence because it encodes as many as 410 of the 695 amino acids that make-up the FSHR. The identification of some SNPs within the exons of the gene has allowed the development of different Restriction Fragment Length Polymorphisms (RFLP) methods that through a simple enzymatic digestion followed by gel electrophoretic run make possible to distinguish individuals homozygous from the heterozygous for each point mutation of the FSHR gene that is intended to be analysed. Another technique is Real-Time PCR (RT-PCR) that allows amplifying and quantifying DNA simultaneously⁽⁶⁾.

Although PCR is the first approach to study the gene for the FSHR, others techniques are able to achieve this goal. After amplification, DNA can be sequenced or used for some analyses in order to identify the nature of the point mutations. The analysis of possible genetic mutations involves the use of different techniques; one of these is the Denaturing High Performance Liquid Chromatography (DHPLC), a very sensitive and accurate technique based on separation of DNA homoduplex from DNA heteroduplex for the detection of mutations and SNPs. Another DNA screening system is the analysis using Single Strand Conformational Polymorphism (SSCP) which consists in the heat denaturation of the DNA fragments followed by a rapid process of cold renaturation to maintain the structural conformation given by the coupling between the bases. This conformation could be altered by an exchange of bases that changes the electrophoretic run of the fragments.

When the mutations have been identified, the region of mutated DNA can be transferred through the process of DNA transfection into host cells, in order to study the differences in expression and binding with FSH between the "wild-type" receptor and the receptor encoded by the mutated gene sequence. Over the years, many cell types have been used as acceptors of these transfection procedures, such as COS cells, 293-T and MSC-1. Once a transfected cell line that expresses the mutated receptor in a stable manner has been obtained, it is possible to proceed with studies of kinetics of the binding with FSH or it can be studied how the mutations result in a variation of the cellular response in terms of synthesis of cAMP or other second messengers⁽⁷⁾.

Follicle stimulating hormone receptor

Molecular structure of FSHR

The FSHR belongs to the family of G Protein Coupled Receptors (GPCR) and consists in a mature protein of 678 amino acids with a molecular weight of 75 kDa. FSHR consists of three domains: an intracellular domain, a 7 α -helical transmembrane domain, and an extracellular domain.

The FSHR portion involved in the interactions with FSH is located towards the extreme N-terminal of the extracellular region. The extracellular domain is composed by 349 amino acids and consists of 10 repetitions of about 24 amino acids with the characteristic LLR motif (leucine-rich-repeats) that allows the interaction both with the transmembrane domain and the hormone. There are several cysteine residues; eight are perfectly preserved even in the luteinizing hormone and thyroid hormone receptors, suggesting their fundamental role in preserving the integrity of the receptor conformation.

In addition, the extracellular domain has three potential glycosylation sites at the level of the 191, 199 and 293 residues important for receptor expression in the plasmalemma and for the binding of FSH. The specificity of binding with FSH is given by the residues of Phe165 and His274 with FS22's Val221.

The transmembrane domain has the typical characteristic structure of the GPCR, made up of 246 amino acids grouped in 7 hydrophobic α -helices alternated by extracellular and intracellular loops. The two residues of Cys, 442 and 517, at the level of the first and second loops, form a sulfide bridge important for the structural stability of the receptor. Following the binding of the receptor with FSH, the signalling pattern is activated by intracellular domain at the C-terminal region of the protein. The intracellular domain consists of 65 amino acids and is rich in Thr and Ser residues that are phosphorylated by intracellular kinases to activate signal transduction.

FSHR isoforms: potential roles in healthy tissues

There are different transcripts of the FSHR gene that appear to be generated by the presence of several polyadenylation sites and by alternative splicing phenomena. The FSHR isoforms that have been identified are:

1. FSHR1 is the most common receptor isoform and has a typical GPCR structure. It is a receptor expressed only in ovary granulosa cells and it is involved in follicle development and granulosa cell differentiation.
2. FSHR2 is a variant of the receptor that lacks the intracellular domain, for this reason it is able to bind the substrate (FSH) but not to activate the G protein. This isoform is also called "dominant negative" because it is thought that two pairs of FSHR2 go to activate the inhibitory G protein instead of the stimulatory G protein.
3. FSHR3 is a receptor isoform that has only one transmembrane domain; it works as type I growth factor receptor because it activates the signalling pathway of MAP kinases and the calcium dependent channels in epithelial cells of the ovarian tissue.
4. FSHR4 is a soluble isoform of the receptor that lacks the transmembrane domain, but the function of this receptor is still unknown.

Signal transduction pathways

The stimulating follicle hormone receptor is synthesized in granulosa cells, glycosylated and then transported and evenly distributed on the plasma membrane of cells. FSHR is activated after a conformational change of its molecular structure; although receptor dimerization is not always necessary, it can stabilize the binding with FSH and ease signal transduction.

Following the binding of the hormone, the receptor interacts with the protein G that binds the cofactor guanosine triphosphate (GTP) and stimulates the enzyme adenylate cyclase to synthesize the second messenger: the cAMP. The cAMP, in turn, activates the protein kinase A (PKA) that recruits the regulatory-extracellular kinase 1/2 (ERK1/2) and phosphorylates structural proteins, enzymes and transcriptional modulators belonging to the family of the cAMP responsive elements (CRE). CRE binding proteins (CREB) are transcription factors that mediate the expression of genes that encode for enzymes that modulate steroidogenesis and signal activation/deactivation, such as steroid acute regulatory protein (StAR) and aromatases that induce the synthesis of steroids and P53 and the cyclins that block their synthesis. Depending on the endocrine status and the stage of maturation of the gametes, different isoforms of the modulators and CRE proteins are preferentially expressed.

Instead, ERK1/2 is involved in the regulation of steroid signals, in receptor desensitization and proliferative events.

In addition to the signalling pathways activated by cAMP/PKA and ERK, there are other intracellular events, such as the increase of intracellular calcium, the activation of protein kinase B (AKT) or Epidermal Growth Factor Receptor (EGFR) and the activation of the mTOR pathway who allows a balance of pro- and anti-apoptotic signals.

The signal is turned off by a negative feedback c-AMP dependent that causes a down regulation of the receptor expression. In particular, after about 1-4 hours from FSH stimulation, the receptor-hormone complex is sequestered in the lysosomes.

Molecular biology of the FSHR gene

The human FSHR gene was mapped on chromosome 2p16.3 [MIM *136435]. The gene occupies a very large region of about 200kb and includes 10 exons and 9 introns. The extracellular domain of the protein is encoded by the first 9 exons of the gene, while the C-terminal part of the extracellular domain, of the intracellular and transmembrane domain are coded by the exon 10.

Low doses of FSH activate signalling pathways that induce FSHR mRNA synthesis, whereas high doses of FSH down-regulate mRNA levels and consequently decrease the number of binding sites for FSH on the receptor.

Studies performed on adult rats during the phases of the estrous cycle have shown that the FSHR concentration increases linearly respect to the follicle maturation, which reaches the maximum peak in the ovulatory phase, and decreases drastically in the luteinizing phase. In humans, studies on perimenopausal patients with irregular cycle have identified a correlation between an increase of serum FSH and a reduction of FSHR mRNA levels associated with follicular atrophy⁽⁸⁾.

In the regulation of FSHR expression are also involved some paracrine factors: activin and Transforming growth factor β (TGF- β) which are potent inducers of gene transcription, and Epidermal growth factor (EGF), insulin-like growth factor (IGF) and Fibroblast growth factor (FGF) which are factors that regulate negatively the transcription to lower the response induced by FSH.

Allelic variants of the follicle stimulating hormone receptor

SNPs in the FSHR promoter

The promoter of FSHR belongs to that class of promoters that lack the conventional TATA box but it has numerous sites of transcription initiation. The promoter core is located respect to the start codon between position 225 and position 1 and presents two responsive elements: the consensus sequence E-box (CACATG) that binds a group of transcription factors called “basic helix-loop-helix” like upstream stimulatory factor (USF) and the Initiator element (Inr), typical region of house-keeping genes.

Comparative studies on different species have shown the presence of a G/A variant in position -29 of the FSHR gene promoter where is present GG / AAA consensus sequence, the binding site for the cE factor- twenty-six specific (c-ETS). The presence of a homozygous AA genotype at position -29 is associated with an increase of serum FSH and consequently a decrease in the receptor response. Another highly polymorphic region at the promoter level results to be the A-stretch oligo (A11-17), but in this case there are no significant changes in receptor functions.

Frequency and ethnic distribution of FSHR polymorphisms

Studies on the frequency distribution of FSHR polymorphisms show contrasting results. This suggests that the ethnic background of the subjects examined can strongly influence these results.

The FSHR gene presents more than 900 SNPs, organized in different blocks in linkage disequilibrium according to ethnic groups.

Considering the distribution of the SNP rs6166 polymorphism (p.N680S), according to the data obtained from the Hap Map Database (<http://hapmap.ncbi.nlm.nih.gov>), the ancestral allele A is predominant in the South-East Asia, while the G allele is prevalent in other populations, particularly in Kalash (North-West Pakistan), Yakuts (Siberia), Surui Paiter (Brazil) and Melanesia (Oceania)⁽⁹⁾.

It is interesting to note that these ethnic groups are geographically isolated and genetically distinct, and this certainly makes us think about events of genetic drift positive selection. While for other populations, such as Europe, Central Asia and Oceania, they show a higher frequency of the G allele, in North America instead the frequency is slightly lower. In particular in the Asian population (including both Japanese and Chinese) the Ser680Ser receptor variant is much less common, with a frequency of 10.5% compared to the Caucasian (21.5%) and

Mediterranean (22.3%) population⁽¹⁰⁾.

Data related to rs6165 polymorphism (p.A307T)^(6,7) show that the ancestral allele G is predominant in sub-Saharan Africa with a MAF of 0.274, while in other populations MAF is similar to rs6166 polymorphism. This suggests that the two SNPs with the exception of the African population are in Linkage Disequilibrium.

The third SNP rs1394205 (c.-29G>A) present on the FSHR promoter does not appear to be in linkage disequilibrium with the two SNPs present on the exon 10. Comparative alignment analyses show that the allele G is the ancestral allele. The homozygous AA genotype, associated with an increase in serum FSH, is more frequent in the United States of America and East Asia than in the European and African populations.

The most studied polymorphism is the Ser680Asn variant because it has been associated with evident effects in the response to ovarian stimulation provided by the IVF protocols⁽¹¹⁾.

The results obtained from different metanalytical studies⁽¹²⁾ suggest that when studying and evaluating the effects of these polymorphisms, it is essential to consider also the ethnic origin and environmental factors that can change from one geographical area to another.

Clinical consequences in reproductive functions

SNP effects in exon 10 of the FSH receptor in normal ovarian functions

The secretion of gonadotropins from the pituitary gland determines the progression of the ovarian cycle and the activity of the gland is controlled by a feedback mechanism from the ovarian hormones. The highest peak in serum levels of stimulating follicle hormone occurs when a group of follicles are recruited at the antral stage, and then allowed to mature. Generally, only follicles with low FSH levels can grow and become Graafian follicle following stimulation with LH.

The biological activity of the hormone depends on the expression of FSHR in the granulosa cells, for this reason numerous studies have focused on the influence of these receptors both in the dynamics of the normal menstrual cycle and in the pathologies linked to the ovarian functions.

Given its functions, FSH is used extensively in ovarian stimulation protocols, both as urinary FSH and as recombinant FSH. The main biomarkers used in clinical practice to predict a normal, low or high ovarian response to the stimulation treatment are: the woman's age, the Anti-Müllerian hormone (AMH), the Antral Follicle Counts (AFC) and the levels of Basal FSH (assessed on the third day of the cycle). AFC and AMH are considered the main predictors of ovarian response with gonadotropins. However, these biomarkers alone do not allow an optimization of IVF therapy for all patients, given that some patients may be over-responsive to a low dose of FSH and hypo-responsive in cases of stimulation with a high dose of gonadotropins⁽¹³⁾. It has been demonstrated that the sensitivity of follicles to exogenous FSH, measured through Follicular Output RaTe (FORT), may be very different between patients with different clinical features⁽¹⁴⁾.

This indicates that genetic factors play a fundamental role

in the ovarian response to gonadotropins. Different studies have analysed the correlation between FSHR polymorphisms, ovarian reserve markers and the outcome of the ovarian response to gonadotropins during in Vitro Fertilization (IVF) or Intracytoplasmic Sperm Injection (ICSI) cycles. Most of these studies focus on the c.2039G>A (p.Ser680Asn) variant [NM_000145.3], as the presence of a G/G (Ser/Ser) genotype appears to be associated with higher basal FSH levels and a reduction in exogenous FSH sensitivity. This was demonstrated by an increase in the total dose of gonadotropins used during IVF ovarian stimulation treatment, by a decrease in estradiol peak following hCG administration and by a decrease in oocyte numbers and a high incidence of G/G genotype in hypo-responsive patients.

This effect was first observed in a non-randomized study and subsequently in a randomized trial involving women undergoing assisted human reproduction. In the non-randomized study ⁽¹⁵⁾ it was observed that the amount of FSH required to induce controlled ovarian hyper-stimulation (COH) and obtain a similar peak of estradiol is low for women homozygous for the Asn680 variant compared to women homozygous for the Ser680 variant. This data were corroborated with a randomized trial in women undergoing COH who received the same dose of FSH ⁽¹⁶⁾, showing that FSH is less efficient in women with the p.N680S variant at the receptor level. However, an increase in FSH dose from 150 to 225 U / day exceeds the low estradiol response in women with Ser / Ser (group II, 7804 ± 983 pmol / l). Even considering the different levels of estradiol, there were not significant differences in the number of follicles or oocytes, variations in fertilization rate and pregnancy. This indicates that some women may receive an excessive amount of FSH, which increases the risk of developing ovarian hyperstimulation syndrome (OHSS), which indirectly depends on excessive stimulation with FSH.

The production of estradiol, an important hormone for the maintenance of normal ovarian functions, depends on the availability of androgen substrates that are LH-dependent. FSH stimulates the expression of LHR. The presence of p.N680S polymorphism in the FSHR induces a low expression of the FSHR and consequently a low expression of LH receptors and/or the expression of aromatases. The modulation of this pathway, given the presence of allelic changes in the FSHR would explain the difference in estradiol levels depending on whether it is a homozygous or heterozygous Asn680 or Ser680 genotype.

This concept has been confirmed by studies performed on women with mono-ovulatory cycles going to monitor a normal menstrual cycle.

SNP effects in exon 10 of the FSHR in ovarian functions under pathological conditions

A change in the reference values of FSH may indicate some pathologies. In non-pathological conditions FSH in males has a concentration of 1.0-17 mIU/mL, while in women it can vary according to the phase of the cell cycle and obviously to the aging: in the follicular phase the concentration of FSH is equal to 1.4-18 mIU/mL, in the ovulatory phase is equal to 9.4-42 mIU/mL, in the lutein phase 1.7-10 mIU/mL and in the menopause 37.0-167 mIU/mL ⁽¹³⁾.

Generally, in women with pathological conditions, high FSH levels indicate primary ovarian failure and low FSH levels indicate secondary ovarian failure due to a hypophyseal or hypothalamic problem. Furthermore, low blood levels of FSH have been associated with an increased risk of ovarian cancer. In man under pathological conditions an increase in FSH levels may be due to primary testicular failure. Low levels instead are consistent with hypophyseal and hypothalamic disorders. A change in FSH levels may in turn be linked to a change in the receptor conformation. For this reason, several studies have been performed on the effects of the main polymorphic variants of the FSHR, such as Asn680Ser and Ala307Thr, in the various pathologies linked to ovarian functions. Among the pathologies considered there are: the OHSS, which may occur following a stimulation of follicular growth and dehiscence and the Polycystic Ovary Syndrome (PCOS) and the Premature Ovarian Failure (POF), which are characterized by hormonal imbalances in reproductive age. Studies have also been carried out on the possible association of ovarian cancer with the presence of FSHR polymorphisms, demonstrated by the fact that in ovarian carcinoma tissues was observed an increase in FSHR expression, compared to healthy tissues.

