12 Male Reproduction: From Pathophysiology to Clinical Assessment

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Couple infertility, defined as the lack of conception after at least 12 months of regular unprotected sexual intercourse aimed at pregnancy [1], is a common clinical condition.

It is a multifactorial disorder affecting one out of six couples in Western countries, and male factor infertility is implicated in about 50% of cases [2].

Male infertility may depend by pre-testicular (for example, hypothalamic or pituitary diseases), testicular, and post-testicular (for example, obstructive pathologies of seminal ducts) causes.

The pathophysiology and the clinical assessment, including treatment strategies, of these situations will be discussed in the next chapters of this book.

However, a large proportion (30–60%) of infertile males does not receive a clear diagnosis. In these cases, generally reported as idiopathic infertility, there is a strong suspicion of genetic factors yet to be discovered, and research in this field will probably reduce the proportion of unexplained infertility in the next years [3].

Furthermore, male fertility may be influenced by a host of lifestyle risk factors such as environment, nutrition, exposure to infections, and smoking. Therefore, lifestyle and environment risk factors may have a role in many cases of idiopathic male infertility.

In this chapter, we will focus our attention on these risk factors, discussing three paradigmatic situations of interference between environment/lifestyle and male fertility, thus providing the pathophysiological basis of their detrimental impact on male fertility: exposure to environmental endocrine disruptors, such as perfluoroalkyl substances (PFAS); exposure to viruses, such as HPV; effect of nutritional status and obesity.

12.1 PFAS Pollution and Male Fertility

PFAS are a class of organic molecules characterized by fluorinated hydrocarbon chains, widely used in industry and consumer products including oil and water repellents, coatings for cookware, carpets, and textiles. PFAS have unique physical chemical properties due to their amphiphilic structures and their strong carbon–fluorine bonds. Consequently, long-chain PFAS are non-biodegradable and bioaccumulate in the environment [4]. PFAS have been found in humans and in the global environment and their toxicity, environmental fate, and sources of human exposure have been a major subject of research. PFAS have

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risen many concerns for their bioaccumulation in body tissues and potential harmful effects in humans [4]. In fact, inhalation of air particles and/or ingestion of contaminated food products and drinking water have been claimed as major routes of exposure to PFAS. Accordingly, PFAS have been found in several human tissues, such as the brain, placenta, semen, and testis even in the presence of acknowledged blood/tissue barriers [5, 6]. Exposure to PFAS has been widely described in several countries, with considerable differences in terms of geographical distribution, ethnicity, molecular weight (long-chain or short-chain PFAS), and degree of fluorination [7–9]. On this matter, perfluorooctanoic acid (PFOA) and perfluorooctanesulfonic acid (PFOS) are the most common and most studied PFAS in toxicological terms.

Epidemiological studies have focused not only on their impact on foetal development, but also on the relationship between PFAS and human fertility, although studies have been focused mostly on female fertility. However, both in vitro and animal studies on PFAS toxicity have shown a negative effect of PFOA and PFOS on testicular function, by the alteration of steroidogenic machinery and subsequent defect of spermatogenesis [10, 11].

Exposure to high levels of PFOS, and of PFOA and PFOS combined, are associated with a reduction in the concentration of morphologically normal spermatozoa in adult men [12, 13]. Furthermore, Raymer et al. reported, in a study of men attending an in-vitro fertilization clinic, that luteinizing hormone (LH) and free testosterone were significantly positively correlated with plasma PFOA [14]. Although conclusive data have not still provided, preliminary data seem to suggest moreover an increased sperm DNA fragmentation in exposed men [15, 16].

Among the endocrine effects of PFOS, it should be underlined that it affects the hypothalamic–pituitary axis activity [17, 18]. A testicular toxicity of PFOS has been demonstrated in rats [18]. High doses of PFOS orally administered in rats for 28 days modify the relative gene and protein receptor expressions of several hormones of the hypothalamus-pituitary-testicular axis (GnRH, LH, FSH, and testosterone) [19].

