Male Infertility and Assisted Reproductive Technology: Lessons from the IVF

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Abstract: Development in ART has been remarkable in the last decades. Several medical conditions affecting the male fertility status are now treated by ART, especially by the combination of *in vitro* fertilization and intracytoplasmic sperm injection. ICSI is now the treatment of choice of most untreatable causes of male infertility, including immunologic infertility, severe oligozoospermia and azoospermia. Sperm retrieval techniques have been optimized and microsurgery offers the possibility of collecting testicular spermatozoa even in the most difficult cases of non-obstructive azoospermia. Nonetheless, the reproductive potential of sperm from men with defective spermatogenesis is decreased in ART and such treatment modalities may carry an increased risk of transmitting genetic and epigenetic defect to the embryo. Efforts should be made to improve the male health status prior to embarking on ART because current evidence suggests that fertility optimization may improve treatment outcomes. Moreover, laboratory management of male infertility cases requires special attention. Spermatozoa collected from men with severely impaired spermatogenesis are often compromised and fragile. Adherence to state of the art laboratory techniques and quality control are recommended to avoid jeopardizing sperm fertilizing potential and the chances of achieving a live birth. In this study, we present and critically review our 10-year experience in the management of severe male factor infertility using ART.

Keywords: Male infertility, assisted reproductive technology, intracytoplasmic sperm injection, azoospermia, oligozoospermia, antisperm antibodies, laboratory techniques.

INTRODUCTION

In a group of 2,383 male infertility patients attending our tertiary center for male reproduction, potentially surgically or medically correctable conditions were identified in 48.4% of the individuals. The other half comprised candidates for assisted reproduction, particularly assisted reproductive technologies (ART) involving *in vitro* fertilization (IVF) coupled to intracytoplasmic sperm injection (ICSI) (Table **1**). In this review, we discuss our 10-year experience in the management of severe male factor infertility using ART.

THE ROLE OF THE MALE PARTNER

Infertility is a common clinical problem that affects approximately 8% of the male population in reproductive age [1]. It is defined as the failure to conceive after one year of regular intercourse without contraception, and traditionally, investigation starts at this point. However, it is recommended that investigation is initiated earlier whenever risk factors are present since longer infertility duration relates to smaller chances of success regardless of the treatment strategy adopted [2, 3]. Determining the couple's fertility potential is a complex process that involves both male and female partners. The cause of infertility may or not be identified and can involve one or both partners.

The cornerstones for the evaluation of a subfertile man include a comprehensive history, physical examination and multiple semen analyses. Advanced paternal age, inadequate diet intake, drug abuse, pesticide environmental exposure, tobacco use, varicocele, medical diseases, hyperthermia, air pollution, genital inflammation or infectious diseases, history of urogenital surgery, and cancer can be cited as possible causes or contributing factors of male subfertility which may be reversed or treated in several cases [4-9]. The seminal

Table 1. Distribution of Diagnostic Categories in a Group of 2,383 Infertile Men Attending a Male Infertility Clinic

Category	N	%
Varicocele	629	26.4
Infectious	72	3.0
Hormonal	54	2.3
Ejaculatory dysfunction	28	1.2
Systemic diseases	11	0.4
Idiopathic	289	12.1
Immunologic	54	2.3
Obstruction	359	15.1
Cancer	11	0.5
Cryptorchidism	342	14.3
Genetic	189	7.9
Testicular failure	345	14.5
TOTAL	2,383	100.0

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analysis is the basis of the laboratory evaluation; it provides information on the functional status of the germinative line, epididymis and accessory sexual glands. An ejaculated semen sample can harbor varying degrees of sperm abnormalities ranging from absence to severe alterations in sperm parameters. Beyond the conventional semen analysis, additional tests have been developed in order to identify functional disorders and other spermatic abnormalities that are not addressed in routine semen evaluation. Such tests include assessment of antisperm antibodies and sperm DNA integrity [10, 11]. Levels of antisperm anti-bodies and sperm DNA integrity are associated with the male fertility potential [12, 13]. An endocrine evaluation is also part of the initial male workup. Genetic testing, such as karyotyping and testing for microdeletions of the Y chromosome or cystic fibrosis gene mutation screening, is indicated in selected cases of azoospermia and severe oligozoospermia, particularly prior to assisted conception.