Ovarian Hyperstimulation Syndrome (OHSS)

OHSS is a disorder linked to ovarian stimulation during the treatment of medically assisted reproduction (MAR). The severe form of OHSS occurs in 0.1-5% of the stimulation cycles and its clinical consequences are: a volumetric increase in the ovaries with the formation of multiple cysts and an increase in the permeability of the capillaries at the ovarian level which in turn increases the transfer of fluids, from the intravascular to the extravascular compartment. This results in a state of concentration of the solid components of the blood which on the one hand exposes the patient to a high risk of thrombosis and emboli and on the other causes a reduction in blood flow in organs with risk of ischemia. Several vascular and inflammatory molecules contribute to the increase in vascular permeability, including interleukin (IL)-6, interleukin (IL)-8, tumour necrosis factor (TNF) and angiotensin II ⁽⁷⁾.

The development of OHSS is mainly associated with the administration of hCG for ovulation induction, because it stimulates the production of VEGF which, in turn, stimulates the formation of new blood vessels and increases vascular permeability. Based on the development of the symptomatology, we can distinguish two types of OHSS: "early" OHSS, with onset in the middle-luteal phase, deriving from the administration of exogenous hCG to stimulate ovulation and "late" OHSS, for the production of endogenous hCG in the initial phase of any pregnancy.

Although the causes of OHSS are not yet fully understood, to date are considered as risk factors: young age at conception, low body weight and lower body mass index (BMI), polycystic ovary syndrome, an increase of estradiol levels and all the stimulations that can compete to vascular damage.

However, there are no absolute predictors to prevent the syndrome which is currently the most serious adverse event in ART.

Recently, three different allelic variants on FSHR associ-

ated with patients presenting recurrent forms of OHSS have been identified. In particular, it has been observed that mutated forms of the FSHR are hyperstimulated at high doses of hCG, whereas instead during pregnancy FSHR levels are either absent or very low. This has prompted several researchers to investigate the possible correlation between FSHR polymorphisms and iatrogenic OHSS.

A recent study has shown that the Ser680 variant occurs very often in women who develop OHSS, but it is the Asn680 variant that is associated with a more severe form of OHSS⁽¹⁷⁾. In another study⁽¹⁶⁾, also the Ala307 polymorphism was associated with a higher risk in iatrogenic OHSS due to a higher sensitization of the FSHR⁽¹⁸⁾. These results indicate that subjects homozygous for the Ala at position 307 (NM_000145.3 (FSHR):c.919G>A (p.Ala307Thr) could make subjects susceptible to the development of OHSS.

Polycystic Ovary Syndrome (PCOS) and Premature Ovarian Failure (POF)

The duration of the fertile age is another parameter that can be considered as a plausible effect of polymorphisms. Among the diseases that may be related to the presence of the most common allelic variants of the FSHR are PCOS and POF.

PCOS is a disorder that affects about 10% of women during reproductive age. It is a syndrome associated with metabolic and endocrinological abnormalities such as hypergonadism, chronic anovulation, abnormal menstrual cycle and polycystic ovaries. Despite numerous studies on the probable genetic causes related to this syndrome, the key factors involved in PCOS are not yet fully understood. The study on the allelic frequency of the Thr307Ala and Asn680Ser variants reveals that the haplotype Ala307/Asn680 is significantly bound with PCOS and could be a risk factor for the disease. A study of 193 PCOS patients shows that women homozygous for the Ser680 allelic variant have a double chance of developing resistance to clomiphene citrate (CC) compared to women carriers of the Asn680 variant⁽¹⁹⁾.

CC is the first choice treatment for patients with PCOS and is a competitive antagonist of 17 β -estradiol at the receptor complex. By blocking the estrogen receptor, FSH and LH can be released allowing follicle growth and ovulation. In about 80% of patients treated with CC ovulation is recovered, while 20% of patients are resistant to the maximum dose of 150 mg of CC. The exact causes of clomiphene resistance cited are still unknown. Among the possible causes, a probable role of polymorphic variants at FSHR level has been hypothesised⁽²⁰⁾. In fact, the presence of SNPs could explain the inability of FSHR to respond to the hormone during ovulation. However, larger-scale studies are needed to further deepen and clarify the correlation between FSHR allelic variants and women with PCOS.

Premature Ovarian Insufficiency (POI)

Another very frequent pathology is premature ovarian failure (POF), recently named as POI, a syndrome that is expressed with ovarian failure and oocyte depletion before the age of physiological menopause. This syndrome is therefore characterized by primary or secondary amenorrhea with anovulation

lasting at least 6 months, elevated serum gonadotropins (FSH > 40 IU/L in at least two doses) and hypoestrogenism in women aged less than 40 years. About 1% of women under 40 and 0.1% of women under 30 are affected by this disease. The causes of POF can be very variable and may be also sought in familial genetics, autoimmune diseases or X-chromosome abnormalities. A recent study shows that there is a clear correlation between POF and the FSHR. In particular, knockout mice for the FSHR receptor have been developed which show the classic POF phenotype with an increase in serum FSH levels and a decrease in estrogen levels. However, after the intra-ovarian injection of an adenovirus expressing a copy of human FSHR, mice are able to induce folliculogenesis, recorded with an increase in the number of follicles and serum estrogen levels and a decrease in Serum FSH⁽²¹⁾. However, a meta-analysis study of 157 cases and 633 controls reveals that the two most important FSHR polymorphisms are not associated with POF in humans, but probably only at a premature appearance of the clinical phenotype of the disease. Ultimately, it appears that the presence of Ala307Thr polymorphism is associated with an early onset of the disease, but longitudinal studies are needed to confirm these findings⁽²²⁾.

Ovarian endometriosis

Endometriosis is a chronic estrogen-dependent inflammatory disease that affects 10-20% of women of childbearing age. It rising up in women between 25 and 35 years and is practically absent in the prepubertal and post-menopausal age. It is so called because it is characterized by the presence of endometrial tissue (the tissue normally found only inside the uterine cavity) in ectopic site that is different from the physiological one. The mechanism that leads to the formation of endometrial tissue is not yet completely clear, one of the hypotheses credited is the passage, caused by uterine contractions that occur during menstruation, of endometrial fragments from the uterus in the tubes and from these in the abdomen, with implant on the peritoneum and on the surface of the pelvic organs. More than 50% of women with endometriosis show changes in fertility. Infertility can be associated to several aetiologies, such as: mechanical factors given by the subversion of the pelvic organs and the formation of adhesions with consequent alteration of the relations between the tubes and the ovaries; or from ovulatory factors in which endometriomas could counteract the normal ovulatory mechanisms that for example could negatively impact with the possibility of implantation of a pregnancy. In recent years, several studies in different ethnic groups have evaluated a possible correlation between the presence of SNPs and the risk of developing endometriosis. These hypotheses emerge from recent discoveries involving allelic variants of FSHR in serum FSH levels and in response to ovarian stimulation. In particular, the 680Asn/Asn variant induces an increase in the aromatase activity compared to the 680Ser/Ser variant, leading to a higher production of estrogens and to the stimulation of the proliferation of the endometrial tissue⁽²³⁾. Through the pharmacological suppression of estradiol it is possible to reduce the symptoms of endometriosis. This suggests that suppression of the functions of FSH in combination with the inhibition of estrogen production could lead to a new therapeutic

strategy for endometriosis. For the first time, a study published in 2018 (24), demonstrates a possible correlation between the presence of the haplotype GG/307Ala580Ser and an increased risk of developing endometriosis. The study was performed on 352 women with endometriosis and 510 fertile women used as controls, all of Brazilian origin. From the analysis it results a non-significant difference in the polymorphisms Ala307Thr and Asn680Ser between the two groups. However, when the group of women suffering from endometriosis was divided according to fertility status and disease stage, a positive association between 680Ser/Ser or GG genotype and fertile women with endometriosis was observed ($P = 0.004$).

Ovarian cancer

Ovarian cancer is one of the most common cancers in the female population. The risk of developing ovarian cancer increases after 35 years and decreases after 59 years. The causes of ovarian cancer are mainly sporadic, but they can also be genetic as in the case of hereditary high penetrance mutations of the BRCA1 and BRCA2 genes. In recent years the hypothesis has been evaluated that the stimulating effects of gonadotropins and female steroid hormones involved in cell proliferation may play a fundamental role in the development of ovarian cancer. This hypothesis was supported by *in vitro* experiments demonstrating that stimulation with FSH leads to growth of the epithelial surface of the ovarian surface and an over-expression of FSHR in Chinese hamster ovary (CHO) (25). In fact, numerous immunohistochemistry studies (26) confirm that there is an increase in FSHR expression not only in ovarian cancer tissue but also in vascular endothelium cells of different cancer tissues. The lack of FSHR expression in many non-cancerous cells indicates that the receptor could play a key role in both diagnostic and therapeutic terms. It appears that FSHR promotes the link between the Vascular Endothelial Growth Factor (VEGF) and its VEGFR-2 receptor, inducing the process of tumour neoangiogenesis (27). Furthermore, activation of the FSH/FSHR pathway induces Gq/11 protein activation in endothelial cells, which in turn induces VEGFR-2 activation even in the absence of VEGF (28). Considering all these mechanisms, one might suspect to block the FSHR pathway and acting against cancer. In fact, some researchers have designed a molecule able to selectively block the FSHR (29). *In vitro* results suggest that it is possible to reduce the growth of FSH-responsive cancers through a small inhibitory RNA (siRNA), which could increase the effect of chemotherapeutics in the treatment of cancer, but many studies are still needed to validate these findings. The SNPs present on the FSHR gene were also investigated, in particular a study carried out on subjects of the Chinese population of Hong Kong examined the possible association between the variant Ala307Thr and Asn680Ser and the different subtypes of ovarian cancer (30). This association study is based on genotyping techniques using DNA restriction enzymes extracted from 202 patients with ovarian carcinoma of different types and 266 controls. The study shows that there is an association between the Ala307/Ser680 haplotype and the susceptibility to ovarian cancer, especially in two cancer subtypes: serous and mucinous ($P < 0.0005$, OR = 2.60, 95% CI = 1, 56 - 4.34; and $P < 0.0005$, OR = 2.89, 95% CI = 1.73 - 4.84). However, the results ob-

tained in Asian patients do not coincide perfectly with the association studies in Caucasian patients, suggesting that the cancer risk may vary depending on the ethnicity of the patient's origin.

SNP effects in FSHR in testicular functions

The stimulating follicle hormone induces the proliferation of Sertoli cells, the mitotic activity of spermatogonia and supports cell differentiation and mitosis up to the spermatid stage. Male infertility affects 7% (<https://www.iss.it/>) of the population and compared to the past today in 1 case out of 2 the difficulty in obtaining a pregnancy depends on male reproductive problems such as insufficient sperm or the qualitatively altered nature of sperm (for reduced motility, altered morphology, damaged DNA) that hinder conception. In about 80% of cases of male infertility the cause of infertility cannot be identified and therefore a specific therapy cannot be used. In case of hormonal alterations, such as hypogonadotropic hypogonadism (a rare inherited condition characterized by deficiency of some hormones) it is possible to intervene with a medical therapy based on hormones. In the most severe situations are used MAR. To date, an increasing number of scientific evidences supports the efficacy of FSH therapy (31, 32). The treatment with FSH allows to increase the count and sperm motility in infertile subjects with idiopathic oligozoospermia (33). FSH levels above the normal range are universally considered to be predictive of a lack of therapeutic response to treatment with FSH, which should therefore not be administered in these cases. The numerous studies in favour of FSH therapy have led scientists to investigate the possible effects that FSHR polymorphisms could have in the hormonal treatment of patients with idiopathic infertility. Contrary to what is observed in women with normal ovarian functioning, the SNPs of FSHR in exon 10 in men have no effect on the serum FSH levels and on other clinical parameters, generating a discrepancy between the two sex genders that remains inexplicable. Various polymorphisms have been investigated in man including the variant at the G-29A promoter level, but the results among them have been very contradictory, suggesting that the correlation between testicular dysfunctions and polymorphisms are evident only in association with particular genetic backgrounds or environmental factors.

The mechanism by which the FSHR haplotype explicates its effect remains to be determined. One of the possible explanations could be that the transcriptional activity of the FSHR promoter is influenced by the SNP present in the promoter's core in the -29 position and in the same way the FSH becomes less efficient in the presence of the Ala307/Ser680 variants and more efficient in presence of the Thr307/Asn680 receptor isoform. Although it has been reported that the polymorphisms of the FSH and FSHR gene may influence the response to therapy, there are no definitive data on the use of a pharmacogenetic approach to identify therapy responders a priori.

Potential therapeutic implications of FSHR

FSHR as a predictive marker of infertility

IVF is a complex process that involves the collection of oocytes after controlled ovarian hyperstimulation, oocyte fertilization,

embryo development and the transfer of embryos produced in utero. All these steps are fundamental to the success of the IVF. However, one of the most critical phases of this complex procedure is precisely the COH, whose objective is to obtain, without risks, a large number of mature oocytes and to allow the selection of embryos suitable for transfer or cryopreservation⁽³⁴⁾. The efficacy and safety of IVF treatments depend substantially on the ovarian response to exogenous FSH. A response to the controlled ovarian stimulation of a normal-responder patient should allow not only adequate ovarian recovery based on the previously assessed ovarian reserve, but also an adequate number of embryos. However, the ovarian response to stimulation with gonadotropins is very variable, a low or high response may occur depending on the patients treated. This heterogeneity may depend on several factors such as patient age, ovarian reserve or endocrine status. Furthermore, the standard dose of gonadotropins may not be suitable for all patients, so using the right dose of gonadotropin could play a very important role in determining the success of COH and then IVF. Based on these considerations, women are generally classified into three groups: high-responders, poor-responders, normal-responders.

Women who are not responsive to stimulation could lead to maturing only a few follicles such that many times they do not respond adequately to treatment. In contrast, women with an ovarian response too high may incur the risk of developing ovarian hyperstimulation syndrome. Optimizing protocols based on individual patient needs would minimize the risk of OHSS and maximize the likelihood of completing IVF programs.

For this reason, the research is turning an increasing interest to the identification of new methods for assessing the state of ovarian reserve of the woman before the beginning of stimulation, and to identify the pharmacological therapy that can guarantee the best outcome of the treatment. Several serum and ultrasound markers were postulated as predictors of ovarian response⁽³⁵⁾. However, these markers seem useful for identifying the appropriate follicle-stimulating hormone dose to be administered but fail to accurately predict the type of response to COH, pregnancy rates or reproductive outcome assisted.

By definition (http://www.fda.gov/cder/genomics/genomic_biomarkers_table.htm), a biomarker is considered valid when it is measured by an analytical test that has well-known yield characteristics and for which there is scientific evidence to demonstrate the physiological, toxicological, pharmacological or clinical significance of the test results. Genetic biomarkers considered “valid” play an important role in the identification of subjects capable or unable to respond to a specific treatment and in the identification of the optimal dose to be administered in order to optimize the efficacy and tolerability of the chosen therapy.

Currently, the AMH level is the most widely used marker, although it has been widely demonstrated that the individual genetic background greatly influences the different responses of women to gonadotrophin stimulation. In fact, it has recently been observed that some genetic variants such as SNPs present in genes encoding LH, estrogen receptors, FSH and the related receptor may affect both the reserve ovarian that the response to COH and, consequently, the IVF outcome.

Although basal serum FSH and/or estradiol concentrations on the third day of the cycle are universally associated with a reduced ovarian reserve and the prospect of low yield in MAR cycles, a significant proportion of hypo-responsive women is not identified by these tests, making the search for new and more sensitive markers crucial. In this regard, FSH remains the most promising factor as responsible for the growth of antral follicles. FSH exists in various isoforms, dissimilar for isoelectric points, for example acidic isoforms appear more secreted during follicular recruitment while the most basic ones appear after the follicle has been selected.

An important role has also FSHR gene that is considered the first candidate to explain changes in ovarian response⁽³⁶⁾.

Numerous studies have evaluated the role of the genetic polymorphisms of FSHR showing that in homozygous ovulatory patients for the Ser680 variant, treated with a fixed dose of FSH to obtain an ovarian response, they show lower estradiol levels than the homozygous patient Asn680 treated with the same dose of FSH. This indicates that a higher dose of FSH is required to obtain an ovarian response in patients homozygous for the Ser680 variant.

