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Male infertility due to spermatogenic failure: current management and future perspectives

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Abstract

Spermatogenic failure has been recognized as the most severe presentation of male infertility in humans. Although it usually results in azoospermia. Approximately 30 to 60% of such men have sparse foci of sperm production within their dysfunctional testes, which can be extracted and used for *in vitro* fertilization techniques to produce a viable offspring. The scope of spermatogenic failure-related infertility covers a wide spectrum from genetic studies to hormonal control, microsurgical and medical therapy to assisted reproduction techniques, as well as innovative stem cell research aiming at creating artificial gametes. From a medical perspective, the management of men with spermatogenic failure seeking fertility involves a series of steps that includes the differential diagnosis of azoospermia, selection of the candidates for surgical sperm retrieval using molecular biology diagnosis, identification of those who could benefit from medical and surgical interventions prior to sperm retrieval, application of the best method to surgically retrieve testicular spermatozoa, and the use of state-of-art *in vitro* fertilization techniques. A coordinated multidisciplinary effort involving urologists, andrologists, geneticists, reproductive endocrinologists and embryologists will offer the best possible chance of achieving a biological offspring to men with spermatogenic failure.

Keywords: azoospermia, diagnosis, genetics, human, male infertility, sperm retrieval techniques, spermatogenic failure, therapeutics.

Introduction

Men in reproductive age deliver, on average, 96 million sperm at each ejaculation (Cooper *et al*., 2010). Approximately 1% of all men and 10 to 15% of infertile males have azoospermia, defined as a complete absence of spermatozoa in the ejaculate without implying an underlying etiology (Esteves *et al*., 2011a; Aziz, 2013). In about 2/3 of these men azoospermia is associated with a spectrum of untreatable testicular disorders that results in spermatogenic failure (SF). Spermatogenic failure (also known as non-obstructive azoospermia) has been recognized as the most severe presentation of infertility in humans (Esteves and Agarwal, 2013b). Although SF invariably results in infertility it does not necessarily indicate absolute sterility. While infertility, defined as the inability of a sexually active couple with no contraception to achieve natural pregnancy within at least one year (World Health Organization - WHO, 2000), implies a reduced but not unattainable potential to achieve pregnancy, sterility is denoted by permanent and complete inability to induce or achieve pregnancy. Of note, it has been shown that 30 to 60% of men with SF have sparse areas exhibiting full spermatogenic activity within their dysfunctional testes. Sperm production, if present, is insufficient for sperm appearance in the ejaculate, and since there are no treatment options to restore fertility in these men, the only alternative is to attempt sperm retrieval with the aim of finding viable testicular sperm to be used for intracytoplasmic sperm injection (ICSI; Silber, 2000; Esteves and Agarwal, 2011; Esteves *et al*., 2011b). Testicular sperm are capable of inducing normal fertilization and embryo development, as well as result in the production of healthy offspring with ICSI (Carpi *et al*, 2009; Belva *et al*., 2011; Esteves *et al*., 2014).

The management of men with spermatogenic failure seeking fertility has been a challenge for urologists, andrologists and reproductive medicine specialists alike. In this review, I present a medical perspective including the lessons I have learned after 15 years dealing with this male infertility condition. Figure 1 depicts an algorithm to guide clinicians on the management of azoospermic men with spermatogenic failure. I hope the information presented here could help clinicians to offer an even better care to men with spermatogenic failure seeking fertility.

Differential diagnosis in azoospermia

Azoospermia is defined based on the absence of spermatozoa in a given ejaculate. Proper laboratory technique is crucial to reduce analytical error and enhance precision when analyzing semen specimens (Esteves *et al*., 2012; Aziz, 2013). Ejaculates of men with spermatogenic failure usually have normal volume and pH, which indicates both functional seminal vesicles and patent ejaculatory ducts. The lower reference limits for ejaculate volume and pH are 1.5 ml (5th percentile, 95% confidence interval 1.4-1.7) and 7.2, respectively (Cooper *et al*., 2010). Retrograde ejaculation should be suspected when ejaculate volume is <1 ml, which can be confirmed by the finding of spermatozoa in the post-ejaculatory urine (Esteves *et al*., 2011a).

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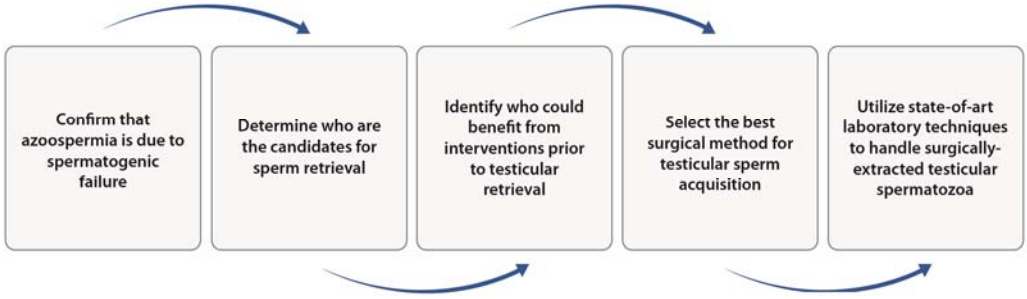

Figure 1. Diagram depicting the main steps to be considered in the clinical management of azoospermic men with spermatogenic failure seeking fertility.

The assessment of an initially normal volume azoospermic ejaculate should be immediately followed by the examination of the pelleted semen to exclude cryptozoospermia, which is defined by the presence of a very small number of live sperm (Aziz, 2013). In one study, centrifuging semen at a low speed of 200 *g* for 10 min revealed that 22.8% of men diagnosed with azoospermia had some spermatozoa in the semen pellet (Jaffe *et al.*, 1998). In addition, when supernatants resulting from low speed centrifugation were centrifuged at higher speeds (>1000 *g*) for longer periods, spermatozoa were also detected (Corea *et al.*, 2005). Therefore, the accuracy of any centrifugation protocol of less than 1000 *g* in pelleting all the spermatozoa in an ejaculate is uncertain (Cooper *et al.*, 2006). The importance of finding such minimal number of sperm lies on the fact that it allows assisted reproductive techniques (ART) to be performed with ejaculated sperm, thus avoiding the more invasive sperm retrieval methods. At our institution, we perform centrifugation at 3000 *g* for 15 min, which is followed by a careful examination of the pellet for the presence of sperm. Moreover, the diagnosis of azoospermia should be based on the examination of multiple semen specimens as transient azoospermia secondary to toxic, environmental, infectious or iatrogenic conditions may occur (Castilha *et al.*, 2006; Keel, 2006). The examination of multiple ejaculates to confirm azoospermia is also important given the large biological variability of semen specimens from the same individuals (Castilha *et al.*, 2006; Keel, 2006; Esteves *et al.*, 2012). Patients should receive clear instructions on how to collect the entire ejaculate and to report the loss of any fraction of the sample. Determination of fructose, a major component of seminal vesicle secretion, is usually not necessary because the presence of a normal volume ejaculate coupled with normal pH practically excludes any problem at the ejaculatory ducts or seminal vesicles levels (Esteves *et al.*, 2011a). In summary, azoospermia should be defined based on the absence of spermatozoa in a microscopic evaluation of multiple semen specimens after centrifugation.

History and physical examination and hormonal analysis (follicle-stimulating hormone and total testosterone serum levels) are undertaken to define the type of azoospermia. Together, these factors provide a >90% prediction of the type of azoospermia (obstructive *vs*. non-obstructive). Obstructive azoospermia (OA) is attributed to a mechanical blockage occurring anywhere along the reproductive tract, including the vas deferens, epididymis, and ejaculatory duct. OA is considered to be one of the most favourable prognostic conditions in male infertility since spermatogenesis is not disrupted, unlike non-obstructive azoospermia (American Society for Reproductive Medicine and Society for Male Reproduction and Urology, 2008; Esteves *et al.*, 2013a). Etiology conditions associated with non-obstructive azoospermia (NOA) include genetic and congenital abnormalities, post-infectious, exposure to gonadotoxins, medications, varicocele, trauma, endocrine disorders, and idiopathic. A detailed medical history should be obtained for any factor that may cause spermatogenic failure. Information not exclusive of the following areas should be collected: a) previous diseases during childhood and puberty such as viral orchitis and cryptorchidism; b) surgeries performed, especially those involving the pelvic and inguinal regions and genitalia; c) genital traumas; d) infections such as orchiepididymitis and urethritis; e) physical and sexual development; f) exposure to gonadotoxic agents such as radiotherapy, chemotherapy and steroid abuse (Carpi *et al*. 2009; Esteves *et al*., 2011a).