In humans, in utero exposure to PFOA was associated later in adult life with lower sperm concentration and total sperm count and with higher levels of luteinizing hormone and follicle-stimulating hormone [20]. In infertile male patients, PFOS levels were higher than fertile subjects. Furthermore, in these patients, a higher gene expression of estrogen receptor (ER) α, Erβ, and androgen receptor (AR) has been reported [21, 22], thus suggesting that PFAS activity might be linked also to the genetic expression of sex hormones nuclear receptors.

Regarding androgen receptor (AR), PFOS and PFOA cause a reduction in its protein expression in hypothalamus, pituitary gland, and testis. This inhibition might reflect PFOS action on post-transcriptional processes of the AR synthesis. AR-dependent gene expression is indeed crucial for male sexual differentiation in utero and male reproductive function and development in adults, including spermatogenesis [23].

Three compounds (PFHxS, PFOS, and PFOA) act as ER agonists in vitro, and five PFAS (PFHxS, PFOS, PFOA, PFNA, and PFDA) act as AR antagonists. Their combined action, as observed in PFCs mixture, induces a synergistic impact on AR function [24]. These findings clearly suggest an antiandrogenic potential of PFAS.

In a recent study, we investigated the possible association between the exposure to PFOA and PFOS and endocrine disruption through the evaluation of developmental alterations and reproductive disorders in a group of 212 young males from the Veneto region, a wide area in the north-east of Italy featured by high environmental exposure to these chemicals [11]. Compared to 171 age-matched controls residing outside of the exposed area, subjects from the contaminated area showed increased levels of circulating testosterone (T) and LH, pointing toward an antagonistic action of PFOA on the binding of T to its natural AR.

Interestingly, most of the exposed male population showed a reduction in testicular volume, penile length, and anogenital distance but not

anthropometric measures. These findings could be explained by considering that anogenital distance and anthropometric measures are differentially determined during fetal and prepubertal development, respectively [25]. Pre-natal exposure to androgens during the "masculinization programming window," a critical window during testicular development, is positively associated with anogenital distance in mammals [26]. On these bases, anogenital distance has been suggested as a putative marker of prenatal exposure to chemicals with a known antiandrogenic effect, or endocrine disruptors in general. As the first report on water contamination of PFAS goes back to 1977 [27], the dimension of the problem is alarming as it affects entire generations of individuals, from 1978 onward.

It has been moreover demonstrated that PFOS has the ability to cross the blood brain barrier [28] and the placenta [29], although the exact mechanism has not been yet clarified. In the same way, PFOS may disrupt the Sertoli cell tight junction-permeability barrier, which ultimately might induce a dysfunction in blood-testis barrier, associated with infertility [30].

In semen of exposed subjects PFOA is more represented than PFOS, despite the pattern of serum concentrations is essentially reversed. In detail, average PFOA levels retrieved in semen samples from exposed subjects were 0.67 ng/mL, ranging from 0 to nearly 6 ng/mL [11]. Furthermore, seminal PFOA levels correlate with seminal pH, thus suggesting a putative interference of PFOA at a prostatic level [11]. The presence of PFAS in seminal plasma suggests either a possible involvement of prostate, that might explain a weak association between PFOS exposure and prostate cancer [31].

Moreover, semen levels of PFOA are significantly correlated with the presence of altered sperm parameters, and namely of motility. This evidence is suggestive of a direct effect of PFOA on gamete function. More recently, we have demonstrated that incubation of sperm cells with PFOA is associated with negative effects on sperm viability, independently from the exposure time and concentration [32]. Progressive motility is significantly impaired by PFOA exposure even at the lowest concentration of 0.1 ng/mL, as reported in Fig. 12.1.

The direct influence of PFOA on sperm motility is related to the impaired metabolic performance associated with a decreased mitochondrial respiratory activity.