For this reason, FSHR polymorphisms could be good predictors for a hypo-response or hyper-response to FSH treatment during ovarian stimulation and FSHR genotype analysis would allow individual modulation of the administration of FSH and therefore to increase the efficacy and safety of the therapy. The most relevant and significant results on the action of polymorphisms in the conditions of female infertility has been obtained by Manuela Simoni studying the ovarian response of patients undergoing COH⁽³⁷⁾. Other scientists have tried to study a possible correlation between allelic variants of the FSHR to other infertility-related disorders, first of all ovarian hyperstimulation, in which it seems that the presence of Ala307Thr variant in homozygous form Ala/Ala induces an increased sensitivity of the receptor which makes the carriers most at risk in the development of OHSS.

A genetic association study on Ala680Ser variant show that this polymorphism, rather than be held as a predictor of risk of incurring OHSS, should be regarded as a marker of the severity of the syndrome. In fact, some studies show that there is a clear and significant correlation between the presence of the variant in homozygous Ser/Ser and the more severe forms of OHSS. Regarding the Polycystic Ovary Syndrome, Endometriosis or ovarian cancer, further studies will be needed to deepen and clarify the role of the genetic polymorphisms of FSHR in these gynaecological syndromes.

While several studies have shown in women the presence of polymorphisms in the FSH-receptor gene that can influence circulating FSH levels and the sensitivity of the hormone receptor, in the male, the meaning of these polymorphisms is still in doubt. The genetic investigation of the variants on the FSHR and FSH genes allows to evaluate a possible treatment of male infertility with the FSH hormone in particular, azoo/oligospermic patients with normal levels of FSH and having at least one serine at position 680 on the gene FSHR (p.Ser680Asn) benefit from the administration of FSH in terms of: number and concentration of spermatozoa, progressive motility and percentage of spermatozoa with normal morphology.

Pharmacogenetics and personalization of MAR therapy

Different subjects respond differently to the same drug; this diversity is linked to the large number of proteins involved in the response to drug therapy.

The variability in the efficacy and tolerability of a drug seems to be, at least in part, the result of the genetic differences of individuals.

Pharmacogenetics aims to study DNA sequence variations that can influence an individual's response to a given drug.

The ultimate goal is to provide the clinician with routine tests to be included in normal clinical practice with the aim of predicting how a patient will respond to a certain therapy both in terms of efficacy and side effects.

The identification, on the basis of a genetic test, of which drug to administer and in what dose would be the solution to a problem both health and socio-economic:

- A safer use of drugs by clinicians, who will be able to choose specific therapies according to the genetic patrimony of the individual patient;
- A research aimed at developing new drugs to be administered to individuals who do not respond or cannot take a specific treatment;
- A more rational use of resources due to the non-use of ineffective drugs and the reduction of hospitalization time due to the side effects linked to the different therapies.

The treatment of infertility is a clear and modern example of personalized medicine, in which the use of the stimulating follicle hormone is widely used for both sexes, although a standardisable approach is still far from being established. The main objective is to obtain the maximum therapeutic result minimizing, at the same time, the risk of potential side effects, including the hyperstimulation syndrome. Given the proven correlation between the amount of FSH necessary to obtain an adequate follicular response, it could be hypothesized to screen the treated patients, so as to provide specific stimulation protocols. In other words, it would be possible to pre-select patients to be treated with higher doses of FSH, with a method that would be more sensitive than the FSH dose in the early follicular phase⁽³⁸⁾. By amplification in PCR of the DNA extracted from the peripheral leucocytes, it is in fact relatively simple to identify polymorphisms of single nucleotides.

Finally, we can also think of the possibility of reducing the incidence and severity of ovarian hyperstimulation syndromes in those patients who, on the contrary, would be negative for screening. The studies of future genetic research will bring about the polymorphisms of FSH and its receptor will be able to broaden the already fascinating panorama of the possible clinical implications that currently exist in the endocrinology of reproduction.

To date, have already been made of genetic testing, can be used in the clinic, which allow the detection of numerous allelic variants, including those of the FSHR, to allow patients who undergo MAR starting a program of stimulation with FSH using a dose that guarantees the success of the therapy.

Despite numerous evidences of the usefulness of pharmacogenetic tests to define an increasingly personalized therapy, the introduction of this discipline in the laboratory routine is still in its infancy.

There are many reasons to explain why pharmacogenetics has a limited impact on clinical practice:

- The studies aimed at evaluating the validity of the identified genetic markers are population-specific and often of small size and not enough statistical power.
- The identified biomarkers, although apparently "valid" (showing that relationship cause-effect between the presence of a specific genetic variation and differences in drug response) are not always significant when compared to individuals from different ethnic groups or in larger series wide.

Pharmacogenetics alone is not sufficient to explain all the inter-individual differences in the efficacy and tolerability of the treatments; environmental factors such as sex, age, organ function, etc., contribute significantly to the success of the therapy. From this it follows that the presence of the same genetic markers in individuals affected by the same pathology and treated with the same drug but with different physiological characteristics, differently influences the response that each of these individuals manifests towards the therapy. The use of genetic tests in the field of infertility in couples has primarily diagnostic purposes but may also allow to formulate predictions on reproductive risk. Therefore, the analysis of the genotype of the FSHR gene allows to individually dose the hormone and thus increase the efficacy and safety of the therapy.

To conclude, the pharmacogenetics in reproductive medicine opens a new and promising era in the concept of personalized medicine of treatment. The known knowledge of the genetic heterogeneity of infertile women can contribute significantly to the design of an appropriate ovarian stimulation protocol for individual patients. At present, the protocols are demitted in accordance with a series of parameters, such as age, body mass index, ovarian reserve and the hormonal profile. The aim of pharmacogenetics is to derive further information from a genetic point of view to allow the right amount of drug to be used in women who respond inappropriately to standard ovarian stimulation protocols. For this purpose, in recent years genetic tests have been developed to which women are subjected before an ovarian stimulation in order to guarantee a greater percentage of success of the therapy.

Ideally, pharmacogenetic studies will project us into a new era in which, on the basis of its own DNA sequence, it will be possible to determine the correct therapy.

The aim is to combine these new genetic tests to the assessment of the parameters traditionally considered (age, AFC, AMH, baseline FSH and patient's history) in order to modulate a therapeutic scheme and obtain the ideal ovarian response that should consist of an optimal balance between high number of oocytes recovered and therefore better clinical outcome and low risk of OHSS.

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HPV infection and ART: the andrologist's point of view

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ABSTRACT

Human papilloma virus infection is a common sexually transmitted disease. In addition to its tropism for epithelial mucosae and skin, HPV can also infect the male urogenital tract and strongly bind the sperm head, causing a reduction of semen quality, with detrimental effects on both natural and assisted fertility. An association between HPV and assisted reproductive technology (ART) programs has been reported, and HPV infection of semen seems to have a negative influence on the outcome of these procedures. None of the standard procedures commonly used for sperm selection before ART showed efficacy in eliminating HPV from the sperm head. Several strategies have been suggested to improve viral clearance in semen of infertile patients, such as counseling and anti-HPV vaccination. Moreover, these strategies were able to increase the natural fertility of couples with HPV semen infection. Finally, a modified swim-up with the addition of an enzymatic treatment, despite reducing sperm motility, was reported to eliminate the virus from the sperm head. In this manuscript we propose a flow chart for the management of infertile couples with HPV semen infection.

KEYWORDS

HPV, HPV semen infection, assisted reproductive techniques, HPV vaccines.

Introduction

Human papilloma virus (HPV) is a term referring to a group of small non-enveloped epitheliotropic viruses with a double-stranded circular DNA genome made up of 8000 bp. The virion of these viruses has an icosahedral shape, with a diameter of 55 nm, and it is constructed of 52 capsomeres, each containing five molecules of the major capsid protein L1, and a smaller number of the minor capsid protein L2^[1]. There are more than 200 HPV genotypes, adapted to particular epithelial tissues, such as anogenital skin and mucosa^[2]. On the basis of its oncogenic potential, HPV can be divided into two different groups: high-risk and low-risk viruses. The former, which include the well-known types 16 and 18, have been classified as oncogenic to humans according to the International Agency for Research on Cancer^[3], and may cause neoplastic transformations in the following epithelial areas: cervical, vulvar, anogenital, penile and oropharyngeal^[4]. The latter, such as types 6 and 11, are responsible for benign diseases such as genital warts^[5]. The morbidity of cutaneous HPV lesions, particularly in immunosuppressed people^[4], also deserves to be mentioned. HPV infections are primarily contracted by direct contact of skin or mucosa with an infected lesion. Genital HPV infection is largely transmitted through sexual intercourse, mostly penetrative, although non-penetrative genital, oral genital, and manual genital types of contact are also possible routes of transmission^[6]. Epidemiologically, there is a clear difference in the prevalence of HPV infection between females and males: while in women the prevalence is high in the first years after they become sexually active and decreases thereafter, in men the prevalence

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remains high throughout life^[7]. A similar difference between females and males can be observed in the prevalence of anti-HPV antibodies: in a 2011 study, Desai *et al.* observed that females had a higher prevalence of antibodies for all the HPV genotypes analyzed^[8].

HPV semen infection

Semen infection is widely accepted, by clinicians and researchers, as a significant etiologic factor of male infertility and it is often associated with poor semen quality even in asymptomatic males. Chronic viral infections of the urogenital tract, especially human immune deficiency virus (HIV) infection, may be significantly associated with chronic low urethral tract inflammation and reduced fertility. Moreover, recent studies have shown that hepatitis B virus and hepatitis C virus semen infection can adversely modify semen parameters^[9].

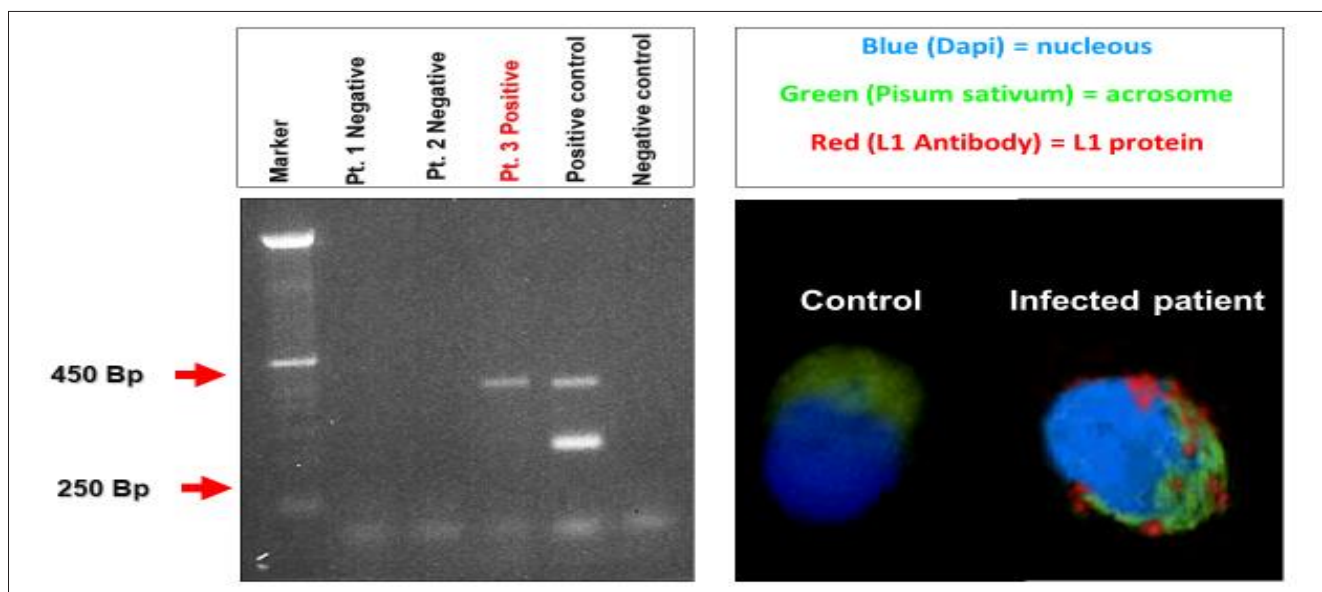
HPV virions can reside not only in the perianal region and external genitalia, including the foreskin, scrotum and glans penis, but also in the urethra, ductus deferens, epididymis, and testis^[10]. Recently, a great deal of interest has been aroused

by the detection of HPV in semen, where it was found both in exfoliated cells and in spermatozoa ^[11]. In a 2019 study, Capra *et al.*, through real-time polymerase chain reactions (PCRs), showed HPV DNA in every fraction of semen: spermatozoa, seminal cells, seminal fluid ^[12]. Figure 1 shows two examples of HPV infection in semen. Part A shows a PCR analysis of non-infected and infected patients. Part B shows the presence of HPV on the sperm surface in the acrosome region.

Because HPV infection in males is usually considered to be transient and without detrimental clinical consequences, its presence in semen has not been adequately investigated. However, some studies, showing a reduction of sperm motility, have suggested that HPV may play a role in male infertility. One of the first clinical studies on this issue showed that in a cohort of 24 patients the incidence of asthenozoospermia (lower total velocity, straight-line velocity, or mean amplitude of lateral head displacement) was significantly higher among patients with HPV 16 and 18 sperm infection than among non-infected controls (75% vs. 8%) ^[13]. In 2010, assessing a cohort of male sperm donors, Foresta *et al.* observed significantly reduced sperm motility in patients with HPV DNA in the semen, independently of the genotype of HPV detected ^[14]. Recently, Moghimi *et al.* observed a significantly higher prevalence of high-risk HPV in infertile men, compared with fertile ones, with the percentage of normal sperm morphology and the sperm motility rate found to be significantly lower in men with HPV infections ^[15]. Furthermore, semen infection has been recognized as a risk factor for the presence of anti-sperm antibodies (ASAs) ^[16,17]. In fact, the prevalence of ASAs seems to be higher in infertile patients than in general population, and their presence is associated with reduced sperm motility ^[18,19]. From these data, HPV semen infection seems to be a significant risk factor for male infertility. In this regard, many studies, reviews and meta-analyses have reported that HPV semen infection is related to reduced fertility both in natural and assisted conceptions ^[20-29]. Although the role of ASAs in reproduction is controversial,

various mechanisms have been proposed, suggesting how they may affect male fertility. These include sperm agglutination, impaired cervical mucus penetration, complement-mediated sperm injury through the female genital tract, and interference with male-female gamete interaction ^[30]. As several kinds of semen infection have been associated with the development of ASAs, different authors have tried to establish whether HPV infection is associated with their presence. In 2013, Garolla *et al.* studied the possible association between the presence of HPV and ASAs and their clearance time in the semen of infected and non-infected infertile patients and in fertile control subjects. They showed that more than 40% of the infected infertile patients had ASAs on the sperm surface. In contrast, sperm auto-immunity was significantly lower in non-infected infertile men and in fertile control subjects. Moreover, infected patients had a higher mean percentage of ASAs compared with non-infected ones. These findings suggested that the presence of HPV DNA on the sperm surface may promote an antigenic stimulus for ASA formation; the authors documented the presence of both viral proteins and immunoglobulins in the same sperm cells of samples with positive sperm mixed antiglobulin reaction test results, and thus confirmed that sperm autoimmunity was HPV dependent. Notably, when immunofluorescence for HPV 16-L1 was present on the sperm surface, they observed co-staining for IgA and IgG. This observation suggests that semen infection could represent a new clinical condition associated with the presence of ASAs. Finally, in infected males, significant viral clearance (approximately 85.3%) was obtained after 24 months of follow up. Interestingly the reduction in sperm infection paralleled the disappearance of ASAs and was significantly related to a progressive improvement in sperm motility ^[31]. Some authors also reported the presence of HPV sperm infection in cryovials from sperm cryobanks ^[25,32,33]. Because these sperm were cryopreserved for future use in ART treatments, the authors/these findings raised concerns about a possible detrimental effect of HPV infection on fertilization

Figure 1 On the left: PCR analysis for HPV DNA performed in semen samples from infertile patients. Patient 3 showed positivity for HPV DNA. On the right: Immunofluorescence at confocal microscope showing an infected sperm with HPV located at acrosome level and a negative control.



and embryo development. In a 2010 study, the prevalence of HPV semen infection was investigated in cryobank cryovials of sperm from two different groups: a group of 98 patients affected by testicular cancer and a group of 60 healthy controls [33]. The percentage of samples with HPV was not significantly different between the patients and controls but, in contrast, the percentage of infected sperm was significantly higher in the patients. Further studies demonstrated that use of infected sperm for ART techniques was associated with a higher rate of treatment failures. Perino et al. reported that, among couples who underwent ART, the abortion rate was higher in HPV-infected couples than in non-infected ones. Furthermore, the risk was higher when the male partner was infected rather than the female [34]. Recently, Depuydt *et al.*, studying the infection in semen samples (sperm donor samples used for ART) from three different cryobanks, observed that no pregnancies were obtained from infected semen samples [25].