Physical examination in men with spermatogenic failure usually reveals normal epididymides and palpable vasa deferentia. Small-sized testes (<15 ml in volume) are often encountered as approximately 85% of the testicular parenchyma is involved in spermatogenesis. Nevertheless, testicular size is not a reliable clinical marker of sperm production. Men with spermatogenic maturation arrest, in whom spermatogenesis is hampered prior to its completion, have well developed and normal-volume testes (Sokol and Swerdloff, 1997; Hung *et al*., 2007).

The serum levels of follicle-stimulating hormone (FSH) are usually elevated whilst testosterone is either low (<300 ng/dl) or within lower limits in men with spermatogenic failure (Esteves *et al.*, 2011a; Gudeloglu and Parekattil, 2013). FSH levels greater than twice the upper normal limit is a reliable indicator of spermatogenic failure (American Society for

Reproductive Medicine and Society for Male Reproduction and Urology, 2008). In one report, low testosterone levels were found in 45% of the males with SF who visited an infertility clinic (Sussman *et al*., 2008). In another study evaluating hormonal data of 736 men with SF who were candidates for sperm retrieval, 346 (47%) had baseline total testosterone (TT) levels <300 ng/dl (Reifsnyder *et al*., 2012). Low testosterone levels often reflect Leydig cell insufficiency, which is accompanied by elevated (or within upper limits) luteinizing hormone (LH) levels (Bobjer *et al*., 2012; Reifsnyder *et al*., 2012). Nevertheless**,** low testosterone levels in men with SF may also result from obesity and metabolic dysfunction (Tchernof *et al.*, 1995; Kumar, 2013). Obesity is associated with an increased serum estradiol levels due to the increased peripheral aromatization of C19 androgens (androstenedione, T) under the influence of aromatase, a product of the CYP19 gene, specially in individuals with high tetranucleotide TTTA repeat polymorphism (TTTAn) present in intron 4 of the CYP19 gene (Hammoud *et al*., 2010). Elevated estradiol levels suppress pituitary LH and FSH secretion and also directly inhibit testosterone biosynthesis (Kumar, 2013). Moreover, Isidori *et al*. (1999) have demonstrated that excess circulating leptin is also an important contributor to the reduced androgens serum levels in male obesity. Their data have indicated that leptin has negative actions on steroidogenesis, mediated by specific receptors in the Leydig cells. Low testosterone levels could also reflect an adaptation to changed SHBG-levels and not testosterone deficiency. As a matter of fact, Strain *et al*. (1994) have reported that, during weight loss, serum SHBG levels increase at an average slope of 0.43 nmol L per unit decrease in body mass index (BMI). Hence, the increased serum TT concentrations as seen after weight loss may be due to a combination of mechanisms that include: (i) an increased binding capacity of SHBG, (ii) an increased amplitude of spontaneous LH pulses, (ii) a decreased androgen aromatization, and (iv) a decrease in circulating leptin and insulin concentrations.

Surprisingly enough, a normal endocrine profile can also be found in men with spermatogenic failure. Control feedback of FSH and LH secretions is based on the number of spermatogonia and Leydig cells, respectively, which is well preserved in men with maturation arrest. It has been reported that patients with diffuse spermatogenic maturation arrest and 10% of those diagnosed with Sertoli-cell-only syndrome (SCOS) present non-elevated endogenous gonadotropins (Sokol and Swerdloff, 1997; Hung *et al*., 2007).

Lastly, it is also important to differentiate azoospermia due to spermatogenic failure from azoospermia due to hypogonadotropic hypogonadism (HH) as both conditions fall in the category of non-obstructive azoospermia. HH is an endocrine disorder characterized by failure of spermatogenesis due to lack of appropriate stimulation by gonadotropins whilst spermatogenic failure comprises the most severe conditions associated with an intrinsic testicular impairment (Fraietta *et al*., 2013). Men with NOA due to HH have remarkably low levels of pituitary gonadotropins (FSH and LH levels below 1.2 mUI/ml) and androgens, and usually have signs of absent or poor virilization. This category of NOA includes not only patients with congenital forms of HH but also men whose spermatogenic potential has been suppressed by excess exogenous androgen administration. Although it is out of my scope to discuss HH in detail, it is worth to mention that patients with HH, albeit rarely seen in the clinical settings, benefit from specific hormonal therapy and often show remarkable recovery of spermatogenic function with exogenously administered gonadotropins or gonadotropin releasing hormone (Fraietta *et al*., 2013).

The 'gold-standard' test to confirm that azoospermia is due to SF is testicular biopsy and histopathology analysis. Hypospermatogenesis, germ cell maturation arrest, germ cell aplasia (Sertoli-cell-only syndrome), tubular sclerosis, or a combination of those, are usually found on the histological examination of testicular biopsy specimens in spermatogenic failure. Biopsies can be performed using percutaneous or open methods. Histopathology results have been used not only to confirm the diagnosis of SF but also to predict the chances of finding testicular sperm on retrievals. In a recent study from our group evaluating 356 men with spermatogenic failure, patients with Sertoli cell-only had lower sperm retrieval rates (19.5%) compared with those with maturation arrest (40.3%; P = 0.007), and both categories had lower sperm retrieval rates (SRR) compared with hypospermatogenesis (100.0%; P < 0.001; Esteves and Agarwal, 2014). Although our data indicate that histopathology phenotypes have prognostic value for sperm retrieval, caution should be applied when interpreting these results because an advanced site of sperm production can be found in approximately 20% of the SCO cases, which represents the worst histopathology phenotype (Verza Jr and Esteves, 2011; Ashraf *et al*., 2013; Esteves and Agarwal, 2014; Esteves *et al*., 2014). Extraction of testicular tissue with the sole purpose of histopathology evaluation could potentially remove the rare foci of sperm production and thus jeopardize the chances of future retrieval attempts (Esteves *et al*., 2011b). Hence, we do not recommend routine testicular biopsy prior to sperm retrieval. We only perform testicular biopsies in the rare cases where a differential diagnosis between obstructive and non-obstructive azoospermia could not be established based on history, physical examination and endocrine evaluation. In these cases, our approach is to perform the procedure either using a percutaneous or an open ''window'' technique without testis delivery (Esteves *et al*., 2011a; Esteves and Verza Jr, 2012). Testicular biopsy specimens are placed in a fixative solution such as Bouin's, Zenker's or glutaraldehyde; formalin should

not be used as it may disrupt the tissue architecture. A fragment is taken for wet examination in addition to conventional histopathology analysis. When mature sperm is found on a wet examination, we routinely cryopreserve testicular tissue using the liquid nitrogen vapor technique (Esteves and Varghese, 2012; Esteves and Verza Jr, 2012).

In conclusion, proper laboratory techniques are needed to reduce the amount of analytical error and enhance sperm count precision when evaluating azoospermic specimens. The finding of an initially azoospermic semen should be followed by the examination of multiple specimens after centrifugation to exclude cryptozoospermia, which is defined by presence of a very small number of live sperm in a centrifuged pellet. Accurate assessment of very low sperm counts is aimed to avoid labeling men with very low sperm counts as azoospermic, and it is particularly relevant in the current era of ART. History and physical examination and hormonal analysis are undertaken to define the type of azoospermia, which will provide high diagnostic accuracy to discriminate azoospermia due to spermatogenic failure from obstructive azoospermia and hypogonadotropic hypogonadism (Table 1). Although the 'gold standard' diagnostic test to confirm spermatogenic failure is the testicular biopsy, removal of testicular tissue with the sole purpose of histopathology evaluation could potentially remove the rare foci of sperm production and thus jeopardize the chances of future retrieval attempts. Testicular biopsy prior to sperm retrieval is therefore not routinely recommended. Testicular biopsy can be performed in selected cases provided a wet prep examination and sperm cryopreservation is available.