Furthermore, it has been demonstrated that PFOA accumulates in sperm membrane, thus dis-

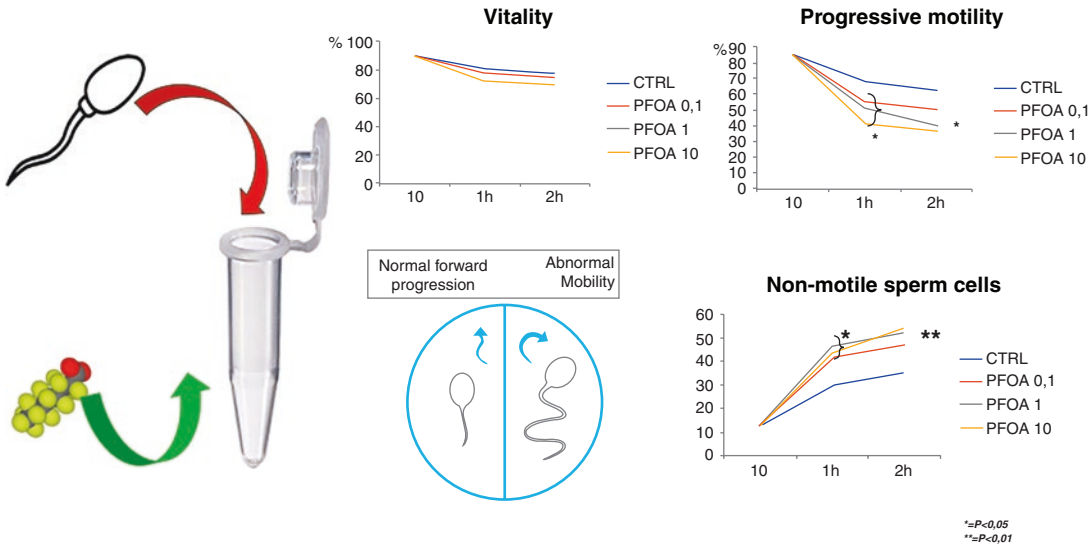

Fig. 12.1 Incubation of sperm cells with PFOA reduces sperm vitality and progressive motility and increases the percentage of non-motile cells

rupting membrane fluidity. Membrane fluidity, acknowledged as a major determinant of sperm motility and fertilization potential, is associated with decreased packing order of phospholipids in the outer layer of the plasma membrane. Plasma membrane is a key organelle, with a pivotal role in sperm physiology and the fine modulation of its composition, from ejaculation to fecundation, has critically effect on the overall efficacy of the fertilization process [33]. During the transit of sperm cell through the female reproductive tract, there is an increase in membrane fluidity, due to cholesterol deprivation by sterols acceptors like albumin of HDL, which is associated with the gain of progressive motility and fusogenic properties [34, 35]. Because of its high hydrophobicity, PFOA might randomly accumulate in sperm membranes, thus altering local pH and permeability to ionic species and, in turn, membrane potential as recently observed also in somatic cell models [36]. Accordingly this model, the local perturbation of membrane composition, may also induce in the production of free radicals, as recently demonstrated for other chemical species such as graphene-oxide [37], possibly explaining the association between PFAS exposure and sperm-DNA damage.

Finally, it can be hypothesized that sperm cells may be exposed to PFAS not only in seminal plasma of exposed males but also in genital secretion of exposed females. It has been in fact demonstrated that cervical mucus from women resident in highly exposed areas have higher levels of PFOA in cervical mucus, compared to control sub-jects [29].

Taken together, these data underline the multifaced role of pollutants, such as PFAS, in male infertility. PFAS exposure acts at different levels impairing the reproductive system: through a modulation of AR expression, both in utero and after the birth; through the alteration of the Sertoli cell tight junction-permeability barrier, which ultimately changes destabilized Sertoli cell BTB integrity; directly altering sperm cell function, causing an impairment of sperm motility, likely relied on the alteration of plasma-membrane potential due to a disruption of membrane fluidity; inducing and increase in sperm DNA fragmentation, which might be due to increased ROS levels; and finally by a putative impairment of prostate function.