On the basis of these data, several authors have suggested that serious consideration should be given to the possibility of screening semen samples for HPV before sperm banking or before their use in ICSI procedures [25,33].

THE mechanism of HPV-sperm binding

From the first studies, it was clear that HPV, unlike what happens when it infects epithelial cells, does not enter sperm cells. No study has shown an increased number of aneuploidies in sperm cells of infected patients [31]. However, some authors have reported an increase in the sperm DNA alteration index [28], while others did not observe this alteration [35].

The exact localization of HPV in sperm was clarified by *in vitro* immunofluorescence. To study the interaction between HPV and sperm cells, HPV type 16 capsids were used. It was shown that interaction of the virus with sperm could be reduced by two HPV infection inhibitors, heparin and carrageenan [36]. These findings were confirmed by others who performed fluorescent *in situ* hybridization (FISH) of infected semen samples from young adult men in order to study HPV DNA adhesion to the sperm cell surface [11]. To better understand the precise localization of HPV in the sperm head, immunofluorescence analysis was also performed using an antibody against the capsid protein L1 in intact sperm, which underwent an acrosome reaction. The signal for L1 was confirmed to be localized exclusively in the sperm head, especially in the equatorial region. After an acrosome reaction, the signal persisted in this region, thus excluding the presence of HPV in the acrosome and suggesting a preferential localization of HPV in the equatorial region. In the same study, a cytometry flow and an immunofluorescence analysis made it possible to demonstrate that binding between HPV and sperm cells involves the L1 capsid protein and the glycosamino-glycan Syndecan-I, expressed in the equatorial region of the sperm head. Moreover, PCR analysis of L1 gene expression confirmed infection of sperm by HPV DNA. Taken together, these data showed that sperm can be infected by HPV and that both HPV DNA and L1 capsid protein are bound to the sperm head, almost exclusively in the equatorial region [37].

In the literature, there is little evidence concerning the possibility that routine sperm washing techniques, used to select sperm before assisted reproduction, can eliminate HPV DNA from semen. On this basis, Foresta et al. evaluated viral clearance in infected sperm from infertile subjects treated with three different standard sperm selection procedures: sperm washing by centrifugation, discontinuous Ficoll-Paque Plus density gradients, and direct swim-up [38]. After sperm washing procedures, HPV detection, performed by *in situ* hybridization analysis, showed that the percentage of infected sperm remained unmodified. Using Ficoll gradients and swim-up procedures, there was a significant reduction in the percentage of infected sperm. However, despite the more efficient clearance obtained by swim-up, complete removal of infected sperm was recorded in only 19% of cases. These results suggest both the presence of very strong binding between HPV and sperm, and low efficacy of conventional sperm selection techniques in eliminating infected sperm.

Infected sperm and ART

While data are available on HIV, no precise data are available concerning the use of HPV-infected sperm in ART procedures. Moreover, little is known about whether infected cells used in ICSI procedures may interfere with the mechanisms of fertilization, implantation and embryo development. The literature is still insufficient to allow final conclusions to be drawn regarding the effect of HPV infection on the most important reproductive outcomes following natural and assisted fertility in women. However, it seems that when the infection is present in the male partner there is a negative effect on the ongoing pregnancy rate and live birth rate, as well as an increase in the rate of miscarriage [34]. Taken together, these findings should be further investigated in order to understand the real impact of HPV semen infection on fertility outcome. Interestingly, the presence of the virus has been associated with increased rates of wastage and miscarriage in both animals and humans, linked to transformation of cells, gene interruption and loss of chromosome heterozygosity or fragmentation of host DNA fragmentation and apoptosis

In vitro studies

Many *in vitro* studies have shown a negative influence of HPV infection on several aspects of human fertility. For example, HPV transfected blastocyst and trophoblastic cells showed a reduction in decidual invasion capacity, potentially responsible for failure of the trophoblastic cell to invade the maternal uterine wall, subsequent placental dysfunction, and adverse pregnancy outcome [39]. Furthermore, several experimental studies have demonstrated that HPV contributes to pregnancy loss by transmitting viral genes to oocytes and determining DNA fragmentation and apoptosis of embryonic cells. The effects on embryo development seem to be stage related: from cellular instability at the two-cell level, through a decrease in blastocyst formation, to inhibition of the blastocyst hatching process [40,41]. However, there is little evidence to support the suggestion that HPV-infected sperm are able to interfere with embryo develop-

ment when injected into the oocyte cytoplasm (such as during *in vitro* fertilization). To better understand this process, Foresta *et al.* performed an *in vitro* study evaluating the ability of a virus-infected sperm to transfer HPV DNA and capsid proteins to the oocyte during fertilization. After transfecting a human sperm with a plasmidic episome containing HPV E6 and E7 proteins, they used the hamster egg-human sperm penetration test to show the ability of infected sperm to transfer the capsid protein L1 to the oocyte, and the expression of E6 and E7 viral protein in the fertilized oocyte^[37]. Their findings demonstrated that both sperm transfected with E6 and E7 genes and sperm exposed to HPV L1 capsid protein are able to penetrate the oocyte. This laboratory data, combined with the observation that HPV DNA is detected in a larger proportion of spontaneous abortions than voluntary terminations of pregnancy^[34], may suggest an active role for HPV (which is carried to the egg by the sperm) in preterm gestation. This phenomenon could lead to an increase in the fragmentation of embryonic DNA resulting in alteration and apoptosis of the embryo^[37]. It should be remembered, however, that the situations described above refer to *in vitro* models that may not reflect the *in vivo* situation, where the entry of viral DNA into the egg has never been proven^[31].

In vivo studies

In experimental murine models it was found that HPV genomes are expressed in fertilized oocyte, blastocyst and trophoblastic cells. The viral genome was frequently associated with cellular changes such as inhibition of the blastocyst hatching process, and DNA fragmentation and apoptosis, often with lethal effects for early embryo development^[42]. These data have been confirmed by further studies, in which authors highlighted that the detrimental effects of HPV on embryo development are stage specific: early exposure of embryos to HPV was linked to death of the two-cell embryo, while delaying exposure, to the late embryo stages, seemed to permit embryo development^[40]. In addition, as reported before, Depuydt *et al.*, studying the infection in semen samples (provided by sperm donors, for use in ART procedures) from three different cryobanks, observed that no pregnancies were obtained from infected semen samples^[25].

Treatment of infertile infected patients

It is well known that HPV infections are frequently long lasting, and it has been suggested that certain anatomical sites could act as viral reservoirs able to support persistence of the infection. Moreover, since there is still no effective and resolute treatment for HPV infection and its related problems, including the consequences of HPV infection on fertility, it is mandatory, pending the development of effective prevention and therapy solutions, to educate and counsel infected couples, especially when they are seeking fertility treatments and ART procedures.

Counseling

It is important to educate and provide careful counseling to couples where at least one partner is infected with HPV. A

2014 study clearly showed the effectiveness of this strategy. In fact, couples in this situation who had carefully followed advice given to them during the first counseling session showed a significant reduction in the persistence of the infection. More specifically, partners were advised to: pay particular attention to hygiene of both of their reproductive tract and their hands; use only their own underwear and towels; avoid oral and anal sex; reduce or quit smoking; have only protected intercourse; and, in the case of HPV-related lesions, treat them and monitor the genital area over time. Couples who complied strictly with the multiple suggestions made during the first counseling session, when monitored at 6, 12, 18 and 24 months, showed significantly reduced viral persistence^[43].

Vaccination

HPV vaccines are produced using recombinant DNA technology. They are prepared from purified L1 structural proteins that self-assemble to form HPV-like empty shells, termed virus-like particles, which mimic the native HPV external coat. Since they are hollow and do not contain viral DNA, they have the potential to elicit an immune response without the ability to cause infection. This makes them completely safe even for immunosuppressed people. Vaccination is recommended before the start of sexual activity, and national immunization programs often target 11-12-year-olds.

Recent evidence suggests that vaccination is a valid tool even in patients who have already contracted the infection^[44]. In particular, adjuvant vaccination in adult males with persistent HPV semen infection is able to reduce the average elimination time of the virus, thus accelerating the healing time. Moreover, it has been shown that patients with semen infection receiving vaccination have a faster rate of seroconversion and greater viral clearance, identified as the percentage of HPV DNA detectable in seminal fluid^[45]. In the study in question, the authors evaluated three groups of patients with HPV semen infection: patients without anti-HPV antibodies (seronegative) at recruitment, who were candidates for vaccination (vaccine arm), patients seronegative at recruitment but not candidates for vaccination (seronegative control arm), and patients who were seroconverted at recruitment (seroconverted control arm). During the two-year follow up, vaccinated patients showed significantly faster healing of disease paralleled by a reduction in the percentage of HPV-positive sperm in the semen and massive development of high-titer seroconversion. Interestingly, a group of 30 patients displaying seroconversion at the start of the study, despite having a much lower serum antibody titer, also showed some improvement of humoral immunity in the anti-HPV response. In view of the aforementioned effects of HPV on oocyte fertilization and embryo development, these data were highly suggestive of a possible impact of male vaccination on both natural and assisted fertility. This issue was addressed in a more recent study in 2018, where 151 infertile couples were retrospectively selected according to detection of HPV DNA in the male partner's semen. In this study, 79 males received the quadrivalent vaccine as off-label vaccine. The remaining male partners from 71 couples refused to receive vaccination and served as controls. Over a one-year follow up, variations in sperm parameters and data on natural

fertility were recorded in both vaccinated and control patients. Compared with the latter, the vaccinated patients showed faster viral clearance paralleled by a progressive improvement of sperm motility and a reduction in the percentage of anti-sperm antibodies. Furthermore, the couples where the male partner received vaccination recorded, compared with controls during the one-year follow-up, a higher number of pregnancies and deliveries associated with a lower miscarriage rate [46].

To assess and clarify any prognostic factor able to predict the effectiveness of the HPV vaccine in promoting viral clearance from the male genital tract, De Toni et al. performed a retrospective cohort study, collecting clinical records from 379 male patients with persistent seminal HPV detection who received therapeutic HPV vaccination. They recorded i) genital HPV-DNA assessment by INNO-LiPA genotyping; ii) semen HPV-DNA analysis using the FISH method; iii) serum antibody titer. All the data were collected before vaccine administration (T0) and 6 months after the vaccination cycle ended (T1). The authors observed complete clearance of genital HPV DNA in 326 (86%) patients and assessed that serum HPV-antibody titer at T1 was the most important prognostic factor associated with HPV DNA clearance, suggesting that anti-HPV antibodies represent a suitable marker of adequate immune response to HPV vaccination in patients with genital infection, and, therefore, of healing [47].

Taken together, these results represent a rationale for a significant improvement in the management of infertile men with risk factors for semen infection by HPV.

Modified swim-up

Recently, studies investigating the role of HPV infection in infertile couples undergoing ART procedures showed a correlation between HPV sperm infection and increased risk of pregnancy loss after ART [23,34]. Considering the high prevalence of HPV semen infection in infertile patients and the absent or poor effect of standard semen selection procedures (centrifuga-

tion, discontinuous density gradient and direct swim-up) in eliminating HPV from sperm, it is of paramount importance to find new techniques to achieve sperm negativization from HPV infection. In an attempt at eliminating HPV from infected sperm samples, a recent study tested a modified swim-up with addition of the enzyme heparinase-III [48]. The rationale for this treatment was to cleave the binding between HPV and its putative ligand, Syndecan-I, on the sperm surface. Compared with the normal swim-up technique, the modified version was able to abolish binding between HPV and sperm in 100% of cases of infected sperm (as shown in Figure 2). This was confirmed by FISH, a technique allowing detection of the HPV virion bound to the sperm head surface [11].

Nonetheless, compared with the state before the treatment, reductions in viability and motility were shown in the sperm of patients who underwent modified swim-up procedures. Even though this reduction was not associated with impaired DNA integrity, potentially allowing the use of selected sperm in ART, the use of heparinase III is not currently approved for use in ART procedures.

Another study used the same swim-up technique modified with another enzyme, hyaluronidase, which is already approved for some ARTs. Given its mechanism, able to cleave the linkages between N-acetylglucosamine and glucuronate, the authors hypothesized that the enzyme might be able to break the linkage between HPV virions and syndecan-like GAG moieties on the sperm surface. In this study the authors collected semen samples from, respectively, five normo-zoospermic fertile volunteers (control subjects), five infertile oligo-astheno-teratoospermic patients (both these two groups with negative detection of HPV-DNA in semen), and 12 infertile patients with HPV DNA detected in semen. Semen samples from the first two groups were incubated with HPV VLPs from the quadrivalent HPV vaccine. The authors investigated the effect of the enzyme at different concentrations both on sperm incubated with HPV VLPs and on real samples from infertile patients with positive

Figure 2 HPV infected semen sample before and after treatment with Heparinase III.