Defining who are the candidates for sperm retrieval

Owed to the untreatable nature of spermatogenic failure, sperm retrieval (SR) and ART are the only options for these men to generate their own biological offspring. Uncertainty of sperm acquisition, however, makes prognostic factors very desirable. Though factors such as etiology, testicular volume, serum levels of pituitary gonadotropins, and testicular histopathology results reflect a global spermatogenic function, they cannot accurately discriminate individuals in whom foci of sperm production will be found upon SR. In a series involving 60 men with SF, we determined the accuracy of commonly used prognostic parameters using a logistic regression analysis (Verza Jr and Esteves, 2011). We confirmed that FSH, testosterone, and testicular volume have low accuracy in predicting a positive sperm extraction as the areas under the receiver-operating characteristic (ROC) curves were 0.53, 0.59 and 0.52, respectively. In another study, Tournaye and cols. combined clinical and laboratory parameters, such as testicular volume and FSH levels and histopathology results, and found that diagnostic accuracy was only 74% (Tournaye *et al*., 1997). Testicular sperm have been obtained in different etiology categories, including cryptorchidism, post-orchitis, Klinefelter syndrome, radio-/chemotherapy and idiopathic, with variable success rates ranging from 25 to 70% (Chan *et al*., 2001; Raman and Schlegel, 2003; Schiff *et al*., 2005, 2006; Esteves *et al*., 2010; Esteves, 2013). In summary, clinical parameters and endocrine profile are unreliable markers for determining the chances of sperm acquisition in men with azoospermia due to spermatogenic failure.

In contrast, the molecular diagnosis and subtyping of Y-chromosome microdeletions (YCMD) have been useful preoperative biomarkers to determine the chances of sperm retrieval in men with azoospermia due to YCMD (Krausz *et al*., 2000; Peterlin *et al*., 2002; Hopps *et al*., 2003; Simoni *et al*., 2008; Stahl *et al*., 2010; Esteves and Agarwal, 2011; Kleiman *et al*., 2011, 2012; Hamada *et al*., 2013). A microdeletion is as a chromosomal deletion that usually spans over several genes, but is smaller in size and cannot be detected using conventional cytogenetic methods such as karyotyping (Navarro-Costa *et al*., 2010; Hamada *et al*., 2013). The long arm of the Y chromosome contains a region at Yq11 that clusters 26 genes involved in spermatogenesis regulation (Repping *et al*., 2002; Simoni *et al*., 2008; Hamada *et al*., 2013; Krausz *et al*., 2014). This region is referred to as "Azoospermia Factor'' (AZF) because microdeletions at this interval is often associated with azoospermia (Fig. 2). The application of molecular biology technology has allowed the recognition of three AZF subregions designated as AZFa, AZFb and AZFc, each one including a major AZF candidate gene (Simoni *et al*., 2008; Krausz *et al*., 2014). It has been estimated that approximately 10% of men with azoospermia due to spermatogenic failure harbor microdeletions within the AZF region that might explain their condition (Simoni *et al*., 2008; Krausz *et al*., 2014).

From the medical point of view the following microdeletions have recurrently been found in men with spermatogenic failure (Navarro-Costa *et al*., 2010; Krausz *et al*., 2014): i. AZFa; ii. AZFb (P5/proximal P1); iii. AZFbc (P5/distal P1 or P4/distal P1); iv. AZFc (b2/b4). The most frequent deletion subtypes comprises the AZFc region (~80%) followed by AZFa (0.5-4%), AZFb (1-5%) and AZFbc (1-3%) regions (Krausz *et al*., 2014). Deletions differentially affecting these AZF subregions result in distinct disruption of germ cell development. AZFa deletions that remove the entire AZFa are invariably associated with the testicular histopathology phenotype of pure SCO with no residual areas of active spermatogenesis. Although partial AZFa deletions have been described and may be eventually associated with residual spermatogenesis, this event is extremely rare (Tyler-Smith and Krausz, 2009). Hence, the diagnosis of a deletion in the AZFa region implies that the chances of retrieving testicular spermatozoa for

ICSI are virtually nonexistent (Krausz *et al*., 2000; Hopps *et al*., 2003; Simoni *et al*., 2008; Kleiman *et al*., 2011; Vogt and Bender, 2013). The clinical feature of complete AZFb and AZFbc (P5/proximal P1, P5/distal P1, P4/distal P1) deletions are similar to AZFa deletions as the chances of finding spermatozoa on the attempts of sperm retrieval are close to zero (Krausz *et al*., 2000; Hopps *et al*., 2003; Kleiman *et al*., 2011). In AZFb and AZFbc deletions the most common testicular histopathology phenotype is spermatogenic maturation arrest, but SCO can also be found. Nevertheless, spermatid arrest and crypto/oligozoospermia have been reported in three patients with complete AZFb or AZbc deletions (Longepied *et al*., 2010; Soares *et al*., 2012). In addition, spermatozoa have been identified in rare cases of partial AZFb and AZFbc deletions (Kleiman *et al*., 2011). At present, however, given the difficulties to explain the biological nature of these unusual phenotypes it is sound to assume that the diagnosis of complete deletions involving AZFb or AZFbc (P5/proximal P1, P5/distal P1, P4/distal P1) implies that the chances of a successful testicular sperm retrieval is virtually zero (Krausz *et al*., 2014). In contrast, the chances of successful sperm retrieval in azoospermic men with SF due to AZFc deletions are 50-70% (Peterlin *et al*., 2002; Simoni *et al*., 2008). AZFc deletions are usually associated with residual spermatogenesis, and therefore testicular spermatozoa can be surgically retrieved and children can be conceived after ICSI (Kent-First *et al*., 1996; Mulhall *et al*., 1997; Kamischke *et al*., 1999; van Golde *et al*., 2001; Oates *et al*., 2002). The probability of fatherhood by ICSI seems to be unaltered by the presence of AZFc microdeletions (Kent-First *et al*., 1996; Mulhall *et al*., 1997; Kamischke *et al*., 1999; Cram *et al*., 2000; Oates *et al*., 2002; Peterlin *et al*., 2002) notwithstanding some authors have reported impaired embryo development in such cases (van Golde *et al*., 2001; Simoni *et al*., 2008). The male offspring born via ICSI from fathers with AZFc microdeletions will inherit the Yq microdeletion and as a result infertility. However, the exact testicular phenotype cannot be predicted as AZFc deletions may jeopardize the Y chromosome integrity, predisposing to chromosome loss and sex reversal. There is a potential risk to the 45,X0 karyotype and to the mosaic phenotype 45,X/46,XY in these offspring, which may lead to spontaneous abortion or genital ambiguity (Patsalis *et al*., 2000; Siffroi *et al*., 2000; Rajpert-De Meyts *et al*., 2011). Genetic counselling is therefore mandatory to provide information about the risk of conceiving a son with infertility and other genetic abnormalities.

Diagnostic testing for YCMD is based on a multiplex polymerase chain reaction (PCR) blood test aimed to amplify the AZFa, AZFb, and AZFc regions of the Y chromosome (Hamada *et al*., 2013). This technique primarily amplifies anonymous sequences of the Y chromosome using specific sequence-tagged sites (STSs) primers that are not polymorphic and are well-known to be deleted in men affected by azoospermia according to the known, clinically relevant microdeletion pattern (Krausz *et al*., 2014). To obtain uniform results, it is necessary to follow validated guidelines, such as those issued by the European Association of Andrology (EAA) and the European Molecular Genetics Quality Network (EMQN; Krausz *et al*., 2014). The basic set of PCR primers recommended by the EAA/EMQN in multiplex PCR reactions for the diagnosis of Yq microdeletion includes: sY14 (SRY), ZFX/ZFY, sY84 and sY86 (AZFa), sY127 and sY134 (AZFb), sY254 and sY255 (AZFc; Fig. 2). While the primer for the SRY gene is included as a control for the testis-determining factor on the short arm of the Y chromosome, the primers for the ZFX/ZFY genes act as internal controls because these primers amplify a unique fragment both in male and female DNA, respectively. A DNA sample from a fertile male and from a woman and a blank (water) control should be run in parallel with the set of primers. According to the current knowledge, once a deletion of both primers within a region is detected, the probability of a complete deletion is very high. The use of this primer set enables the detection of almost all clinically relevant deletions and of over 95% of the deletions reported in the literature in the three AZF regions (Krausz *et al*., 2014). However, as partial AZFa, AZFb and AZFbc deletions have been described and their phenotypic expression is milder than the complete ones (Krausz *et al*., 2000; Kleiman *et al*., 2011;), the definition of the extension of the deletion is now recommended in sperm retrieval candidates and should be based on additional markers as described by Krausz and colleagues (Krausz *et al*., 2014).