12.2 HPV Infection and Male Infertility

A large spectrum of viruses may infect the testis and other male genital organs, thereby impairing male fertility [38]. Human Papillomavirus (HPV) is the etiological agent of the most common sexually transmitted infection worldwide, with an estimated 6.2 million new cases annually [39]. HPV comprises a group of small non-enveloped epitheliotropic viruses with a double-stranded circular DNA genome made- up of 8000 bp. Its virion has an icosahedral shape, of 55 nm diameter, constructed of 52 capsomeres, each containing five molecules of the major capsid protein L1 and a smaller number of the minor capsid protein L2 [40]. HPV consists of more than 200 genotypes, adapted to specific epithelial tissues, such as anogenital skin and mucosa [41]. According to the basis of oncogenic potential, HPV can be divided into two different groups: high-risk (HR-HPV) and low-risk (LR-HPV). The former ones, that include the well-known 16 and 18 types, have been classified as oncogenic to humans according to the International Agency for Research on Cancer [42], and may cause neoplastic transformations in the following epithelial areas: cervix, vagina, vulva, anus, penis, and oropharynx [43]. The latter ones, such as 6 and 11 types, are responsible of benign diseases such as genital warts [44]. HPV infections are primarily contracted by direct contact of the skin or mycoses with an infected lesion. Genital HPV infection is largely transmitted through sexual intercourse, mostly insertive intercourses, although non-penetrative types of contact (i.e., genital-genital, oral-genital, and manual-genital) represent possible routes of transmission [45].

Recently, it has been clearly confirmed that, in addition to the well-known external genital areas, HPV virions may also be detected inside the male reproductive tract. In detail, it has been detected in male accessory glands, where it can represent

a possible cause of male accessory gland infection [46]. This localization in accessory gland is reflected in changes in seminal parameters such as an increase of pH and viscosity and a reduction of seminal volume [47, 48].

Finally, HPV was found in semen, both in exfoliated cells and even bound to spermatozoa [49] as reported in Fig. 12.2.

Several studies have underlined the possible role of HPV in causing male infertility [50]. In fact, several authors have confirmed the presence of the virus in the seminal fluid of men suffering from idiopathic infertility [47, 51]. These data, combined with the higher prevalence of sperm HPV-infection in infertile subjects compared to general population [52], suggested a role for HPV as a cause of sperm damage and, consequently, of male infertility.

Several studies reported that HPV infection is related with a reduction in seminal parameters. Five studies reported a relation between HPV seminal infection and a reduction in sperm motility [49, 53–56]. Moreover, Piroozmand showed also a significant reduction of the sperm count [53]. An increased sperm DNA fragmentation index has been moreover reported by Boeri et al. when semen infections involved high-risk HPV genotypes [57] while Yang et al. demonstrated that HPV seminal infection is associated with a reduction in sperm normal morphology [58].

More recently, Moghimi et al., observed a significantly higher prevalence of high-risk HPV in infertile men, compared to fertiles, associated with an impairment of sperm morphology and motility [59].

HPV infection in semen represents moreover a risk factor for the development of anti-sperm antibodies (ASAs). In fact, the prevalence of ASAs is higher in infected infertile patients compared to non-infected infertiles and general population. Moreover, in infected infertile subjects, presence of antibodies is associated with a further reduction of sperm motility [60]. In detail, more than 40% of HPV infected infertile patients had ASAs on the sperm surface. Moreover, infected patients had a higher mean percentage of ASAs compared with non-infected ones [55]. These findings suggested that sperm autoimmunity could probably be HPV-dependent. In order to confirm this finding, Garolla et al. documented the presence of both viral proteins and immunoglobulins in the same sperm cells of samples with positive sperm-mixed antiglobulin