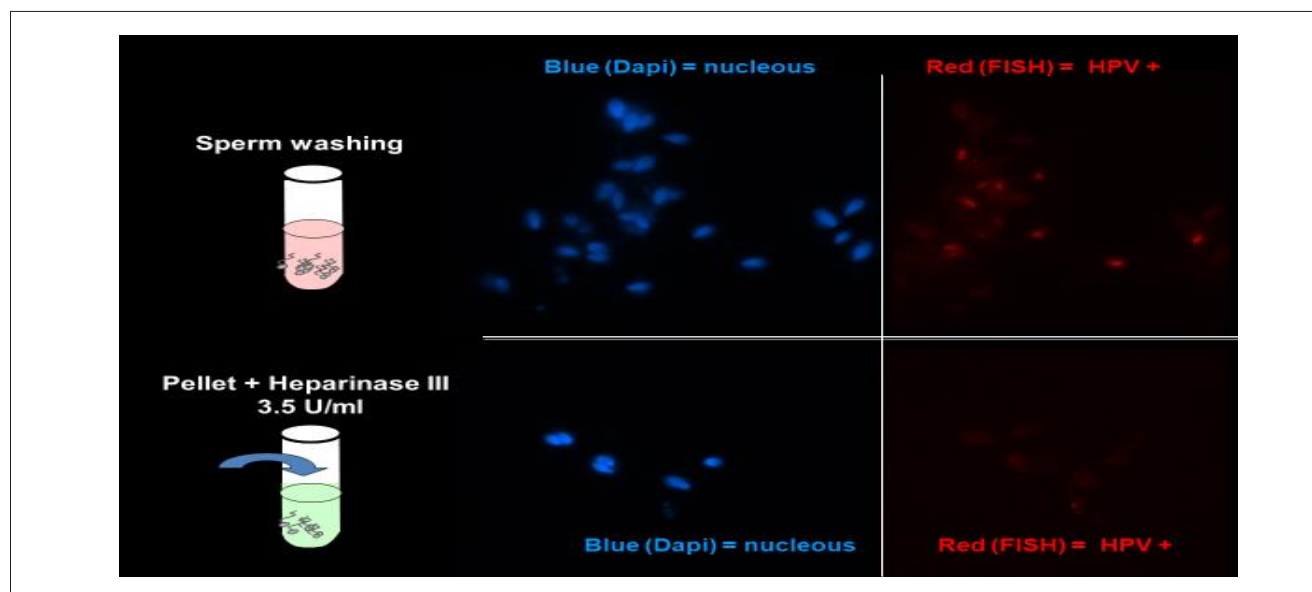
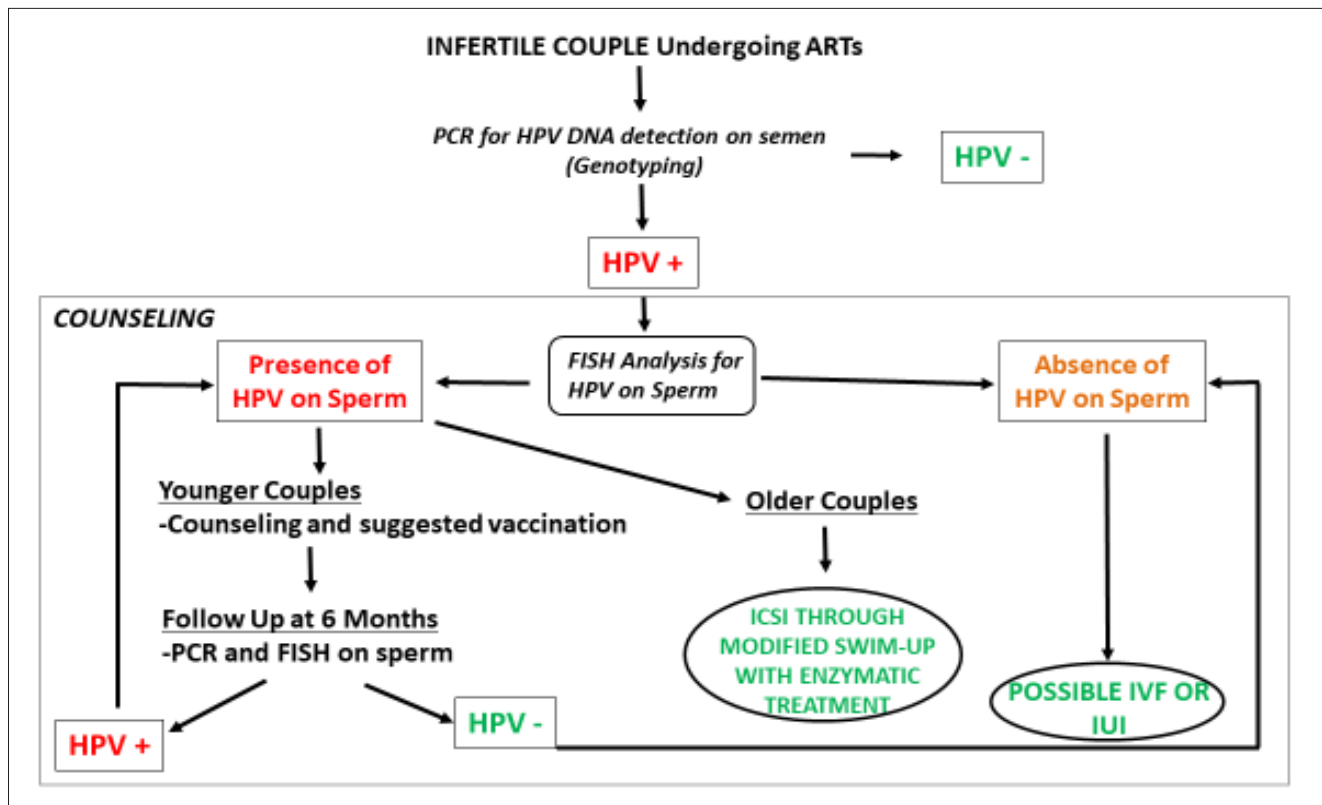


Figure 3 Flow chart for the management of infertile couples undergoing ART based on the presence or absence of HPV semen infection (HPV: Human Papillomavirus; PCR: Polymerase chain reaction; ICSI: Intra cytoplasmic sperm injection; FISH: Fluorescent in situ hybridization; ARTs: Assisted reproductive technologies)



semen detection of HPV DNA. Total removal of virions was observed at the highest concentration of the enzyme (80 UI/ml). Although this treatment, too, led to significant alteration of sperm motility, this effect was not sufficient to compromise use of the spermatozoa in ART. In support of this consideration, no alterations of sperm morphology, DNA integrity or markers of capacitation and acrosome reaction were observed.

New experiments, aimed at testing other enzymes capable of removing HPV from the sperm surface without detrimental effects on sperm quality, are in the trial phase in our lab ^[49].

Conclusions

Because HPV sperm infection may be a major cause of male infertility and seems to play an important role in spontaneous abortions, semen samples from patients undergoing ART should be always tested for the presence of HPV. Figure 3 shows a flow chart summarizing the main steps in the management and treatment of infertile couples undergoing ART in the presence of HPV semen infection.

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Report on viral infections in ART

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ABSTRACT

Human immunodeficiency virus (HIV), hepatitis B virus (HBV) and hepatitis C virus (HCV) are widespread infectious agents. In developed countries, effective therapies have turned potentially lethal HCV, HBV and HIV infections into chronic diseases with a good quality of life, also in terms of reproductive chances. Serodiscordant status is the condition in which only one partner is HIV, HBV and/or HCV positive. In fertile serodiscordant couples, natural conception should be encouraged following specific prophylactic strategies, while infertile serodiscordant couples should be referred for treatment performed using assisted reproduction technology (ART). ART centers working with serodiscordant couples must set up an internal management and control system to guarantee the safety of operators, uninfected patients, gametes and embryos, and also of the fetus, especially during the cryopreservation step.

Herein we summarize general features of HIV, HBV and HCV infection, their possible impact on human reproduction, and laboratory recommendations to achieve safe management of serodiscordant couples seeking pregnancy through ART.

KEYWORDS

Viral infections, HBV, HCV, HIV, contamination.

Introduction

Human immunodeficiency virus (HIV), *hepatitis B* virus (HBV) and *hepatitis C* virus (HCV) are widespread infectious agents. In Italy, over one million people are estimated to be infected by HCV, 332,000 by HBV, and almost 4,000 by HIV. Nevertheless, anti-viral therapies have turned potentially lethal HIV, HBV and HCV infections into chronic diseases with a good quality of life, also in terms of reproductive chances. Serodiscordant status is the condition in which only one of the partners in a couple is positive to one or more of these viruses. Serodiscordant status has social and psychological implications; in particular, it limits sexuality and can undermine the couple's welfare, causing feelings of bewilderment, fear, guilt, responsibility, and inadequacy, and leading to breakdowns in communication. Serodiscordant couples therefore need psychological support, especially after the discovery of the infection^[1]. Fortunately, fertile serodiscordant couples can nowadays seek a pregnancy by natural conception thanks to several therapeutic strategies for both the infected partner and the non-infected one^[2,3]. Infertile serodiscordant couples should instead be offered treatment involving assisted reproduction technology (ART). Even though serodiscordant status is not a discriminatory factor for access to infertility treatments, specific procedures are required to protect operators, healthy patients, and the future fetus. A higher risk of cross-contamination and infection may be present during cryopreservation and gamete donation, as suggested by literature data reporting the transmission of viruses and other microorganisms via liquid nitrogen^[4-6]. For this reason, screening and suitable operating procedures are mandatory in order to avoid contamination between cryopreserved

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samples and safeguard the health of patients and the future unborn child.

In this report we summarize recommendations, outcomes, and good laboratory practices allowing safe management of serodiscordant couples seeking pregnancy by ART.

HCV

HCV is a blood-borne RNA virus responsible for hepatitis C. It is transmitted primarily through parenteral exposure (blood products, shared needles, needle stick injuries), although it may also be found in other biological fluids such as saliva, urine, semen, vaginal secretions, and breast milk. Sexual and vertical transmission are considered secondary modes of transmission^[7]. HCV infects over 1% of the world's population, with intravenous drug users, hemophiliacs, homosexual men, sex workers and sexual partners of HCV-infected subjects being the categories at highest risk^[8]. The virus is highly pathogenic: 80% of infected patients develop chronic liver disease, 35% develop cirrhosis, and 5% of the 35% will progress to hepatocellular carcinoma^[9]. There is no vaccine available.

In the ART field, HCV can be transmitted both to operators

and to healthy patients, for example via semen. Good management of HCV serodiscordant couples should begin with counseling aimed at informing the infected partner about the risk of infecting their healthy partner, members of the healthcare team, and the fetus. To prevent HCV infection during ART, sperm washing is strongly recommended in order to reduce the viral load in the semen sample [10]. Moreover, in serodiscordant couples with male infection, employment of elective intracytoplasmic sperm injection (ICSI) is recommended even though conventional IVF has also been demonstrated to reduce the risk of HCV transmission [11].

Data on the impact of HCV on ART outcomes are discordant. Several studies suggest an adverse effect of viral infection on sperm parameters, hormone levels, and pregnancy rates [11-14], while others show no differences in ART results between serodiscordant couples and negative ones [15-17]. Other studies investigating ART outcomes in serodiscordant couples with an HCV-positive female partner suggest that the virus may affect ovarian response to stimulation, fertilization, implantation, and pregnancy rates; however, data are contradictory [18,19].

HBV

HBV is a double-stranded DNA virus responsible for hepatitis B, a major cause of acute and chronic hepatitis, cirrhosis, and hepatocellular cancer. With about 350 million infected people worldwide, HBV is one of the world's most common infectious diseases. The virus can be parenterally, sexually and vertically transmitted. Moreover, HBV can be transmitted via mucosal exposure (blood, semen, vaginal secretions, and saliva) and during ART [20-22]. Several efficient strategies for prevention of HBV transmission are today available. The HBV vaccine, introduced in 1982, is the most important method of preventing the spread of HBV, both in the general population and among healthcare workers [22]. Good evidence is available supporting the administration, within 12 hours of birth, of HBV immunoglobulins and vaccine in infants born to HBsAg-positive patients, and an appropriate immunoprophylaxis allows breastfeeding of babies born to infected patients [11-23].

In the safe management of serodiscordant couples seeking pregnancy by ART, any treatment should be started only after vaccination of the negative partner, documented by the presence of anti-hepatitis B surface antibodies [1]. Sperm washing is not required if the female partner is immunized against HBV.

HBV infection in the ART setting is an important issue not only for the risk of transmission, but also for the possible effects on gamete competence. Several reports suggest an association between the presence of this virus and a poor reproductive prognosis both in natural and in assisted conception due to increased chromosomal instability in sperm, even without worsening of semen parameters. Sperm progressive motility and fertilization rate both seem to be significantly lower in patients affected by HBV. For these reasons, in serodiscordant couples with an HBV-positive male partner, a mixed (half ICSI, half IVF) approach could be proposed in the first ART cycle, with the aim of evaluating the possible impact of the virus on sperm competence and HBV could be also detected in the oo-

cytes or ovarian tissue. The impact of HBV on ART outcomes such as embryo development, implantation rate, and pregnancy rate are currently under investigation [24-27].

HIV

HIV is a retrovirus that depletes CD4+ T cells and is responsible for *acquired immune deficiency syndrome* (AIDS). HIV is characterized by a single-stranded RNA and by the presence of reverse transcriptase, an enzyme that allows RNA to be copied into DNA. HIV in fact uses a DNA "copy" to infect the host. The first step in the infection is fusion of HIV with the host cell. Then, the viral RNA is converted into DNA and the virus uses the host cell's machinery to replicate itself by reverse transcription. New copies of HIV leave the host cell and go on to infect other cells. There are nearly 37 million people living with HIV/AIDS around the world, the vast majority in developing countries, and more than half are women. Two-thirds of all people newly infected with HIV live in Sub-Saharan African countries, where three-quarters of all AIDS-linked deaths occur [28,29]. HIV is mainly transmitted via unprotected sexual intercourse and facilitated by infections and ulceration of the genital tract, trauma or bleeding during sexual practices, and absence of male circumcision [30]. HIV transmission is directly associated with peripheral blood viral load, with a lower risk in subjects with peripheral viral loads <10,000 copies/mL [31]. Interestingly, the seminal HIV viral load roughly correlates with the plasma one, but is much more variable and isolable. It should also be stressed that infected men with low or undetectable viral loads could have HIV copies in their seminal plasma [32-34].

In the ART field, several women were infected with HIV after donor insemination between 1980 and 1984, before strict donor screening and semen quarantine practices were implemented. Moreover, a significant reduction of the infection has been documented following sperm-wash via density gradient centrifugation and swim-up allowing separation of motile sperm from round cells and seminal plasma [35,36]. In the safe management of HIV serodiscordant couples with a positive male partner, there is good evidence supporting the efficacy of sperm-wash in significantly reducing the risk of infection. In addition, the non-infected partner must be checked periodically for HIV during the treatment and during any resulting pregnancy. Moreover, the storing of HIV-infected samples (i.e., semen, oocytes and embryos) in specially designed tanks is now a widespread practice, making it possible to significantly reduce the risk of viral transmission via liquid nitrogen. Finally, vertical transmission can be reduced thanks to the use of antiretroviral drugs during pregnancy and/or labor, elective cesarean section, and avoidance of breastfeeding. These measures can reduce the risk of HIV transmission to less than 2% [37-39].

Discussion

In developed countries, effective therapies have allowed lethal infections like HCV, HBV and HIV to be turned into chronic diseases associated with good life expectations, also

in terms of reproductive chances. For fertile serodiscordant couples, natural conception following specific prophylactic strategies should be encouraged, while infertile serodiscordant couples should be offered treatment with ART. In recent years, serodiscordant couples have increasingly turned to ART centers. Their desire for parenthood is identical to that of any other couple, even though their management must involve specific counseling and recommendations. Counseling is crucial to inform patients about the risk of sexual and vertical transmission of the infection; it should include information on safe sexual practices, such as the use of condoms during the infertility treatment and the pre- and post-pregnancy period, as well as guidance on appropriate lifestyle choices. Moreover, in serodiscordant couples, serial diagnostic testing of the uninfected partner is important, also during the first year following the child's birth. A clear and explicit informed consent process is essential, in which it must be made clear that the risk of transmission cannot be entirely eliminated, even when all the necessary precautions are taken and the best risk reduction strategies are implemented. Appropriate psychological, medical and obstetric assistance should ideally be provided by a multidisciplinary medical team, for the protection of patients, and also of the future unborn child.

In the management of serodiscordant couples seeking pregnancy by ART, is important to remember that a risk of viral contamination has been documented in ART clinics and blood banks. Even though this risk can be reduced, it cannot be entirely eliminated. For this reason, ART centers working with serodiscordant couples must set up an internal management and control system designed to guarantee the safety of operators and uninfected patients, and also of the fetus. Correct and safe management of serodiscordant couples should follow recommendations, such as the treatment of infected samples in a separate laboratory or in a designated space within the main laboratory to minimize the risk of cross-contamination. The risk of cross-contamination during ART is probably greatest during the cryopreservation step because viruses and other microorganisms can survive in liquid nitrogen. In order to protect the cryopreserved samples against the risk of cross-contamination, the use of separate tanks and sterile nitrogen is strongly recommended. Other possible strategies to avoid cross-contamination via liquid nitrogen are the use of "closed" freezing devices or the use of "double enveloping". Another possibility is to store cryopreserved samples in nitrogen vapor, although the literature is conflicting with regard to this approach as it has been demonstrated that contaminants persist in the vapor phase^[40-45].

An important aspect of ART in serodiscordant couples, recently discussed by Borges *et al.*^[46], is the cost of microbial contamination during infertility treatment. Although data on the economic impact of microbial contamination in the global ART industry are scarce, this impact is found to be considerable when estimated on the basis of the prevalence of contamination (about 0.7%, according to literature), the number of IVF cycles per year, and the cost of the procedure. For instance, of the approximately 285,000 IVF cycles performed in the USA in 2017, nearly 2000 cycles presented microbial contamination and a decline in cell quality, without successful embryo transplantation in most cases. This translates into a mean cost per

patient of US\$ 10,000 (according to current US market prices) and an overall cost of about US\$ 20 million per year, excluding the ancillary costs linked to couples who do not achieve pregnancy due to potentially undetectable contamination of embryo cultures. Similarly, in 2017, over 75,000 cycles of IVF were carried out in 119 licensed ART clinics across the UK where the cost of IVF is around £5000 (US\$ 6500) per cycle of treatment. Applying the same parameters used to estimate the cost of microbial contamination in the USA, the overall cost in the UK corresponded to about US\$ 3.4 million. In Brazil, each IVF cycle costs, on average, R\$ 15,000 (US\$ 4000). On the basis of the 36,370 IVF cycles reported in 2017 and the estimated 4.8% rate of microbial contamination reported in national registers, the total annual cost of microbiological contamination in ART laboratories and clinics reaches nearly R\$ 26.1 million (US\$7 million), a non-negligible cost for IVF clinics and patients.