In conclusion, patients with azoospermia due to spermatogenic failure who are candidates for sperm retrieval and ICSI should be screened for Y chromosome microdeletions because the diagnosis of a deletion has prognostic value and can influence therapeutic options (Table 1). Retrieval attempts are not recommended in cases of complete deletion of the AZFa region. Sperm retrieval in azoospermic carriers of deletions of the AZFb or AZFbc regions may be eventually attempted. However, the patient should be fully informed about the very low/virtually zero chance to retrieve spermatozoa. Owed to reports of deletion carriers among men with non-idiopathic SF, including cryptorchidism, post-chemo-/radiotherapy, varicocele, and Klinefelter syndrome, the presence of any of these diagnostic categories, accompanied by azoospermia should be an indication for YCMD screening testing (Krausz *et al*., 1999; Mitra *et al*., 2006). Genetic counselling should be offered to men with AZFc deletions who are candidates for sperm retrieval because testicular spermatozoa used for ICSI will invariably transmit the deletion from father to son. Although the likely result is azoospermia, AZFc microdeletions might be associated with an increased risk of miscarriage and other genetic abnormalities in the offspring.

Table 1. Interventions and recommended actions in the clinical management of azoospermic men with spermatogenic failure seeking fertility.

Clinical management phase	Intervention	Action	Interpretation
Differential diagnosis of azoospermia subtype	Medical history, physical examination, endocrine profile (FSH and testosterone levels at a minimum; LH, prolactin, thyroid hormones and estradiol are added as needed), and examination of pelleted semen in multiple occasions. Testicular biopsy could be considered in the few cases in which the differential diagnosis is not determined	Confirm that azoospermia is due to spermatogenic failure, and exclude the men with severely impaired spermatogenesis in whom sperm is found in ejaculates	A differential diagnosis between obstructive azoospermia, hypogonadotropic hypogonadism and spermatogenic failure should be performed as treatment strategies and outcomes vary according to the type of azoospermia
Determination of the individuals who are candidates for an sperm retrieval attempt	Y chromosome microdeletion screening using multiplex (PCR) blood test. The basic set of PCR primers recommended by the EAA/EMQN to be used in multiplex PCR reactions for the diagnosis of Yq microdeletion includes: sY14 (SRY), ZFX/ZFY, sY84 and sY86 (AZFa), sY127 and sY134 (AZFb), sY254 and sY255 (AZFc)	Deselect men with microdeletions involving subregions AZFa, AZFb and AZFb+c	Approximately 10% of men with azoospermia due to spermatogenic failure harbor microdeletions within the AZF region. The chances of sperm retrieval in men with YCMD involving the subregions AZFa, AZFb and AZFb+c are virtually nill and such patients should be counseled accordingly. The chances of a successful sperm retrieval in men with AZFc deletions range from 50-70%. Genetic counselling should be offered to men with AZFc deletions because testicular spermatozoa used for ICSI will invariably transmit the deletion from father to son
Identification of the patients to whom interventions prior to testicular retrieval can be offered	Determination of the serum levels of total testosterone and estradiol	Medical treatment with gonadotropins, aromatase inhibitors or clomiphene citrate should be considered for the patients with hypogonadism (TT < 300 ng/dL) or T/E ratio < 10	Patients should be counseled that the evidence of a positive effect of medical treatment is limited, and such interventions are at present considered empirical
	Physical examination to identify the presence of clinical varicocele and analysis of testicular biopsy results (if available)	Microsurgical repair of clinical varicocele	Microsurgical varicocele repair is associated with better outcome concerning recurrence and postoperative complications. Patients with testicular histopathology indicating Sertoli cell-only are unlikely to benefit from varicocele repair. Evidence of a positive effect of varicocele repair is limited, and patients should be counseled accordingly
Selection of the most effective surgical method for testicular sperm acquisition	Analysis of testicular biopsy results (if available) and of whether sperm have been obtained in previous treatment and by which method	Microdissection testicular sperm extraction. Conventional testicular sperm extraction may be considered in cases of previous success with TESE, particularly when testicular histopathology indicates hypospermatogenesis	Micro-TESE in SF is associated with a more favourable sperm retrieval rate ranging from 42.9 to 63% compared with 16.7 to 45% in conventional TESE. The lower tissue removal facilitates sperm processing and lessens testicular damage
Application of state-of-art laboratory techniques to handle surgically-extracted testicular spermatozoa	Extraction of a minimum volume of tissue by micro-TESE facilitates tissue processing and search for sperm. Testicular tissue preparation techniques include mechanical and enzymatic mincing and erythrocyte lysis.	Sterile techniques, stable pH and temperature, and high laboratory air quality conditions useful to optimize micromanipulation efficiency and safety assurance. Excess sperm not used for ICSI should be cryopreserved for future attempts	Spermatozoa collected from men with SF should be handled with great care because they are often compromised in quality and are more fragile than ejaculated counterparts. The reproductive potential of such gametes used for ICSI is differentially affected by SF.

ICSI: intracytoplasmic sperm injection; micro-TESE: microdissection testicular sperm extraction; PCR: polymerase chain reaction; T/E: testosterone to estradiol ratio; TESE: testicular sperm extraction; TT: total testosterone.

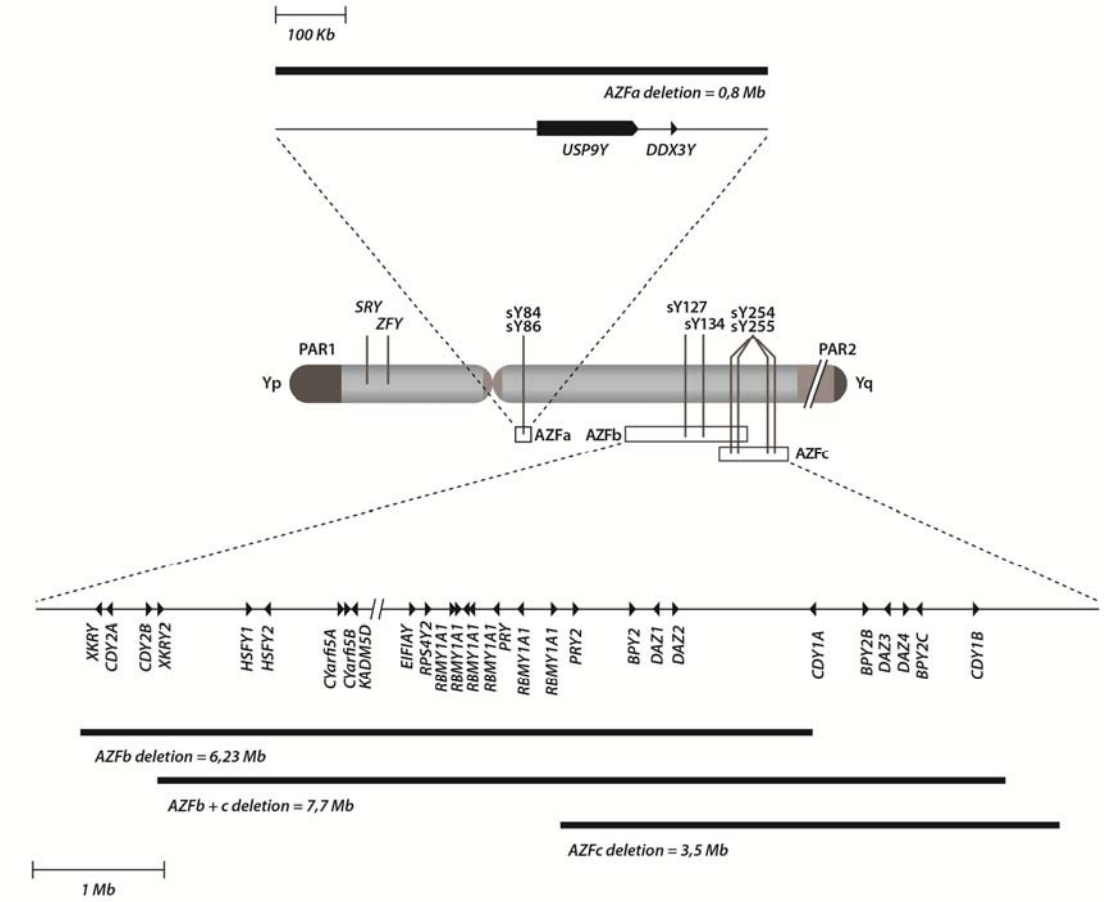

Figure 2. Human Y chromosome map depicting the AZF subregions and gene content. The AZFa region is maps from approximately 12.9 to 13.7 Mb of the chromosome and contains two single copy genes, USP9Y and DDX3Y. AZFb spans from approximately 18 to 24.7 Mb of the chromosome and AZFc from aproximately 23 to 26.7 Mb. Both regions contain multiple genes as depicted in the botton of the figure. The location of the basic set of sequence-tagged sites primers to be investigated in azoospermic men with spermatogenic failure, according to the European Association of Andrology (EAA) and the European Molecular Genetics Quality Network (EMQN) 2013 guidelines (Krausz *et al*., 2014), are identified by solid vertical lines.