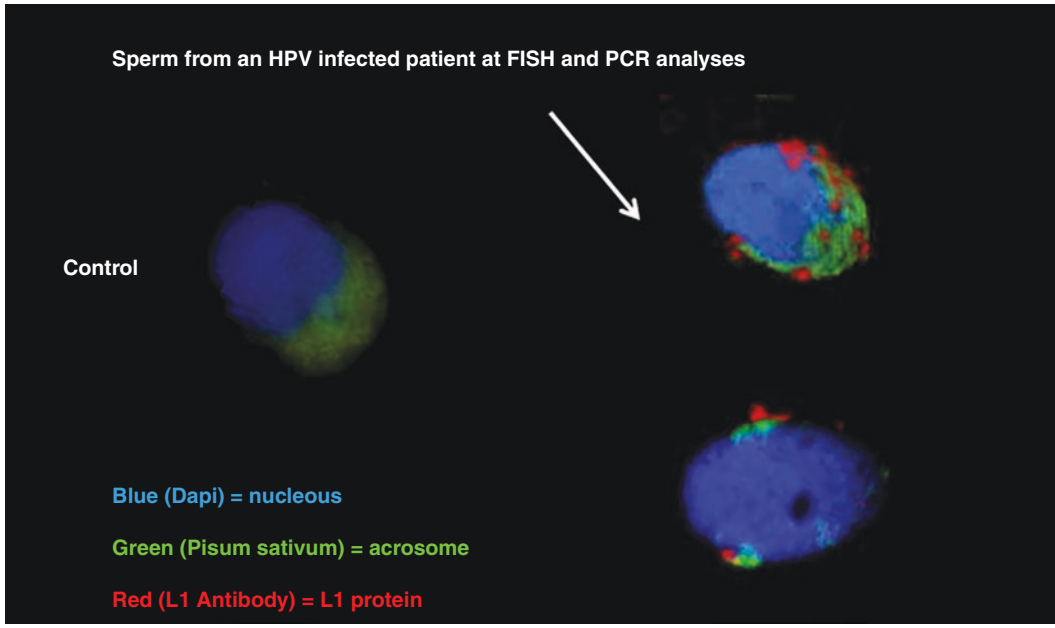

Fig. 12.2 Immunofluorescence for HPV L1 capsid protein (red) in sperm cells

reaction (Mar) test results. 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 represents a new clinical condition associated with the presence of ASAs. In infected males, a significant viral clearance (approximately 85.3%) is obtained after 24 months of follow-up and the reduction in sperm infection paralleled the disappearance of ASAs and with the progressive improvement of sperm motility [55].

Several studies have analyzed the effect of semen HPV infection, in terms of reduction of fertility both in natural [61] and in assisted reproduction [61–64]. An increase in miscarriage rate has been moreover reported by Garolla et al. [61].

To understand the pathophysiological role of HPV in fertilization, we performed an in vitro study evaluating the ability of the virus-infected sperm to transfer HPV DNA and capsid proteins to oocyte during fertilization. After transfecting a human sperm with a plasmidic episome containing HPV E6 and E7 proteins, the hamster egg-human sperm penetration test has been performed to show the ability of infected sperm to transfer the capsid protein L1 to oocyte and the expression of E6 and E7 viral protein in the fertilized oocyte [65]. We may therefore conclude that both spermatozoa transfected with E6 and E7 genes and exposed to HPV L1 capsid protein are able to penetrate the oocyte. These laboratory data, combined with the observation that HPV DNA is found in a larger proportion of abortions rather than voluntary termination of pregnancy [62], may suggest an active role for HPV (which is carried to the egg by the spermatozoa) in the etiology of premature term gestation. This phenomenon could lead to an increase in the fragmentation of embryonic DNA, thus resulting in alteration and apoptosis of the embryo [65].

On the basis of these evidences, we recommend testing for HPV in the male partner of the infertile couples in the following cases: male affected by unexplained couple infertility (not related to known male or female factors), asthenozoospermia, presence of ASA, positive medical history for HPV infection or evidence of ongoing HPV-related diseases [50].