Of greater concern, IVF failure has the potential to be an emotionally and financially exhausting experience for patients. This has enormous implications for couples of increasingly higher age, when compared to the mean age of the women undergoing ART procedures. Furthermore, longitudinal studies should be carried out to identify possible adverse effects of the presence of microorganisms in contact with embryos conceived *in vitro*, and the possible implications for adult life. Meanwhile, there is also a need to discuss new ways to improve or modify the preparation and cryopreservation of gametes. These discussions should be developed with a view to improving the safety of ART by reducing the impacts, both subtle and overt, of contamination in the embryo culture and cryopreservation steps.

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Cross-contamination risk management in biobanking. Lesson from the pandemic

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ABSTRACT

Millions of human gametes are stored worldwide. Cryostorage presents many potential risks to the cryopreserved cells/tissues, including loss of viability and most of all contamination. Assisted reproductive technology (ART) clinics need to develop effective strategies to manage these risks. Human ART is the only medical discipline where human gametes and embryos with the potential to produce live births are routinely stored in liquid nitrogen or nitrogen vapor (LN₂/NV).

The risk of infection is real, especially when new, highly infectious agents arise. Airborne contaminants come into contact with LN₂/NV and are cryopreserved. While some industrial sectors (drug manufacturing, food and beverage sterile packaging) carry out raw filtration of LN₂ before use, this precaution is not yet mainstream, although it is increasingly used in the field of human ART. During the COVID-19 pandemic, manufacturers of drugs/disposables/culture media tried to mitigate contamination risk by sourcing raw materials from low-risk regions, but this approach is not feasible in the case of LN₂/NV, which can thus become potential vectors of contamination. The risks associated with the use of contaminated LN₂/NV are virus awakening and contamination of thawing cells, the environment, and operators.

KEYWORDS

Cryopreservation, cryostorage, sterile liquid nitrogen, risk management, contamination, personalized virus-free vitrification.

Cryostorage-associated risks

Cryopreservation of gametes and embryos has now become mainstream in assisted reproduction (AR). The exponentially growing inventory of cryopreserved cells around the world has many implications. On the practical side, the primary concerns are related to the safety and longevity of the cells while in storage.

Duration of storage

Recent studies, including those focusing on oocytes subjected to slow freezing, support the conclusion that long-term cryostorage is generally safe [1–4]. However, the shift in the past decade from slow freezing to vitrification necessitates studies focusing on vitrified cells specifically. Vitrified cells are suspended in minute volumes of cryoprotectant (less than 1 microliter) and for this reason they are more sensitive to temperature shocks because they are not protected by the ice layer typical of slow freezing. Furthermore, the majority of oocytes and embryos are vitrified with open carriers, which exposes the cells to potential contamination. It has been demonstrated that cryostorage of vitrified oocytes for up to 3.5 years has no effect on the chromosomal integrity of the resulting embryos [4] and that cryostorage of vitrified blastocysts in open devices (Cryotop) for up to 8 years does not impact clinical and neonatal outcomes [5]. Furthermore, cryostorage duration has no impact on the viability of biopsied blastocysts [6]. Thus, current evidence does not suggest that duration of cryostorage, per se, threatens

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oocyte or embryo integrity or viability. However, the conditions under which the cells are stored and handled over time can undoubtedly influence these outcomes.

Handling and transportation

Vitrified cells are highly sensitive to temperature shocks. Temperature during handling, shipping and storage should not rise above a safe threshold of –180°C even though glass transition temperature is approximately –130°C. Despite the predominant use of liquid nitrogen (LN₂) for storage, shipment of cells/tissue is generally performed with tanks or dry shippers that use nitrogen vapor (NV). Failure of these shippers is not uncommon according to formal and informal surveys [7]. However, risks associated with handling and transport of vitrified human eggs and embryos have scarcely been investigated through experimentation [8]. McDonald *et al.* [9] showed that the survival rate of vitrified shipped oocytes was lower than that of their non-shipped counterparts or shipped slow-frozen oocytes. They speculated that this effect was likely due to one of many abnormal conditions during shipping, including vessels being

exposed to elevated temperatures and air pressure, vibration/ other physical shocks, and horizontal storage.

In a study by Parmegiani *et al.* [10], shipping temperature and type of transportation appeared to impact survival of vitrified oocytes transported from Spain to Italy. It was the experience of these investigators that road courier was the safest means of transportation; in their case, the shippers were filled with LN₂ rather than “charged” with NV, although this is not necessarily common practice globally. According to the data logger, shippers maintained the temperature below −196°C for the duration of the 24-hour journey. During transport by air (where LN₂ onboard is not permitted due to its hazardous material designation), despite the shipper temperature being recorded as below the safe threshold of −180°C in both cargo and cabin baggage, both locations seemed to negatively affect oocyte survival rate, which decreased by up to 20% compared with that of oocytes transported by road.

Shipment of cryopreserved samples is complex, comprising multiple critical steps. What can be expected is that during transfer between storage vessels, moving from liquid to vapor phase of nitrogen and vice versa, temperature and humidity can change in uncontrolled ways, and these fluctuations may be harmful to vitrified oocytes. Devitrification and recrystallization of intracellular water can occur and have been shown to lead to cryoinjury during warming of vitrified samples [11].

Multiple handling steps are involved in the shipment of samples from one laboratory to another. It is not yet known how temperature fluctuations during shipment and handling may affect cell survival and quality.

Cross-contamination

During both cryopreservation and storage, particular attention should be paid to avoidance of bacterial or viral contamination. Depending on the storage vessel type, both LN₂ and NV may be used for cryostorage of human gametes and embryos. Storage in either phase can be potentially hazardous because some pathogens can survive at cryogenic temperatures and may contaminate the frozen cells or surfaces of the cryostorage containers [12]. To date, there have been no cases of disease transmission via transferred cryopreserved human embryos [13], but microorganisms at any stage during culture and transfer of embryos can ultimately reduce the chances of a successful pregnancy [14]. Vitrification, in conjunction with so-called open devices, requires direct exposure of the sample to LN₂, and this exposure poses additional contamination risks [15]. However, the hypothetical risk of culture contamination at warming cannot be excluded even when using some “closed” vitrification systems [16].

Precautions

Some precautions can be routinely taken in AR laboratories to minimize the risk of cross-contamination during cryopreservation. For example, cryostorage in hermetically sealed containers and the use of a secondary sleeve (straw-in-straw) has

been recommended for both vitrified and slow-frozen cells [17]. Another step that can be taken is sterilization of LN₂ to prevent contamination [18]. Sterile LN₂ can easily be obtained through UV irradiation. Periodic refilling of storage dewars with sterile LN₂ and annual decontamination of the cryotanks (which requires removal of the specimens) can help minimize the potential risk of cross-contamination. It is also possible to decontaminate frozen human specimens before warming [19]. This procedure consists of washing the specimens with sterile LN₂ and has also been shown to efficiently decontaminate vitrification cryo-devices even under extreme experimental conditions, in which the concentration of microorganisms was more than ten thousand times higher (108 to 109 CFU for bacteria and > 105 CFU for fungi) than any observed under ordinary conditions.

Regulations

There are currently no regulations regarding device type, nor any mandates for sterility of LN₂ /NV in the USA, although such regulations could be considered by the US FDA, since it does regulate medical devices and is sensitive to the issue of infectious disease transmission. European authorities, in their effort to ensure safety and quality of tissues, may also consider regulating both the devices and the environment in which reproductive cells are stored. From the regulatory point of view, human reproductive cells are treated in the same manner as other non-reproductive tissues; for this reason, even though there is consensus among practitioners that vitrification with open devices using non-sterile LN₂ is safe, best practice guidelines, as well as current and future regulations, must make provision for maximum care and concrete steps to reduce the risk of contamination during or after cryopreservation and cryostorage. Indeed, some European countries have specific requirements geared at reducing the risk of contamination at cryopreservation [20,21].

Cryopreservation in the age of COVID-19

During the COVID-19 pandemic, scientific societies in the field of fertility released guidelines geared at minimizing the risk of contamination in IVF laboratories.

The Italian Society of Embryology, Reproduction and Research (SIERR) pointed out that LN₂/NV can be a source of infection, especially during a pandemic involving a respiratory virus. They highlighted the need for safer and more protective measures, including LN₂ sterilization, to minimize viral exposure during cryopreservation and cryostorage.

The Spanish society ASEBIR released recommendations to minimize risk to reproductive cells at cryopreservation during the COVID-19 pandemic. In this document ASEBIR suggested increasing the number of washes of oocytes and embryos with culture medium and making dilutions in LN₂ before passing the sample to the final tank (Recomendaciones para la seguridad y reducción de riesgos ante la infección por coronavirus [SARS-CoV-2] en las clínicas de reproducción asistida). The procedure for vitrification carrier washing with sterile LN₂ was originally described by Parmegiani *et al.* in 2012 [19]. This procedure is also

recommended by the Practice Committee of the American Society for Reproductive Medicine in its “Recommendations for reducing the risk of viral transmission during fertility treatment with the use of autologous gametes” [22].

Finally, the World Health Organization (WHO), in its 2021 “WHO laboratory manual for the examination and processing of human semen” (Sixth Edition), recommended some precautions to take to avoid or limit viral contamination:

- sterilization of LN₂ to prevent contamination – useful in case of vitrification where the sample is directly immersed in LN₂;
- periodic refilling of dewar storage flasks or tanks with sterile LN₂ and annual decontamination of the cryotanks;
- decontamination of frozen specimens with sterile LN₂ before warming.

Articles suggesting precautions for the pandemic

Recently many authors have suggested implementing “good manufacturing” practices in the field of ART to minimize the risk of LN₂-mediated contamination. Maggiulli *et al.* [23] suggested the use of single-personalized-disposable vitrification containers; Arav [24] and Alteri *et al.* [25] recommended the sterilization of LN₂ before use. Hickman *et al.* [26] and Shapiro *et al.* [27] advocated for the washing of cryopreserved specimens with sterile LN₂ before thawing/warming. Pomeroy and Schiewe [28], Scarica *et al.* [29], and Vajta *et al.* [30], in their recent articles, encourage using precautions for the safe use of LN₂, namely sterilizing it before use.

Risk management when using LN₂

De Santis *et al.*, on behalf of SIERR, observed that the safety of cryopreservation in the context of reproductive medicine is a crucial topic to mitigate any risk of cross-infection. For this reason, they suggested changing perspectives with regard to the use of LN₂ in IVF labs, arguing that it is necessary to overcome the old diatribe between open and closed vitrification carriers, given that LN₂ and NV can themselves potentially be a source of infection. Since the use of contaminated LN₂/NV increases the risk of virus awakening, as well as that of contamination of warmed samples, the local environment and operators, LN₂/NV sterilization is recommended [31].

Adoption of single-use medical devices for personalized vitrification/warming

In a recently published detailed risk analysis Maggiulli *et al.* suggested using sterilized LN₂ and disposable LN₂ trays to mitigate risk [23]. Washing the polystyrene boxes used in IVF laboratories for vitrification and warming is difficult due to the porosity of the material; it is also time consuming and, most of all, difficult to verify. It therefore seems obvious that, in the future, vitrification and warming performed in single-use sterile containers will become the “best practice”. Furthermore, these disposable containers should be certified medical devices, giv-

en that they are used to contain human reproductive cells to be reimplanted.

Specimen washes with sterile LN₂ before warming/thawing

LN₂ can be effectively sterilized with UV-C radiation [18], and “vitrification carrier washing” with UV-sterilized LN₂ before warming has been demonstrated to eliminate vitrification carrier contamination in extreme experimental conditions. In a 2021 study, Parmegiani *et al.* used a concentration of microorganisms over 10,000 times higher than any observed in LN₂ IVF storage vessels and the washing procedure fully removed the carrier contamination mediated by LN₂ [19]. Scientific society guidelines and various authors advise using this procedure to eliminate viral pathogens [22,26,27,32].

Setting up a virus-free vitrification program

In healthcare, blockchain technology can be used to address challenges around sensitive data sharing and traceability of medical and laboratory procedures. Recently, Parmegiani *et al.* described the first application of this technology in IVF for obtaining incorruptible traceability of a “virus-free” vitrification/warming procedure involving the combined use of UVC-sterilized LN₂ and CE medical devices (CE-MDs) [33]. They reported 2346 Ethereum blockchain data transactions for IVF laboratory procedures mined from 1st October 2019 to 31st December 2021. The procedures involved oocyte/embryo vitrification, warming and handling in LN₂ after cryopreservation. For each vitrification/warming procedure, a UVC-sterilized batch of LN₂ was associated with the code assigned to the vitrification/warming procedure and with the lot number of the single-use sterile vitrification box (N-Sleeve). The clinical results obtained from warmed oocytes/embryos were observed as the outcome of this process. A blockchain-trusted “virus-free” vitrification/warming program was set up using a specially designed CE-MD N-Bath-System (Nterilizer, Italy).

Each procedure was traced by the CE-MD software and a dedicated web application. Finally, data were made incorruptible using Ethereum blockchain transactions. Before oocyte/embryo warming, vitrification carrier washing with UVC-sterilized LN₂ was performed in accordance with recent international anti-COVID guidelines. Of the 2346 blockchain transactions, 1268 were vitrification and cryopreserved specimen handling procedures; 1078 transactions were frozen cell warmings (308 oocytes and 770 embryos) performed in 799 patients. To date, 445 pregnancies have been obtained (pregnancy rate: 41% per cycle; 56% per patient) and 219 babies have been born. This is the first evidence of the application of blockchain technology in IVF and many others will probably follow. Blockchain immutable records of LN₂ sterilization combined with procedure codes and disposable lots represent incorruptible traces of “virus-free” vitrification/warming. During the pandemic period (December 2019 to December 2021) 219 babies were born from blockchain-powered “virus-free” cryopreservation procedures.

Conclusion

The risk of using contaminated LN₂ has been underestimated for many years. However, in the wake of the SARS-CoV-2 pandemic, even authors who more than ten years ago considered the risk of cross-contamination in ART cryotanks negligible are now suggesting sterilizing LN₂ [28]. The solution to minimize LN₂ contamination risk is straightforward and easy to implement. It is hoped that LN₂ sterilization will become a mainstream practice before the first cases of specimen cross-contamination or operator infection occur in IVF. LN₂ sterilization and adopting single-use medical devices for personalized vitrification/warming are best practices suggested by many authors and scientific societies, after the recent SARS-CoV-2 pandemic.

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Male infection: cross-contamination risk management and impact on sperm parameters

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ABSTRACT

Chronic viral infections are considered a risk factor for male fertility given their ability to infect semen. Several studies have shown that semen parameters and gamete DNA integrity can be compromised by human immunodeficiency virus (HIV), hepatitis B virus (HBV) and hepatitis C virus (HCV) infections. These effects on male fertility are also associated with other types of infection, such as human papillomavirus. To date, European directives for viral screening of couples undergoing assisted reproduction treatment requires evaluation only for the three most common infections: HIV, HBV and HCV. In all these cases it is essential that all operators involved in the laboratory are aware of the risk to themselves and to the patients treated, and also of the risk associated with the possibility of cross-contamination with other patients. In IVF centres, specific procedures must be established and respected in the case of serodiscordant couples, in order to guarantee correct management and treatment of biological samples. We can reduce or minimise the risk of cross-contamination by applying specific laboratory protocols for the safe handling of gametes and embryos. This mini review aims to describe the impact of viral infection on sperm and male fertility and the protocols and recommendations for the management of gametes from serodiscordant couples with infectious diseases.

KEYWORDS

Serodiscordant, IVF, cross-contamination, procedures, male infertility, sexual transmission, sperm, viruses.

Introduction

Before starting treatments, assisted reproduction centres should implement effective measures to identify patients infected with blood-borne viruses (BBVs). In the past, systematic screening for human immunodeficiency virus (HIV), hepatitis B virus (HBV) and hepatitis C virus (HCV) infection was performed only by some centres^[1,2]. To date, European Union directives for viral screening of couples undergoing *in vitro* fertilisation (IVF) treatment require that they be evaluated for the presence of the abovementioned infections^[3]. In recent years, the improvement of living conditions linked to the evolution of medical techniques and procedures for the treatment of viral diseases has led to an increased desire for parenthood among people with infections.