Defining who can benefit from interventions prior to sperm retrieval

After identifying who are the candidates for SR by excluding those patients with complete AZFa, AZFb and AZFbc microdeletions, the next step is to select the azoospermic patients with SF who could benefit from medical and surgical interventions prior to SR. While a positive clinical outcome has been observed after gonadotropin treatment in azoospermic men with hypogonadotropic hypogonadism, it is generally believed that medical therapy would be ineffective in SF due to the presence of high plasma levels of gonadotropins. Treatments that could improve sperm production in men with SF are highly expected since nearly half of them will be halted in their attempt to conceive due to the absence of testicular sperm on retrievals (Carpi *et al*., 2009; Esteves *et al*., 2011b; Kumar, 2013; Esteves *et al*., 2014).

Given that approximately 50% of men with azoospermia due to spermatogenic failure have hypogonadism, characterized by low endogenous levels (<300 ng/dl) of total testosterone (Sussman *et al*., 2008; Reifsnyder *et al*., 2012), recent studies have examined the effect of therapies that could boost testosterone production as potential targets for medical intervention. Testosterone is essential for spermatogenesis (Quigley *et al*., 1995; McLachlan *et al*., 2002), and it has been shown that its levels are more than 100-fold greater in the testes as compared with the serum (Jarow *et al*., 2001). Although the mechanism by which testosterone regulates the spermatogenic process in humans is not fully understood, intratesticular testosterone action on target cells seems to involve a paracrine mechanism on androgen receptors (ARs; Boukari *et al*., 2009; Kato *et al*., 2014). Enhancing testosterone production using medication could allow the restoration of adequate levels of intratesticular androgenic bioactivity that are

essential to sustain spermatogenesis in combination with adequate Sertoli cell stimulation with FSH (Coviello *et al*., 2004). Drugs that have been utilized include clomiphene citrate, gonadotropins (human chorionic gonadotropin and FSH), and aromatase inhibitors (Pavlovich *et al*., 2001; Schiff *et al*., 2005, 2006; Ramasamy *et al*., 2009, 2012; Reifsnyder *et al*., 2012; Ashraf *et al*., 2013; Hussein *et al*., 2013; Kumar, 2013).

Clomiphene citrate is a selective estrogen receptor modulator that competitively binds to estrogen receptors on the hypothalamus and pituitary gland. As a result, the pituitary perceives less estrogen, which leads to the secretion of both FSH and LH. The latter binds to LH receptors that are present in the Leydig cells and induces androgen secretion and a consequent rise in testosterone levels (Kumar, 2013). Human chorionic gonadotropin (hCG) is a glycoprotein similar to the native LH, but with a higher receptor affinity and half-life compared with LH (Kumar, 2013; Leão and Esteves, 2014). HCG binds to the same LH receptor at the Leydig cell level, thus stimulating the production of androgens. Aromatase inhibitors, on the other hand, block the aromatase enzyme, which is present in the adipose tissue, liver, testis and skin, and is responsible for converting testosterone and other androgens to estradiol. Aromatase inhibitors have been particularly used in obese/overweight men who often have aromatase hyperactivity and consequently, elevated estradiol levels (Hammoud *et al*., 2010). Estradiol suppresses pituitary LH and FSH secretion and also directly inhibits testosterone biosynthesis. This results in an imbalance in the testosterone and estradiol (T/E) ratio, which may be reversible by oral administration of aromatase inhibitors (Pavlovich *et al*., 2001; Reifsnyder *et al*., 2012). The aforementioned drug categories have been used in combination or alone.

In an early study, including 43 men with SF associated with various etiologies, Pavlovich and colleagues reported that an increase in T/E ratio was obtained after treatment with aromatase inhibitors, but none of the treated men experienced a return of sperm into the ejaculate (Pavlovich *et al*., 2001). In a series involving 42 men with non-mosaic Klinefelter syndrome and azoospermia, Schiff and colleagues administered aromatase inhibitors alone or in combination with clomiphene citrate or hCG prior to sperm retrieval (Schiff *et al*., 2005, 2006). The authors observed an increased SR rate in men who had received medical therapy. In a later series from the same group also involving non-mosaic Klinefelter patients, the authors reported that the SR rates were increased by 1.4-fold in the men who responded to medical therapy, determined by an elevation of 150 ng/dl testosterone from baseline, compared to the ones who did not (Ramasamy *et al*., 2009). In a recent study involving a large cohort of 442 men with azoospermia due to spermatogenic failure who received medication (clomiphene citrate and hCG) prior to SR, the investigators aimed at achieving 600 to 800 ng/dL posttreatment serum testosterone. In this study, SR rates were significantly higher in the group of patients who achieved the desired hormonal level post-medical therapy (57 *vs*. 33.6%; Hussein *et al*., 2013). Contrary results, however, have been observed in a large cohort of unselected men with SF treated with aromatase inhibitors, clomiphene citrate and hCG (Reifsnyder *et al*., 2012). In this aforementioned series involving 736 men, the authors observed that SR rates were not significantly different between treated and untreated individuals (52 *vs*. 53%) despite of a high positive response to medical therapy in terms of boosting endogenous testosterone levels.

Recently, Shinjo *et al*. (2013) demonstrated that the Leydig cells of men with SF produce increased amounts of intratesticular testosterone (ITT) in response to exogenous hCG stimulation even under a hypergonadotropic condition. The aforementioned authors studied a group of twenty men with SF and found that ITT levels were significantly higher after hCG treatment (pre: 273.6 ± 134.4; post: 1348.1 ± 505.4 ng/ml; $P < 0.0001$). LH secretion is determined by the frequency, amplitude and duration of its secretory pulses (Spratt *et al*., 1987). In men with SF, the relative amplitude of LH pulses is low because the basal LH levels are high (Shiraishi *et al*., 2012), thus indicating that the endogenous gonadotropin stimulation of Leydig and Sertoli cells is paradoxically weak (Keenan and Veldhuis, 2004). Not surprisingly, the percentage of Sertoli cells showing androgen receptors is significantly higher in the men with SF compared with those with normal spermatogenesis (23.7 *vs*. 18%, $P < 0.05$), despite of similar absolute number per seminiferous tubules (Kato *et al*., 2014).

It has been observed that endogenous FSH were suppressed below pre-adolescent levels through a negative feedback mechanism of elevated serum testosterone in over half of the azoospermic men with SF treated with hCG (Shiraishi *et al*., 2012). Such an effect could be beneficial since it has been shown that high plasma FSH levels, determining a down-regulation of FSH receptors, impair tubular function, and that an improvement in Sertoli cell function in men with severely impaired spermatogenesis was achieved after reduction of high FSH plasma concentration by administration of a GnRH analogue (Foresta *et al.* 2004). Sertoli cells have been considered to be a major target for testosterone signalling via the activation of nuclear androgen receptors (Griswold, 2005; Kato *et al.*, 2014). The Sertoli cells support male germ cell development and survival, and their function can be restored by elevated intratesticular testosterone (O'Shaughnessy *et al*., 2010). Interestingly, Shinjo *et al.* (2013) showed that basal ITT was lower in men with SF who responded to hormonal treatment and had sperm retrieved than those in whom any spermatozoa could be retrieved. Human chorionic gonadotropin treatment may

thus increase not only intratesticular testosterone, but also reset FSH action.

Although the exact mechanism underlying the beneficial effect of hCG therapy remains unclear, it has been speculated that hCG acts by stimulating spermiogenesis and spermatogonia DNA synthesis in patients with SF whose histological examination reveal hypospermatogenesis or late maturation arrest (Matthiesson *et al*., 2006; Aggarwal *et al*., 2009; Wistuba *et al*., 2010; Shinjo *et al*., 2013). These effects could result in the formation of well-differentiated seminiferous tubules that would be detected during sperm retrieval (Shiraishi *et al*., 2012).