When HPV is detected in at least one of the members of an infertile couple, it is important to provide careful counselling. A 2014 controlled study showed the effectiveness of this strategy. Couples in which both partners had HPV infection at genital site were carefully counselled to follow some strict advices aimed to clear the virus (such as hygiene of both of their reproductive tract and their hand; using personal underwear and personal towels only; avoiding oral and anal sex) and monitored at 6, 12, 18, and 24 months. Counselled couples had a significantly higher clearance rate and shorter time of viral persistence, compared to non-counselled infected controls [66].

Recent evidence has moreover suggested that HPV vaccination is a valid tool even in patients who have already contracted the infection. In fact, it has been demonstrated that HPV infected patients receiving vaccination have a faster rate of seroconversion and greater viral clearance compared to infected patients who did not receive vaccination [67]. In detail, vaccinated patients showed a higher viral clearance that paralleled an improvement of sperm motility and a reduction in the percentage of anti-sperm antibodies. Furthermore, couples where the male partner received vaccination recorded higher pregnancy and delivery rates and a lower miscarriage rate [68].

In addition to counselling and adjuvant vaccination, different techniques of sperm selection (centrifugation, discontinuous density gradient, and direct Swim-up) have been tested aimed to remove HPV from the sperm surface. However, all techniques had very poor or even absent effect in the complete removal of the virus. Very recently, our group tested a modified swim-up technique with the addiction of hyaluronidase enzyme obtaining the complete elimination of HPV from infected samples [69]. The rationale of this treatment was to cleave the binding of HPV to its putative ligand, Syndecan-I, located on the sperm surface. Compared to normal swim-up technique, the modified swim-up with hyaluronidase was able to abolish the binding between HPV and sperm in 100% cases of infected sperm, confirmed by negative fluorescent in-situ hybridization

(FISH) for HPV, without any significant impairment of either motility or DNA fragmentation in the spermatozoa.

12.3 Obesity and Male Infertility

Obesity is defined as an abnormal or excessive accumulation of fat. According to the World Health Organization (WHO), a body mass index (BMI) that is greater than or equal to 25 kg/m^2 is classified as overweight, a BMI greater than 30 kg/m^2 is considered obesity, and a BMI greater than 40 kg/m^2 is considered severe obesity [70].

The worldwide prevalence of obesity has risen dramatically in the last decades, so that 1.9 billion adults worldwide are overweight and around 650 million have obesity [71].

Couples with an obese male partner have a significantly higher risk of infertility than couples with normal-weight male partners [72]. Moreover, male obesity negatively affects the success of assisted reproductive technology (ART) [72]. Mushtaq et al. reported in 2018 that male obesity is associated with a significant reduction in pregnancy and live birth rates in intracytoplasmic sperm injection cycles [73].

Previous population-based studies reported a reduction of semen parameters in overweight and obese men [72, 74, 75]. Further studies demonstrated the association between obesity and asthenozoospermia or teratozoospermia [76]. A systematic review and meta-analysis performed by Sermondade et al. in 2013 reported the association between obesity and azoospermia/oligozoospermia [77].

Several mechanisms have been hypothesized to explain obesity-induced sperm dam- age [78].

A pivotal role is played by heat-induced damage. Testicular thermal stress increases in obese men and is mainly due to fat accumulation in the suprapubic region and around the pampiniform plexus. Spermatogenesis is a temperature-dependent process, and an increase in scrotal temperature can disrupt its progression. In detail, obese men have both right and left scrotal temperatures that are significantly higher than control subjects. Healthy controls had a mean scrotal temperature of 34.738 °C, about two degrees below core body temperature. Moreover, in these subjects, fluctuations of scrotal temperature were characterized by a pattern similar to a circadian rhythm, with high fluctuations between day and night, between daily activities, and between different body postures. In contrast, obese men showed continuously increased mean scrotal temperatures (35.388 °C), with temperature fluctuations reduced in number and amplitude, and circadian patterns less evident or even absent, as reported in Fig. 12.3.