Reproductive care aims to minimise the transmission risk both to an un-infected partner and to the future child; this imposes the need for semen processing of men infected with HIV or HCV. Moreover, pre-treatment screening enables the identification of patients representing a risk to staff and other patients. It also minimises the risk of possible transmission in the operating theatre and laboratory environments.

Infected patients seeking treatment can be divided into two groups: i) those who have no fertility disorders and require procedures (i.e., sperm washing) aimed at reducing the risk of viral transmission; ii) infertile couples requiring assisted reproduction treatment (ART) in order to conceive.

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Managing samples derived from virus-positive patients requires significant effort in applying laboratory safety procedures to prevent any form of contamination, especially in relation to the handling of embryos and gametes.

In this respect, team management is crucial; in fact, an employee training programme must be provided and continuously implemented in order to ensure that measures are always in place to safeguard the safety of gametes and embryos and minimise the risk of cross-contamination, even though it is known that the mere presence of measurable viral DNA or RNA is not sufficient to cause infection^[4]. Good communication among clinical staff with reference to procedures, trials, test results, precautions and care offered to the patient is also pivotal. A checklist of good laboratory practice to reduce transmission risk when handling gametes from infected patients was compiled by ESHRE in 2021^[5]. The ESHRE paper lists 78 recommendations on the prevention of viral transmission (horizontal and vertical) before, during and after medically assisted reproduction.

Relevant regulations should describe universal precautions

and personal protection equipment, how to minimise aerosol formation during sample processing and, possibly, how to decontaminate a working area ^[6].

Cross-contamination

Specific laboratory protocols have been developed to safely treat virus-positive patients requiring ART. The measures concern the handling of biological material representing a potential risk of transmission of the infection to staff, to other patients' biological material, or to the partner ^[5].

Infected sample as source of staff risk

The main risks to IVF staff are through needle puncture or splash injuries. HBV is a well-recognized occupational risk for all health care operators involved in handling blood, body fluids and clinical waste, although exposure risk has been significantly reduced since the introduction of a vaccine. Unfortunately, on the contrary, there are no effective vaccines for HIV or HCV. It is well known that the cross-contamination risk in the IVF laboratory setting can be minimised by using universal precautions when handling infected samples. This involves handling with latex gloves, using eye protection and, where possible, avoiding sharps. The Centres for Disease Control and Prevention ^[7] recommend universal precautions, i.e., handling of all specimens as if they were hazardous, and that these precautions be applied in all laboratories and in all patients ^[8].

Clearly, sanitisation and sterilisation carried out routinely in all assisted reproduction laboratories, irrespective of the viral status of the patient, is a primary measure. However, an IVF laboratory is a complex structure where all components are designed and used to promote adequate conditions for cell survival and culture. Unfortunately, the same conditions also facilitate viral and bacterial growth.

There are specific procedures designed to ensure personnel safety and prevent cross-contamination:

- Vaccination of all personnel against hepatitis B, and other viral diseases for which vaccines are available, is highly recommended.
- Patients must be rigorously screened for infectious diseases.
- Staff must be promptly informed of a scheduled treatment of a viral-positive patient and made aware of the risks of handling infected biological material.
- Procedures should be implemented to manage situations where infection might occur.
- Scheduling full-blown infected patients at the end of a working session of the operating theatre is highly recommended, thus allowing the necessary time for a thorough disinfection of allocated areas and equipment.

Infected samples as source of patient risk

One of the main concerns in managing infected samples in assisted reproduction is the cross-contamination risk to un-

infected samples. Although relative numbers are small, samples from HCV- and HIV-positive women clearly represent a cross-contamination risk in the laboratory setting. Although all follicular fluid samples should be handled as potentially infected and universal precautions should be adopted at all times, the risk to uninfected samples is inevitably higher when samples known to be infected are handled within the same laboratory area. Patients may be exposed to risk if specific protocols are not designed and adopted to prevent cross-contamination of samples. Unsafe conditions and procedures include lack of information, improper labelling of containers, inadequate sanitisation between patients, improper handling of body fluids, and use of blood products contaminated with infectious agents.

Cross-contamination between infected and uninfected patients and samples can potentially occur during oocyte retrieval and embryo transfer as well as during subsequent laboratory procedures such as insemination, injection, incubation and cryopreservation ^[9]. According to the American Society for Reproductive Medicine (ASRM) guidelines on management of the risk of viral transmission during fertility treatments, samples from infected and non-infected patients need to be treated separately ^[10].

The European Society of Human Reproduction and Embryology (ESHRE) Committee of the Special Interest Group on Embryology published guidelines for good practice in IVF laboratories ^[11] and recommended treatments for patients infected with HIV, HBV or HCV ^[5]. The guideline indications include the use of dedicated laboratory space, and processing using disposable instruments within a separate biosafety cabinet for every type of viral infection to prevent cross-contamination of patient specimens and also contamination of healthy samples ^[5].

Recommendations already exist for handling gametes exposed to viral infection in the IVF laboratory.

- Consider scheduling patient treatment at the end of the daily workflow so as to isolate the patient in time.
- Use separate, dedicated instruments and equipment and consider culturing the gametes and embryos in a separate incubator so as to isolate the patient in space.
- Use disposable contact materials and, in particular, use disposable counting chambers for semen.
- At the time of oocyte retrieval, the virus may be present in follicular fluid and may adhere to granulosa cells surrounding the oocyte, hence the need to denude oocytes completely and inseminate via intracytoplasmic sperm injection (ICSI). Remove granulosa and cumulus cells and wash oocytes thoroughly in fresh medium drops to dilute the viral load.
- Semen treatment consists of a modified sperm washing step, separation of sperm from leukocytes and other seminal constituents by centrifugation over a gradient, and separation of motile sperm from the resulting pellet. This final step is achieved by a swim-up step in which washed sperm are moved to a clean tube and allowed to migrate into an overlay of fresh medium.
- Oocytes and embryos are cultured in mini-styled incubators with separate case-specific compartments. This strategy decreases any risk of cross-contamination.
- It is important to wipe down surfaces with 6% hydrogen peroxide followed by distilled water.

- It has been experimentally demonstrated that cross-contamination between liquid nitrogen (LN₂) and embryos may occur when infectious agents are present in LN₂ and oocytes/embryos are not protected by a hermetically sealed device [112]. Since HIV, HCV, HBV and possibly other viruses can survive in LN₂, treat and cryopreserve the samples in heat-sealed cryopreservation straws to prevent direct contact of cryo-containers with LN₂. Sanitisation of instruments to restore standard operating conditions and separation of frozen storage according to virus (HIV, HBV, HCV) is mandatory.

Cross-infection between partners

IVF procedures must aim to reduce infection of patients. Modified sperm washing protocols are available for the treatment of infected patients. After treatment and before cryopreservation, if possible, viral screening is recommended [110].

Viral infection: impact on male fertility, treatment protocols and recommendation

It is known that chronic viral infections can infect sperm and are considered a risk factor in male infertility [113]. Recent studies have demonstrated that the presence of BBVs in semen impairs DNA integrity and other sperm parameters. In fact, infections can result in temporary or permanent infertility, impairing hormone levels, testicular function and spermatogenesis [114].

HIV

Impact on male fertility

HIV RNA and leukocyte antigens are detected in the testis, epididymis, prostate and seminal vesicles [115]. Patients infected with HIV-1 can develop chronic orchitis and, consequently, progressive hypergonadotropic hypogonadism, which suggests that testicular steroidogenesis is impaired [116,117].

Sexual transmission plays a major role in the spread of HIV-1. The semen of infected men may contain high levels of HIV-1, and virus can be recovered from seminal cells or seminal fluid. Seminal cells are mixtures of spermatozoa, precursors of germ cells, T lymphocytes, macrophages and epithelial cells. HIV-1 proviral DNA has been detected in several of these cell types, mainly lymphocytes, monocytes and macrophages [118].

In vitro studies have demonstrated that HIV attaches to spermatozoa through heparin sulphate proteoglycans [118] but there is no evidence of the virus's transmission by sperm to the foetus (vertical transmission).

HIV treatment aims to suppress viral replication and maintain plasma HIV-1 below detection levels with antiretroviral therapy. Antiretroviral therapy, however, has gonadotoxic effects that are difficult to distinguish from the direct impact that HIV-1 has on male fertility. The latter can affect sperm parameter values and lead to decreased total sperm count, decreased progressive motility, and an increase in sperm counts with abnormal morphology [119].

Protocols and recommendation

If the male partner is HIV positive, and patients are good candidates for intrauterine insemination (IUI), HIV viremia should be minimised (peripheral blood viral load less than 10,000 copies/mL) through the use of highly active antiretroviral therapy, to reduce levels of HIV in semen, followed by a special washing of sperm, which is a safe, cost-effective and widely used treatment method [120].

An efficient sperm washing procedure was proposed by Semprini in 1992 [121]. It appears to be a safe and effective method for achieving pregnancy in HIV-discordant couples in which the man is HIV infected. The procedure consists of:

1. initial dilution of the seminal fluid 1:2
2. 30 minutes centrifugation (1600 RPM) with silica-based discontinuous density gradient, which allows infected leukocytes to be eliminated
3. double washing followed by a second centrifugation
4. 1h sperm swim-up at 37°C with 5% CO₂

A systematic review and meta-analysis published in 2016 [122] demonstrated that this sperm washing protocol appears to significantly reduce the risk of transmission in HIV-discordant couples requiring ART, regardless of viral suppression in the male partner. In fact, no HIV transmission occurred in 11,585 cycles of assisted reproduction using washed semen among 3,994 women.

Cumulative evidence suggests that ART is safe and effective for avoiding horizontal and vertical transmission in HIV serodiscordant couples [123].

HBV

Impact on male fertility

During HBV infection, the virus can be found in many secretions, including semen, and in other tissues beyond the liver and blood [124,125]. HBV is able to not only pass through the blood-testis barrier and enter the male germ cells but also integrate into their genomes [126].

Current data show an increased incidence and risk of infertility in men infected with HBV. Moreover, they showed that exposure to hepatitis B virus S protein induced oxidative stress in sperm cells up to the stage of apoptosis, as revealed by phosphatidylserine externalisation, caspase activation, and DNA fragmentation. In view of these results, it is possible to conclude that HBV-infected males have significantly impaired sperm quality compared with that of control men [127].

HBV can be vertically transmitted, and spermatozoa infected with HBV are susceptible to apoptosis and have reduced fertilisation capacity [128].

The prevalence of virus shedding in semen was found to be around 68% in a population of chronically HBV-infected men [129].

Protocols and recommendation

In couples with a male partner infected by HBV, if the female partner is vaccinated against HBV, sperm washing is considered unnecessary for preventing the risk of sexual transmission. Therefore, ART should be postponed until the couple has re-

ceived the necessary prevention measures, i.e. vaccination ^[10]. If the female partner is HBV-positive, the couple must be informed of the need for specific vaccination of their newborn ^[30].

HCV

Impact on male fertility

HCV infection is associated with alterations in semen quality, including decreased sperm count, reduced motility, and abnormal sperm morphology ^[24,31].

It is noteworthy that treatment with ribavirin and interferon further worsened semen quality in HCV-infected patients ^[32]. In chronically HCV-infected men, viral replication is associated with worsening of all sperm functional tests; this worsening includes high levels of seminal ROS and high sperm DNA fragmentation.

Protocols and recommendation

Unlike HBV, there is no vaccine against HCV. Therefore, it is essential to employ risk-reduction measures during assisted reproduction involving affected patients.

For HCV-positive males with detectable viral load in serum, the density gradient with swim-up protocol ^[33,34] can be used before IUI or ICSI to reduce the viral concentration in the final sperm sample.

The infected partner can be treated with peginterferon alfa and ribavirin to reduce viral load before assisted reproduction treatment ^[35]. It is recommended that this treatment be administered for 48 weeks.

During this period, two forms of contraception should be used followed by a six-month washout period to reduce the teratogenic and embryotoxic risks of ribavirin, a pregnancy category X drug.

There is a small, but measurable, risk of HCV transmission via semen. When the male partner is HCV infected, sperm washing can reduce the viral load in semen. It is also recommended to reduce the risk of transmission to his partner. Using IVF with ICSI has also been demonstrated to reduce the risk of transmission of HCV when the male is seropositive ^[34].

As described elsewhere ^[36] ejaculates obtained after a sexual abstinence of 3-5 days are allowed to liquefy, and then diluted 1:1 (vol:vol) with sperm medium.

They are then pelleted at 400 g for 10 min, and the supernatants are discarded. A volume of sperm medium equal to the initial volume of semen is added, and then layered onto a triple concentration gradient (90, 70 and 45%, PureSperm; Nidacon, Sweden) of 1-1.2ml of each layer, and centrifuged 20 min at 300 g. Each pellet thereby obtained is then washed with sperm medium and re-pelleted again. Supernatants are discarded and another swim-up of 0.5-0.7ml is executed. After 45 min, the upper 0.35 ml of each tube supernatant is collected, pooled and split into two samples.

One half is immediately plunged into liquid nitrogen for polymerase chain reaction (PCR) determinations, and the other half is frozen with sperm freezing medium, according to the manufacturer's instructions, and finally stored until use, following a negative result for viral presence ^[36].

HPV

Impact on male fertility

HPV is a non-enveloped, double-stranded circular DNA virus, a member of the *Papillomaviridae* family ^[37].

It is the most common sexually transmitted virus in humans, with over 80% of the sexually active adults being infected by one HPV type at least once in their lifetime.

Human papillomavirus is frequently detected in semen and urethral swabs from asymptomatic men. In the past, HPV semen infection was always considered transient and without clinical consequences. In a recent study, nested PCR showed the presence of HPV DNA sequences in 10% of semen samples from asymptomatic young adult males who had had unprotected intercourse ^[38].

HPV can be localised at different levels: in sperm, in exfoliated cells or in both sites. Curiously, infertile patients had both a prevalent infection in sperm and a higher percentage of infected sperm, while exfoliated cells were significantly more affected in patients with other risk factors. In fertile control subjects HPV infection was never found in sperm cells ^[39].

The proposed mechanisms underlying male infertility associated with HPV infection include direct modification of semen quality, damage to sperm DNA integrity, and production of anti-sperm antibodies that can interfere with sperm motility and sperm-oocyte binding ^[40].

The hypothesis of a direct viral effect on sperm is substantiated by the findings of high sperm DNA fragmentation rates and decreased progressive sperm motility in infertile men with asymptomatic chronic high-risk HPV infection ^[41].

Overall, some questions remain unanswered and many studies are required to clarify the mechanisms underlying the effects of HPV in both the female and the male reproductive system, and to determine the role of HPV persistence in fertility alterations. It is also known that oncogenic or high-risk HPV plays a significant role in the development of vulvar, vaginal, penile, anal and oropharyngeal cancers.

Protocols and recommendation

Human papillomavirus infection has been detected in the sperm of a large percentage of sexually active males. Standard sperm selection techniques are ineffective in removing HPV. The application of an efficient swim-up procedure is therefore recommended. Garolla *et al.* ^[42] suggested a "direct" swim-up method, which reduced the number of HPV-infected sperm by ~24% ($p < 0.01$), and a "modified" swim-up procedure, which is able to remove HPV DNA completely from both naturally and artificially infected sperm. In this modified protocol, enzymatic treatment with heparinase-III coincided with a decrease in sperm motility, sperm viability and DNA integrity, but these effects were not statistically significant. Unfortunately, heparinase-III is not licensed for assisted reproduction in humans. In a study published by the same group in 2021 ^[43], the authors tested the possibility of using hyaluronidase as an innovative method for removing HPV virions bound to sperm cells before proceeding with ART ^[44]. The experimental treatment is associated with complete loss of HPV-VLP in association with a reduction of sperm viability and progressive motility.

The sperm sample is first incubated for 1 h at 37°C in fresh SWM with HPV at the ratio of 1 mg HPV-VLP per 1×10^6 spermatozoa per mL. After the incubation the sample is incubated with hyaluronidase at the concentration of 80 IU/mL at 37°C for 30 min.

After this second incubation sperm is washed in order to eliminate excess of reagents and processed with the standard swim-up procedure.

HPV virions bound to sperm cells are detected by immunofluorescence. Using the 80 IU/mL concentration, total removal of HPV virions was observed without any alterations of cell morphology, DNA integrity, capacitation and acrosome reaction according to 2010 WHO criteria^[43,44].