Varicocele, found in approximately 5% of men with SF, has been also a target of intervention prior to sperm retrieval (Weedin *et al*., 2010; Miyaoka and Esteves, 2012). While it is still debatable whether varicocele is merely coincidental or contributory to spermatogenesis disruption, the surgical repair of clinical varicoceles, particularly using microsurgical techniques, has been carried out in an attempt to improve sperm production in such men (Esteves and Glina, 2005; Weedin *et al*., 2010; Miyaoka and Esteves, 2012). The goals are to allow the appearance of small quantities of sperm in the ejaculate or increase the chances of retrieving sperm from the testis. Sperm production restoration, albeit minimal, will facilitate sperm injection procedures. In an early study, we evaluated a group of 17 men with clinical varicocele and azoospermia due to SF who underwent microsurgical subinguinal varicocele repair (Esteves and Glina, 2005). In a mean postoperative follow-up of 19 months, 35.3% (6/17) of the patients had motile sperm in ejaculates with a mean sperm count of 0.8 million/ml (range 0.1-1.8). A testicular biopsy obtained for analysis revealed that the histopathology phenotype was associated with the surgical outcome. Viable sperm was identified in the ejaculates of 72.7% (8/11) of the patients with hypospermatogenesis or maturation arrest, in contrast to none (0/6) of those with SCO (Esteves and Glina, 2005). Subsequently, a meta-analysis of eleven case series, including our own, involving 233 patients with clinical varicocele and azoospermia showed similar results (Weedin *et al*., 2010). At a mean postoperative follow-up of 13 months, motile sperm was found in ejaculates of 39% of the subjects. With a mean sperm count of 1.6 million/ml, natural and assisted conceptions were obtained in 26% of the men. Analysis of testicular biopsies taken either prior or during varicocele repair revealed that hypospermatogenesis and maturation arrest were significantly more likely to be associated with the presence of sperm in the postoperative ejaculate compared with Sertoli cell-only (odds ratio 9.4; CI 95% 3.2-27.3; Weedin *et al*., 2010)

Although the aforementioned studies indicate that improvements in sperm production can be achieved in approximately one third of men with azoospermia after varicocelectomy, most of the treated individuals will either remain azoospermic or have an inadequate number of sperm in the ejaculated for ICSI (Schlegel and Kaufmann, 2004). In such cases, sperm retrieval will be the only alternative, and therefore the validity of a varicocele operation has been questioned. In one study, Schlegel and Kaufmann reported that 22% of the patients had sperm on a post-varicocelectomy semen analysis at an average follow-up of 14.7 months, but only 9.6% had adequate motile sperm in the ejaculate for ICSI to avoid a surgical sperm extraction (Schlegel and Kaufmann, 2004). In this aforementioned retrospective study involving 138 patients who had undergone SR, similar retrieval rates of 60% per attempt were obtained regardless of whether or not varicocelectomy had been carried out. In contrast, two retrospective series have shown a beneficial effect of varicocelectomy applied to azoospermic patients with SF and clinical varicocele. Inci and colleagues, studying a group of 96 men, observed that retrieval rates were significantly higher in treated (53%) compared with untreated men (30%; $P = 0.03$), which represented a 2.6 fold increase in the chances of identifying sperm at a retrieval attempt (odds-ratio [OR]: 2.63; 95% confidence interval [CI] of 1.05-6.60; Inci *et al*., 2009). Along the same lines, in a study involving 66 men, Haydardedeoglu and cols. reported higher retrieval rates in men who have had varicocele repair prior to SR (61%) compared with untreated men (38%; $P < 0.01$; Haydardedeoglu *et al*., 2010).

In conclusion, interventions prior to SR including medical therapy to boost endogenous testosterone production and microsurgical varicocele repair can be offered to selected patients with azoospermia due to spermatogenic failure (Table 1). Although an overall beneficial effect has been observed, the evidence is currently limited and based mostly on case series. Hence, a firm conclusion on the role of medical and surgical intervention therapy in men with spermatogenic failure and azoospermia cannot be drawn yet. Randomized controlled trials are needed to precisely assess the impact of such interventions on sperm production and sperm retrieval outcomes.

Defining what is the best method of sperm retrieval for azoospermic men with spermatogenic failure

Sperm retrieval techniques should be aimed at offering the highest possible chance of obtaining an adequate number of good quality testicular sperm, which can be immediately used for ICSI or cryopreserved for future ICSI attempts. Retrieval methods should also minimize testicular damage, thus preserving androgen activity and the chance of repeated retrieval attempts.

The method of choice for sperm acquisition in azoospermia due to SF has been the conventional testicular sperm extraction (TESE), with a mean reported SRR of 49.5% (Donoso *et al*., 2007). In TESE, open single or multiple testicular biopsies are randomly

taken, processed and examined for the presence of sperm (Tournaye *et al*., 1997; Carpi *et al*., 2009; Esteves *et al*., 2011b, 2013b). Since the prediction of both the existence and the geographic location of the islets of normal spermatogenesis are not possible prior to SR, more than one specimen is usually required until sperm is found. TESE with multiple biopsies resulted in higher SRR than fine-needle aspiration (TEFNA), a variation of testicular sperm aspiration (TESA), particularly in the cases of SCO and maturation arrest (Donoso *et al*., 2007). A disadvantage of TESE is that removal of large fragments of testicular tissue might jeopardize the already compromised androgen production, in a transient or permanent way, and therefore results in severe hypogonadism (Schlegel and Su, 1997). Also, laboratory processing of such large quantities of testicular tissue taken by TESE is time-consuming and labor intensive (Schlegel 1999; Esteves and Varghese, 2012; Esteves and Verza Jr, 2012).

Microdissection testicular sperm extraction (micro-TESE) is a microsurgical method of sperm retrieval that has been proposed as a better alternative to TESE in cases of spermatogenic failure (Schlegel, 1999). The reasons are the greater success at obtaining sperm, ranging from 43 up to 70%, and the lowest tissue removal that facilitates sperm processing and lessens testicular damage (Schlegel, 1999; Amer *et al*., 2000; Okada *et al*., 2002; El-Haggar *et al*., 2007; Tsujimura, 2007; Esteves *et al*., 2011b; 2013b). The rationale of micro-TESE is to identify focal areas of sperm production within the testes, based on the size and appearance of the seminiferous tubules, with the aid of the operating microscope (Schlegel, 1999;). Such areas are selectively extracted thus allowing minimal tissue removal, which has been shown to be 50 to 70-fold less when compared with conventional TESE (Schlegel, 1999; Esteves *et al*., 2011b, 2013b). The use of optical magnification also reduces the chances of vascular injury by proper identification of testicular blood supply, thus reducing the chances of hematoma formation and testicular devascularization (Esteves, 2013). Although a decrease in serum testosterone has been documented after removing testicular parenchyma by micro-TESE, especially in men with severely compromised androgen activity such as those with Klinefelter syndrome (Schiff *et al*., 2005, 2006), testosterone levels return to pre-surgical values in 95% of the subjects within 18 months following surgery (Ramasamy *et al*., 2005;).

In micro-TESE, a large incision is made in an avascular area of the tunica albuginea under 6-8X magnification, and the testicular parenchyma is widely exposed. The parenchyma is then dissected at 16 to 25X magnification to enable the search and isolation of seminiferous tubules exhibiting larger diameter in comparison with non-enlarged or collapsed counterparts. These enlarged tubules are more likely to contain germ cells and eventually normal sperm production (Fig. 3). Microsurgical-guided biopsies are performed by carefully removing such tubules, which are sent to the laboratory for examination. In addition to minimizing testicular damage, a smaller amount of tissue extracted facilitates laboratory processing and sperm search thus increasing the process efficiency (Schlegel 1999; Amer et al., 2000; Tsujimura, 2007; Esteves *et al*., 2011b, 2013b; Esteves and Varghese, 2012; Ashraf *et al*., 2013; Esteves, 2013).

In a controlled study of our group involving sixty men with SF, we compared SRR between micro-TESE and conventional single-biopsy TESE (Verza Jr and Esteves, 2011). The SRR was significantly higher with micro-TESE (45 *vs*. 25%; P = 0.005) both overall and after stratifying the patients by testicular histopathology phenotype (hypospermatogenesis: 93 *vs*. 64%; maturation arrest: 64 *vs*. 9%; Sertoli cell-only syndrome: 20 *vs*. 6%; P < 0.001). Controlled studies have corroborated our results showing that micro-TESE is associated with a higher sperm recovery and lower complication rates (below 5%) than conventional TESE (Amer *et al*., 2000; Okada *et al*., 2002; El-Haggar *et al*., 2007; Tsujimura, 2007). We have recently reported an updated experience involving three hundred and fifty-six patients with SF who have undergone Micro-TESE. SRR was 41.4% overall (Esteves *et al*., 2014), and 100.0, 40.3 and 19.5%, according to the histopathology phenotypes of hypospermatogenesis, maturation arrest and SCO, respectively (Esteves and Agarwal, 2014). Micro-TESE has been shown to rescue approximately one third of the cases that failed in previous retrieval attempts with conventional TESE and TESA, and is particularly useful for men with spermatogenic failure presenting the worst-case scenarios (Schlegel, 1999; Ashraf *et al*., 2013). Lastly, a recent systematic review involving seven comparative studies and 1062 patients confirmed that micro-TESE in SF was associated with a more favourable sperm retrieval rate ranging from 42.9 to 63% compared with 16.7 to 45% in conventional TESE (Deruyver *et al*., 2014).