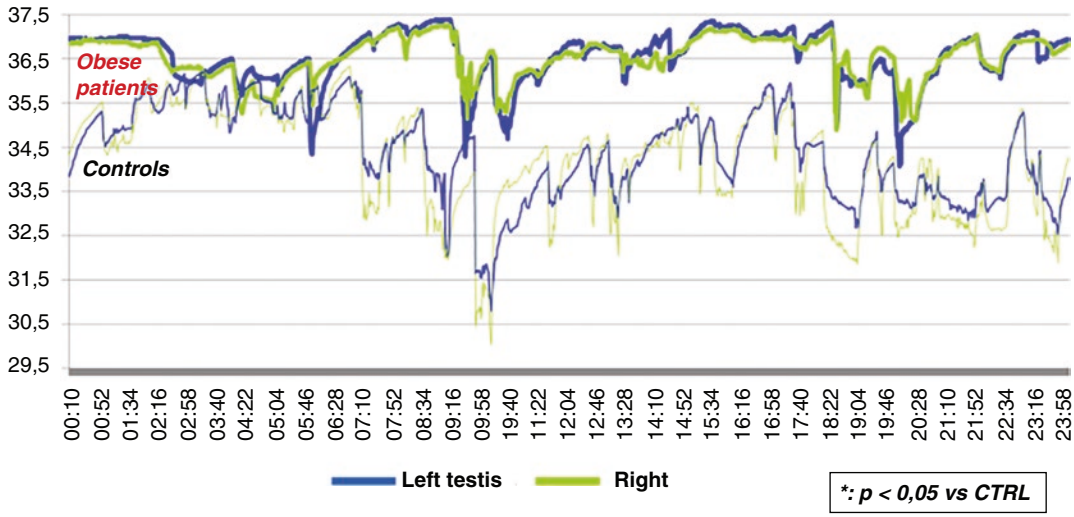

Fig. 12.3 Scrotal temperature in obese subjects and controls

These data underline that in obese men, the spermatogenic alteration is primarily related to hyperthermia due to the excess of adipose tissue [79].

Furthermore, several hormonal alterations have been described and contribute to impairing spermatogenesis in obese patients.

Most obese males have impaired reproductive hormonal profiles compared to normal-weight men. The excessive visceral fat decreases the serum sex hormone-binding globulin (SHBG), total and free T, and inhibin B levels and increases the conversion of T into 17ß-estradiol due to greater aromatase activity [80].

We have recently demonstrated in explants of subcutaneous adipose tissue (SAT) from obese males the presence of higher levels of intracellular T and E2, compared with lean subjects. In addition, after adrenergic stimulation, T release is reduced in obese SAT. Testosterone accumulation resulted in even lower expression in androgen-responsive genes, further contributing to adipose tissue dysfunction and to systemic hypogonadism, as evidenced by higher estrogens determined by increased aromatase expression in obese SAT [81]. Aromatase activity indeed increases with the body fat mass and further increases fat accumulation, creating a vicious cycle [82]. In obese male patients, the higher aromatase activity leads to a decreased T/E2 ratio [83].

Furthermore, obesity is associated with higher serum SHBG, so furtherly reducing free testosterone levels [84]. In addition, the associated hyperinsulinemia has a direct inhibitory effect on spermatogenesis, increasing nuclear and mitochondrial DNA damage [85].

12.4 Conclusions

In conclusion, exposure to environmental risk factors, such as pollutants or infections, or adverse lifestyle, i.e., causing obesity, may interfere at different levels with male fertility, and might play a major role in situations of idiopathic infertility. We have provided three examples, describing their role as a cause of male infertility, from pathophysiology to clinical assessment. As a consequence, their role should be considered in the clinical workflow of male infertility.

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