Conclusion

Semen as a vector for sexual and vertical viral transmission must be studied further; nevertheless, the mere presence of viral nucleic acids in semen does not indicate infection. Further research should be aimed at detecting accurate viable viral loads and particles in semen capable of causing disease.

It is essential that all operators are aware of the risk of cross-contamination, not only between serodiscordant partners but also between patients and operators. To ensure adequate safety measures, infected patients should be treated only in IVF laboratories with dedicated areas and equipment. Alternatively, the treatment of infected patients must be carried out in defined working time frames, provided that, following the procedures, the areas and equipment are fully disinfected. In IVF centres specific procedures must be established and followed for serodiscordant couples, to ensure correct management and treatment of biological samples. Samples should, in any case, always be considered and treated as potentially contaminated. Patients and their gametes/embryos properly treated for HIV and HCV pose no infection risk to their partners or other patients undergoing assisted reproduction. Laboratories can simply and effectively mitigate risk from individuals with active blood-borne viral infection by individual preparation and treatment of gametes.

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Candida contamination of embryos in the ART laboratory: a case report and literature review

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ABSTRACT

Objective: To report a case of yeast contamination with adhesion of hyphae to the zona pellucida. **Design:** A case report and brief literature review. **Setting:** Nextclinic Fertility Center, Merano (Italy). **Patient(s):** A 32-year infertile patient facing microbial contamination of her embryos after thawing. **Results:** Due to the heavy contamination of the embryo by yeast, embryo transfer (ET) was not carried out, resulting in cancellation of the cycle. **Conclusions:** A very rare case involving embryos contaminated by *Candida* is here described. Strategies and practical measures to prevent the risk of cross-contamination in the storage tanks of cryobanks are illustrated. Zona pellucida removal may be a possible method to rescue contaminated embryos for ET.

KEYWORDS

In vitro fertilization, yeast contamination, cryopreservation, zona pellucida.

Introduction

Despite strict quality control protocols and aseptic techniques, microbial contamination of assisted reproductive technology (ART) cultures is a known possibility. Only a few studies report the incidence of this phenomenon, which can affect from 0.29% ^[1] to 0.68% ^[2] of embryonic cultures. There are even fewer data available on the incidence of yeast contamination, which can be up to 0.18% ^[3].

The main sources of contamination are follicular fluid and semen, therefore intracytoplasmic sperm injection (ICSI) is an established method to minimize the risk of microbial contamination ^[2]. Most reported contaminants are caused by bacteria strains, with *E. Coli* ^[1] accounting for 83% of cases.

We report a case of contamination caused by *Candida spp.*, which resulted in cancellation of the planned embryo transfer (ET).

Case report

A 32-year-old patient with primary infertility was referred to our clinic in February 2022. She had been trying to conceive for 7 years. Her baseline follicle-stimulating hormone, luteinizing hormone, estradiol and anti-Müllerian hormone levels were normal. Her husband's semen analysis showed moderate asthenozoospermia (sperm concentration 42 x 10⁶/mil; motility 28%; normal morphology 33%, leukocytes 340000) (Table 1). She had already undergone a cycle of ovarian stimulation in another IVF clinic, where a sperm culture was not performed before the fresh cycle. A low-dose gonadotropin antagonist protocol was used to obtain a controlled ovarian stimulation.

Article history

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Table 1 Patient's baseline data.

MATERNAL	VALUES	PATERNAL	VALUES
Baseline FSH (U/L)	6,6	Sperm concentration	42 x 10 ⁶ / mil
Baseline LH (U/L)	7,5	Motility	28
Baseline E2 (U/L)	5,6	Normal morphology	33%
AMH (ng/mL)	4,47	--	--

FSH = follicle-stimulating hormone; LH = luteinizing hormone; E2 = estradiol; AMH = anti-Müllerian hormone. Levels of FSH, LH, E2 on day 3 during the patient's menstruation.

After oocyte retrieval, 18 oocytes were collected, 16 of which were mature. Eight oocytes were cryopreserved for later use and 8 were fresh fertilized using the ICSI technique, which resulted in four embryo transfers (ETs) at the blastocyst stage, but only one biochemical pregnancy was achieved.

From subsequent ICSI fertilization of the cryopreserved oocytes, four blastocysts were obtained that were still available for ET. The embryos were cryopreserved using an open system (Cryotop, Kitazato).

The couple decided to perform ET in our clinic due to per-

sonal preference. Transport of the four embryos was organized and documentation and protocols regarding the embryos were collected and evaluated in order to ensure compliance with national and our internal quality regulations ^[4].

To achieve a personalized ET, we performed an Endometrio test (Igenomix, Italy) which demonstrated a low endometrial microbiome. Vaginal lactobacillus (Avidif CV) was prescribed during preparation for ET. No pathogens were detected and the endometrium appeared to be in the receptive stage.

In May 2022, during thawing of the first embryo the embryologist immediately noticed the presence of a mass, presumably hyphae, attached firmly to it. The mass appeared tightly adhered to the outer surface of the zona pellucida and covered a significant part of the blastocyst itself (Fig. 1A). The embryologist attempted to detach the mass by repeatedly rinsing the blastocyst with a pipette with a diameter of 300 micrometers, but without success.

In order to ensure a transfer for the couple and avoid cancelling the cycle, all the remaining blastocysts were thawed (Fig. 1B) and similar findings were observed in all except for the last one which had no evident contamination with hyphae. The embryos were classified according to the Gardner classification ^[5] which showed a good embryo grading for all four blastocysts: B14BB, B13BB, early B1, B13BB.

No additional information regarding embryo cultures before freezing was available (single/double embryo culture, evidence of contamination during the embryo culture, presence of hyphae before loading blastocysts on the cryotops, etc.), and therefore, after consultation with the couple, we decided not to perform ET.

The embryo culture media were sampled and sent to the microbiology laboratory for further investigation.

Following the sampling, all blastocysts were vitrified using a closed device (Vitrisafe).

The workstations were thoroughly cleaned with a surface disinfectant (Oosafe). After 15 minutes microbiological samples were taken from the work surfaces to ensure that contamination had not spread.

Candida contamination was confirmed by the microbiology laboratory for the embryo culture media of three blastocysts, while the fourth was found to be contaminated by *E. Coli*. The microbiological samples from the work surfaces were negative, confirming the effectiveness of the laboratory's sanitization procedures. The relevant surveillance institute (Centro Nazionale Trapianti - CNT) was immediately notified of the serious adverse event, as was the original laboratory that had performed the insemination, so that they could also take the necessary precautions.

Discussion

Although ART procedures have to be carried out in accordance with strict standards, microbiological contamination can occasionally still occur, with serious consequences for the patients. Literature regarding this occurrence is rare and refers mainly to aspecific contamination of cultures by bacteria, yeast or viruses.

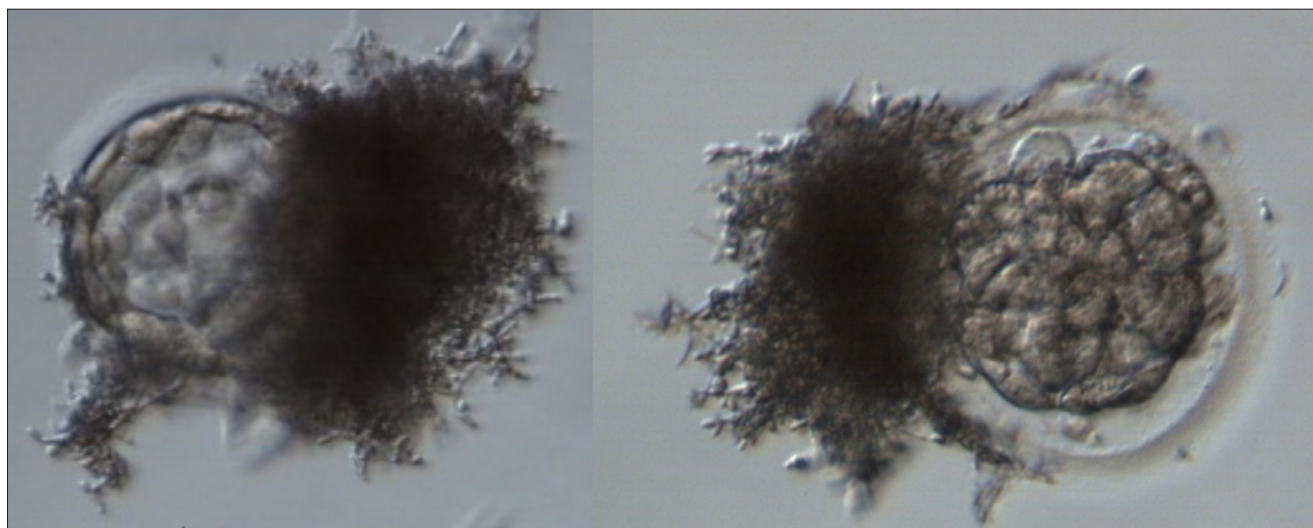
Over a period of 8 years, Kastrop and his study group analyzed 13977 ART cycles, finding a contamination rate of 0.68%; contamination was due to *E. Coli* in 59% of cases and to *Candida* species in 25% ^[2].

A decade later, Li-Ling et al. retrospectively reviewed 4118 ART cycles and found 12 cases of microbial contamination, extrapolating an incidence of nearly 0.29%. The contamination was mainly caused by *E. Coli*, while no other microorganisms such as yeast species were isolated ^[1].

When we sought literature specifically concerning yeast contamination in ART cultures, the available data were found to be even scarcer. To our knowledge, there are only two scientific publications on this topic: in 1996 Ben-Chetrit et al. found yeast colonization in 5 dishes out of 729 cycles ^[6]; in 2007, Klein *et al.*, overviewing 11816 cycles, reported an incidence of 0.18% ^[3].

In all of the mentioned publications, microbial contamination was suspected after microscopic evaluation of the culture

Figure 2 *Candida* contamination: adhesion of hyphae to the zona pellucida. A: First thawed embryo. B: second thawed embryo.



dish revealed a turbid liquid which was then sampled and sent for confirmatory microbiology assessment. In our case, hyphae of *Candida* were directly visible and firmly attached to the zona pellucida of the thawed blastocysts. In all three visibly contaminated embryos, the contamination was confirmed by microbiological analysis.

Reviewing the limited existing literature, it becomes clear that microbial contamination is much more frequent when the fertilization technique involved is IVF. The ICSI technique is proposed as a strategy to minimize contamination. Considering that seminal fluid is considered to be the main contaminant ^[1], the fact that ICSI involves isolation of a single spermatozoon from the solution may explain the low risk of contamination associated with this procedure ^[2].

Our clinical case is therefore a rare event in itself, given the minimal risk of *Candida* contamination in ART laboratories; however, the fact that the contamination was found in embryos derived from fertilization by ICSI performed on thawed oocytes makes it even more unusual. This aspect underlines the importance of ensuring that operators observe all the necessary measures to prevent contamination of cultures, such as the use of disposable personal protection, a controlled air system in the laboratory room, and regular cleaning of the laboratory equipment, forceps, tongs, canisters, canes, and so on.

In an important review on cryostorage management of reproductive cells and tissue in ART, published in 2023, the authors remark that temporary storage tanks, dewar flasks and devices used for transporting straws or devices between long-term storage tanks and the IVF laboratory bench should be cleaned after use by rinsing with a cleaning solution and then left to dry upside down in order to prevent contamination ^[7].

There is still no unanimous consensus regarding the transfer of embryos from contaminated culture. In 1996, a positive effect deriving from the co-culture of embryos with *Candida* was even hypothesized thanks to a reduced concentration of oxidative free radicals ^[6]. On the other hand, a later report suggested that earlier contamination, at day one, has a detrimental effect on embryo development ^[3]. The authors of this report still proceeded with ET after adequate counseling of patients regarding the decreased likelihood of clinical success. They reported no statistically significant differences in clinical outcomes between the control cycles and the yeast cycles. However, they noticed that all live births resulted from cycles in which contamination was noted on day 3 or 4. No live birth occurred when contamination was noted on day one, due to poorer embryo quality ^[3].

In our specific case, we decided, together with the couple, not to proceed with ET of the contaminated blastocysts due to the patient's favorable ovarian reserve and the favorable prognosis for the couple in a subsequent cycle. Moreover, the contamination we detected was not limited to the culture medium, but more extensive, being firmly attached to the blastocyst itself, even though the quality of the embryo did not appear to be compromised. We were concerned about the possibility that the embryo might undergo degeneration under the faster and thriving development of the hyphae, and about the danger of introducing a considerable amount of yeast into the uterus, with the risk of causing infections. We notified the original center of

the contamination because we deemed it implausible that such substantial contamination could have occurred within a brief time frame, meaning that the contamination likely transpired at the source center.

Since Italian law prohibits the destruction of embryos, the blastocysts had to be cryopreserved again. Initially they were stored in an open device (Cryotop, Kitazato). In this regard, it should be noted that there is a risk of both cross-infection and cross-contamination of embryos and gametes in liquid nitrogen tanks ^[8].

Our clinic has always worked with a closed carrier device to guarantee a safe procedure ^[9].

As this device is hermetically sealed, there is no contact with the liquid nitrogen during cooling, storage and warming. This device offers the highest protection during the entire vitrification process compared with open or semi-closed cooling devices ^[10].

Despite this, the embryos that turned out to be contaminated only at the time of thawing had been stored in the cryobiology room of our clinic in an open device in liquid nitrogen containers. This arrangement might result in contamination of the liquid nitrogen itself. One of the main biosafety measures to prevent contamination is segregation of samples. It is therefore recommended that samples from infected or suspected donors be quarantined, tested, and if contamination is found, stored in separate containers ^[11]. In this case, however, it was not possible to use the quarantine tank as *Candida* infection is not among the contaminants regularly tested in the transport of gametes and embryos between fertility clinics.

Since the majority of commercial liquid nitrogen is not sterile, long-term storage tanks also require periodic cleaning. An interesting strategy, proposed by the working group of Parmegiani *et al.*, involves the use of UV radiation to sterilize the liquid nitrogen and the tanks ^[12]. This procedure requires that all cryopreserved samples be transferred in a witnessed way to a similar cryostorage vessel, after which the long-term tank is sterilized by UV radiation.

The damage caused by microbial contamination in ART procedures has a direct impact on laboratory and fertility clinics' costs ^[13]. The greatest damage, however, is suffered by couples trying to achieve a pregnancy: ART treatments place an extremely high emotional and financial burden on patients. The destiny of oocytes and embryos is a sensitive issue, especially if they have to be stored indefinitely and can no longer be used. For this reason, although we initially wondered whether an alternative solution might be found to salvage the ET, given that the transfer of embryos contaminated by *Candida* is generally considered possible ^[3,6], in our case the contamination was not limited to the culture medium, and therefore ET was not deemed feasible.

From a later evaluation of our clinical case and after a careful review of the international literature, we found two publications regarding two interesting approaches to this problem.

In 2016, Shu *et al.* reported live births after removing the zona pellucida from contaminated blastocysts. The two cases they analyzed concerned the transfer of blastocysts contaminated by bacteria ^[14]. After thawing, the authors performed zona pellucida removal with laser pulse at the fifth day of embryo

development, in order to avoid microbial growth.

In 2022 Ruiqi et al. performed a similar procedure but with different timing: they removed the zona pellucida using acidic Tyrode's solution on zygotes, and obtained one ongoing pregnancy. Again, in this case, the contamination was caused by bacteria, specifically *Klebsiella pneumoniae* [15].

With hindsight, we can state that laser removal of the zona pellucida might perhaps have been used to perform a rescue ET for our couple. However, we have not found any specific reports of zona pellucida removal in cases of *Candida* infection with adhesion to the blastocyst. Moreover, considering the impracticality of conducting an extended culture period, given that we only discovered the contamination at the blastocyst stage, we would not have been able to definitively exclude residual contamination.

Conclusions

In conclusion, this report describes a very rare case of *Candida* contamination of embryos derived from fertilization by ICSI performed on thawed oocytes. Two aspects can be highlighted: first, in order to minimize the risk of contamination in IVF laboratories the application of aseptic techniques is of fundamental importance. Second, zona pellucida removal might be a feasible method to rescue *Candida* contaminated embryos, but more studies are needed to safely implement the technique.

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