In conclusion, the efficiency of sperm retrieval in azoospermia due to SF varies according to the method of sperm acquisition. Micro-TESE should be the method of choice for SR in such cases because it not only increases the chance of retrieving testicular sperm for ICSI but also minimizes testicular damage (Table 1).

Laboratory handling of testicular sperm

After sperm retrieval procedures, the extracted testicular parenchyma is immediately transferred to the embryology laboratory for sperm search after tissue dissection. The laboratory management of surgically retrieved gametes requires special attention because spermatozoa collected from men with SF are often compromised in quality and are more fragile than ejaculated counterparts (Verza Jr and Esteves, 2008). Both sperm DNA fragmentation and aneuploidy rates

are higher in testicular sperm obtained from men with spermatogenic failure compared with ejaculated sperm obtained from infertile men with various etiology categories (Meseguer *et al*., 2009; Vozdova *et al*., 2012). As a result, a lower fertilization, embryo development and pregnancy rates have been achieved when the gametes retrieved from men with SF are used for ICSI (Verza Jr and Esteves, 2008; Esteves *et al*., 2014).

The extraction of a minimum volume of tissue by using advanced surgical techniques, such as micro-TESE, is advantageous because the processing of TESE specimens may be incredibly labor-intensive. The searching process in large testicular tissue volumes may miss the rare spermatozoa in the sea of cells and non-cellular elements. Hence, the lower the amount of tissue to be processed the easier the sperm processing and search procedures (Esteves and Varghese, 2012). Testicular tissue preparation techniques designed to increase sperm retrieval rates have been used to handle these specimens, including mechanical and enzymatic mincing. These methods ensure tubular wall break down and cellular content loss (Baukloh, 2002; Aydos *et al*., 2005; Esteves and Varghese, 2012). After proper disintegration of the seminiferous tubules, specimens are processed to eliminate surplus tissue elements and red blood cells. This step can be achieved by using erythrocyte lysing solution and density gradient centrifugation, respectively (Esteves and Varghese, 2012; Ozkavukcu *et al*., 2014). Lastly, a series of Petri dishes are prepared containing oil-covered microdroplets of sperm culture media loaded with aliquots of processed testicular tissue. It offers the opportunity of an effective examination of the specimens by the embryologist, thus allowing the identification and retrieval of testicular spermatozoa (Esteves and Varghese, 2012). This final step is carried out at the ICSI workstation. Throughout the aforesaid processes, the temperature and pH of working solutions should be kept constant. Moreover, state-of-art laboratory practice standards, including sterile techniques and laboratory air quality conditions, are of utmost importance to optimize micromanipulation efficiency and safety assurance (Esteves and Varghese, 2012; Popal and Nagy, 2013). At our center, we perform sperm retrieval and all related-laboratory steps involved in the handling of testicular specimens in controlled environments. The latter includes tissue processing, microinjection of surgically extracted sperm, culture of embryos generated from such procedures, and cryopreservation. Our facility, comprised of reproductive laboratories (IVF and Andrology), an operating room where microsurgical sperm extractions and oocyte collections are carried out, and embryo transfer rooms, was constructed according to clean room standards for air particles and volatile organic compounds filtration. Not surprisingly, we observed a significant increase in IVF treatment effectiveness after having implemented clean room technology (Esteves and Bento, 2013).

After a successful SR in NOA, cryopreservation of surplus testicular sperm is highly recommended because such patients often require more than one ICSI attempt until a pregnancy is established, but repeated retrieval attempts are not always possible. Some centers prefer to retrieve and intentionally cryopreserve testicular sperm for future use while other coordinate sperm retrieval and oocyte collection to occur simultaneously. In many cases, only immotile spermatozoa will be available for sperm injection after thawing, which could negatively impact ICSI outcomes. A comprehensive review of the advantages and disadvantages of performing sperm injections with fresh or frozen-thawed testicular sperm and the methods of selecting viable immotile sperm for ICSI can be found elsewhere (Esteves and Varghese, 2012).

In conclusion, adherence to state-of-art laboratory techniques and quality control are recommended not only to avoid jeopardizing the sperm fertilizing potential, but also to improve ICSI outcomes when handling testicular specimens extracted from men with azoospermia due to spermatogenic failure (Table 1).

Results of assisted reproductive technology in azoospermic men with spermatogenic failure

The clinical outcomes of ICSI using surgically extracted testicular sperm from men with azoospermia due to SF are lower than ejaculated counterparts (Palermo *et al*., 1999; Verza Jr and Esteves, 2008; He *et al*., 2010; Esteves and Agarwal, 2013a). The results are also lower when the former is compared with epididymal and testicular sperm obtained from men with obstructive azoospermia (He *et al*., 2010; Esteves *et al*., 2014). These findings seems to be related to the higher tendency of spermatozoa obtained from men with SF to carry deficiencies such as the ones related to the centrioles and genetic material, which ultimately affect the capability of the male gamete to activate the egg and trigger the formation and development of a normal zygote and a viable embryo (Meseguer *et al*., 2009; Vozdova *et al*., 2012).

In an early series involving 330 patients with different infertility conditions, including 53 azoospermic men with SF, we examined the ICSI outcomes, according to the source of spermatozoa and the type of azoospermia. We found that normal fertilization rates were significantly lower when testicular sperm of men with SF was compared with ejaculated and testicular/epididymal sperm of men with obstructive azoospermia (52.2, 71.1 and 73.6% in SF, ejaculated sperm and OA, respectively; $P < 0.05$). Embryo development and pregnancy rates were also negatively affected by SF (Verza Jr and Esteves, 2011). In two recent series involving a larger cohort of azoospermic men with SF, we compared the outcomes

of ICSI and analysed the health of offspring, according to the source of sperm and the type of azoospermia. In one study, one hundred and eighty two women underwent ICSI using sperm from partners with SF, and the outcomes were compared with a group of 182 and 465 women whose partners had OA and non-azoospermia male infertility, respectively. Live birth rates after ICSI were significantly lower in the SF group (21.4%) compared with the OA (37.5%) and ejaculated sperm (32.3%) groups (P = .003). A total of 326 live births resulted in 427 babies born. Differences were not observed among the groups in gestational age, preterm birth, birth weight and low birth weight, although we noted a tendency towards poorer neonatal outcomes in the azoospermia categories (Esteves and Agarwal, 2013a). In another series, we compared three hundred and sixty-five azoospermic men with SF who underwent micro-TESE with forty men with SF who used donor sperm for sperm injections due to failed retrieval and 146 men with OA who underwent percutaneous sperm retrieval. The sperm retrieval rate in SF was 41.4%, and the results were lower than the OA group (100%; adjusted odds ratio: 0.033; 95% CI: 0.007-0.164; P < 0.001). Live birth rates after sperm injections were lower in men with SF (19.9%) compared with donor sperm (37.5%; adjusted odds ratio: 0.377 (95% CI: 0.233-0.609, P < 0.001)) and obstructive azoospermia (34.2%; adjusted OR: 0.403 (95% CI: 0.241-0.676, P = 0.001). Neither the miscarriage nor the newborn parameters (gestational age, birth weight, malformation rate, perinatal mortality) of infants conceived were significantly different among the groups (Esteves *et al*., 2014). Although the data on the health of resulting offspring after ICSI using sperm of men with azoospermia due to SF are reassuring, only five studies have compared to date the neonatal profile of such babies (Vernaeve *et al*., 2003; Fedder *et al*., 2007; Belva *et al*., 2011; Esteves and Agarwal, 2013a; Esteves *et al*., 2014). The limited population analyzed calls for continuous monitoring, and studies on the physical, neurological, and developmental outcomes of children conceived are still lacking.

In conclusion, the chances of obtaining sperm on retrieval and achieving a live birth after ICSI are reduced in men with spermatogenic failure. The short-term profile of infants conceived after sperm injection does not seem to be negatively affected by spermatogenic failure.

Complete aspermatogenesis: a glance towards the future

Aspermatogenesis, defined as a severe impairment of spermatogenesis in which germ cells are completely lacking or present only in an immature form, results in sterility in approximately 25-45% of patients with spermatogenic failure (Aponte *et al*., 2013). *In vitro* fertilization with immature germ cells and *in vitro* culture of these cells have been proposed as an approach to overcome the cases where no mature spermatozoa is retrieved. ICSI with immature germ cells, including elongating and round spermatids, has yielded conflicting results and despite deliveries of healthy offspring have been reported, the method has very low efficiency as currently used (Vloeberghs *et al*., 2013). In addition, there is uncertainty whether this approach can be considered a safe treatment option. Ethical and safety concerns related to the potential transmission of genomic imprinted disorders have been raised leading to the ban of spermatid injection in the United Kingdom. Human spermatozoa are highly specialized cells with the purpose of not only delivering competent paternal DNA to the oocyte, but also providing a robust epigenetic contribution to embryogenesis. The latter requires that chromatin contains layers of regulatory elements sufficient to drive genes towards activation or silencing upon delivery to the oocyte. Changes in epigenome are known to affect gene expression, and several genes participating in spermatogenesis are epigenetically regulated (Kumar *et al*., 2013).

Because assisted reproduction techniques require mature germ cells, research efforts are now focused on the differentiation of preexisting immature germ cells or the production/derivation of sperm from somatic cells. In this regard, biotechnology has been investigated as a valuable tool for rescuing fertility while maintaining biological fatherhood. Breakthrough advancement in this field has been accomplished by Japanese scientists who used stem cells from mouse embryos to create primordial germ cells, which differentiated in spermatozoa after testis transplantation in mice (Sato *et al*., 2011). In humans, the formation of human haploid-like cells has already been obtained from pluripotent stem cells of somatic origin using the novel technique of *in vitro* sperm derivation. Haploidization is another technique under investigation as an option to create gametes based on biological cloning technology. Despite being promising, these methodologies are experimental and the production of human gametes in the laboratory is a highly complex process which is yet to be fully translated to humans (Aponte *et al*., 2013).

In conclusion, biotechnology techniques have been investigated as an alternative to rescue fertility in men with complete aspermatogenesis. At present, these methods remain largely experimental and still require extensive research, which should address, among other concerns, ethical and biosafety issues, such as gamete epigenetic status, ploidy, and chromatin integrity.

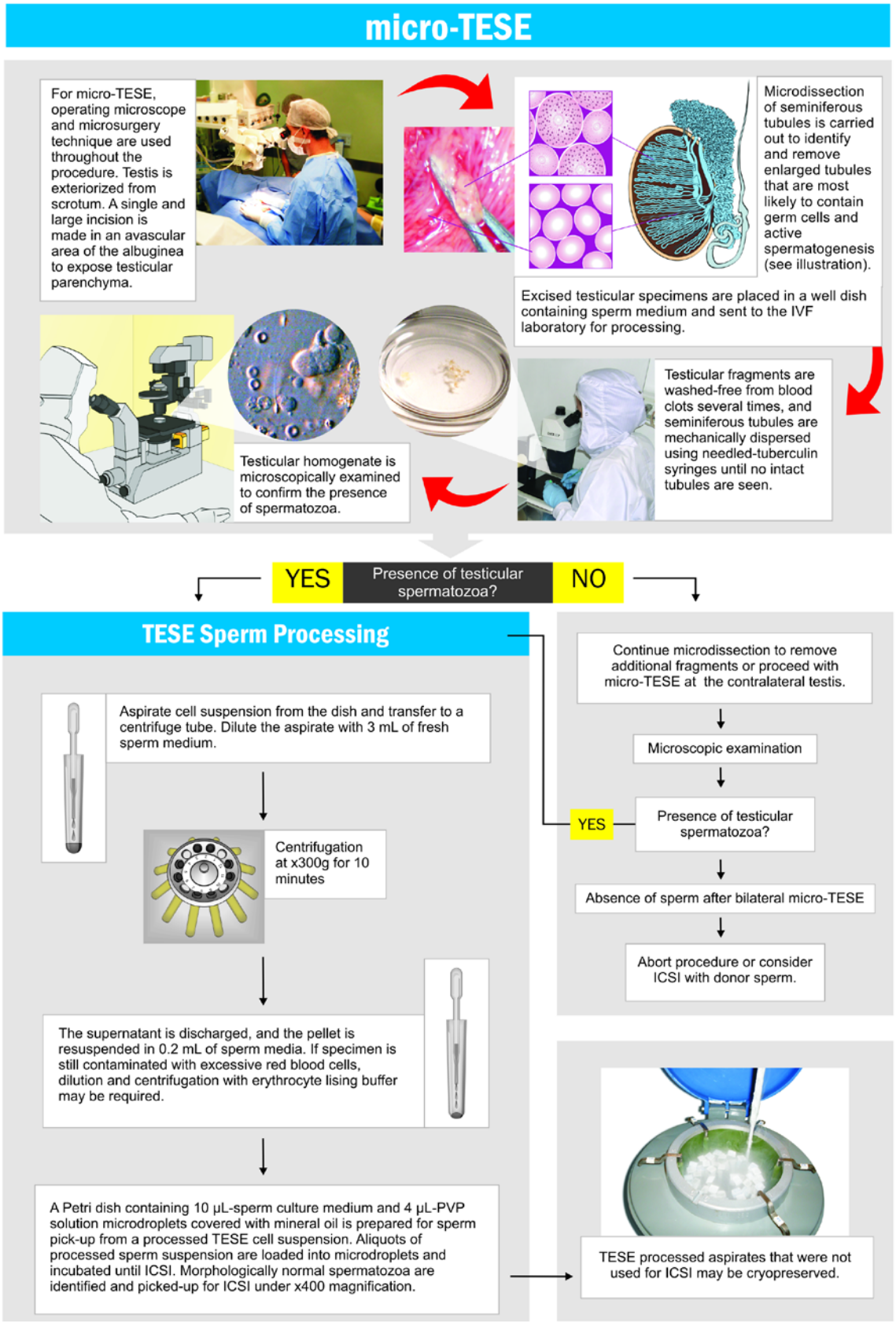

Figure 3. Microdissection testicular sperm extraction. Flow chart illustrates the consecutive steps from the microsurgical procedure to the laboratory processing of testicular specimens.

Conclusions

The clinical management of azoospermic men with spermatogenic failure seeking fertility starts with a proper diagnosis work-up that allows the differentiation between SF and other types of azoospermia. Azoospermia should be confirmed based on the absence of spermatozoa on multiple semen examinations after centrifugation of complete semen specimens using microscopic analysis. The combination of history and physical examination and hormonal analysis will differentiate with high accuracy spermatogenic failure from hypogonadotropic hypogonadism and obstructive azoospermia. Testicular biopsy with the sole purpose of histopathology diagnosis is not recommended because removal of testicular tissue might remove the rare foci of sperm production and thus jeopardize retrieval attempts.

Patients with azoospermia due to SF who are candidates for sperm retrieval should be screened for Y chromosome microdeletions because the diagnosis of a deletion has prognostic value and can influence therapeutic options. While retrieval attempts are not recommended in the complete deletion of the AZFa region, SR in azoospermic carriers of AZFb or AZFbc deletions may be eventually attempted, but patients should be fully informed about the very low/virtually zero chance to retrieve sperm. The presence of AZFc deletions represents a good prognostic factor for positive sperm retrieval because this deletion subtype is usually associated with residual spermatogenesis. Nevertheless, genetic counselling should be offered to these men because testicular spermatozoa used for ICSI will invariably transmit the deletion from father to son.

Before a sperm retrieval attempt, medical therapy to boost endogenous testosterone production and microsurgical repair of clinical varicoceles can be offered to men with hypogonadism and clinical varicocele, respectively. Although some individuals will ejaculate minimal quantity of sperm after such interventions, the majority remains azoospermic and will require SR. Micro-TESE should be the method of choice for sperm retrieval in spermatogenic failure because it not only increases the chance of retrieving testicular sperm for ICSI but also minimizes testicular damage.

After sperm retrieval, the extracted testicular parenchyma is immediately transferred to the embryology laboratory for sperm search following tissue dissection. Adherence to state-of-art laboratory techniques and quality control are recommended not only to avoid jeopardizing the sperm fertilizing potential, but also to improve ICSI outcomes when handling testicular specimens extracted from azospermic men with SF. The chances of obtaining sperm on retrievals and achieving a live birth after ICSI are reduced in men with SF, but the short-term profile of infants conceived after sperm injection does not seem to be negatively affected by SF.

Biotechnology techniques, including sperm derivation and haploidization, have been investigated as an alternative to rescue fertility in men whose germ cells are completely lacking or present only in immature forms. Although these methods at present remain largely experimental, they can become a valuable tool for rescuing fertility while maintaining biological fatherhood.

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