ASSISTED REPRODUCTIVE TECHNOLOGY FOR THE MALE

Treatments or procedures that involve *in vitro* handling of human oocytes and sperm or embryos for the purpose of establishing a pregnancy are included in the ART category. In the context of male infertility, ART procedures include *in vitro* fertilization (IVF), IVF with intracytoplasmic sperm injection (ICSI) and sperm and embryo cryopreservation. ART does not include assisted insemination (artificial insemination) using sperm from either a woman's partner or sperm donor [14]. Two major breakthroughs occurred in the area of male infertility in the last years. The first was the development of intracytoplasmic sperm injection (ICSI) for the treatment of male factor infertility due to severely abnormal semen quality [15]. The second was the extension of ICSI to azoospermic males and the demonstration that spermatozoa derived from either the epididymis or the testis were capable of normal fertilization and pregnancy [16, 17].

INTRACYTOPLASMIC SPERM INJECTION TREATMENT STEPS

Controlled Ovarian Stimulation (COS)

In our center, COS for ART is performed using either the long pituitary desensitization gonadotropin-releasing hormone agonist (GnRH-a) or the GnRH antagonist protocol [18]. For ovarian stimulation, initial daily doses of 150–375 IU of gonadotropins (human menopausal gonadotropin [hMG], high-purified hMG or recombinant human follicle stimulating hormone [r-hFSH]) are used. The initial dose of gonadotropin is determined by the treating physician taking into account female characteristics such as age, body mass index, serum FSH on day 2 or 3 of the menstrual cycle, baseline ovarian volume on transvaginal ultrasound (TVUS), and number of pre-antral follicles between days 2 and 3 of the menstrual cycle. For cycles involving long pituitary desensitization, treatment is initiated with 400 μg daily intranasal administration of gonadotropin-releasing hormone agonist (nafarelin acetate), starting on the twenty-first day of the menstrual cycle and is maintained until the day before human chorionic gonadotropin (hCG) administration. Ovarian stimulation commences only after confirming pituitary down-regulation by both serum estradiol levels <50 pg/mL and the absence of ovarian follicles >10 mm in diameter on TVUS. For cycles involving GnRH antagonist, ovarian stimulation is initiated either on day 2 or 3 of the menstrual cycle after confirmation of the absence of ovarian follicles >10 mm on TVUS. A daily dose of 0.25 mg subcutaneous administration of GnRH antagonist is started when the lead follicle reaches 14 mm or ≥5 follicles are between 11-13 mm (usually between days 5 and 7 but no later than day 8 of stimulation) and is continued up to and including the day of hCG administration. Ultrasound assessment between the sixth and eighth days of stimulation is performed to determine if gonadotropin dose adjustments are required. Human chorionic gonadotropin (hCG) is administered when two or more ovarian follicles reach a mean diameter of 18 mm.

Oocyte Retrieval, Sperm Processing, and ICSI

Oocyte retrieval is performed under intravenous sedation and guided by TVUS, 34–36 hours after hCG administration. Cumulus-corona-oocytes complexes are denuded, classified according to nuclear maturity and are maintained in culture until sperm microinjection. Ejaculated spermatozoa are processed by discontinuous colloidal gradient or swim-up techniques [19]. In the cases of azoospermia, either percutaneous testicular or epididymal sperm aspirations, or microsurgical testicular sperm extraction are performed for sperm retrieval [20]. Selection and immobilization of the spermatozoon, and microinjections, are performed under a 400X magnification [21]. The injected oocytes are transferred to a closed culture system and incubated for 16–18 hours at 37°C and 5.5% CO_2, until confirmation of fertilization. Fertilization was considered normal when oocytes with two pronuclei are seen.

Embryo Culture, Cleavage Check, Transfer, and Luteal Support

Fertilized oocytes are maintained in culture until transfer of the embryos to the uterine cavity on the third or fifth day of embryo culture [22]. Embryo quality is scored daily, and the number, symmetry, and expansion of the blastomeres, multinucleation, anomalies of the zona pellucida, the rate of cytoplasmic fragmentation and blastocyst formation are recorded. The embryos are classified as top quality when they had three to four symmetrical blastomeres on the second day of culture and seven to eigth symmetrical blastomeres on the third day, with no multinucleation, grade I (no fragmentation) or grade II fragmentation (up to 20% of the perivitelline space with fragments), and no abnormalities in the zona pellucida [23]. One to four embryos are transferred to the uterine cavity depending on the couples' clinical profile and regulatory guidelines. Abdominal ultrasound guidance is used during all embryo transfers, which are performed three to five days post-oocyte retrieval. Luteal phase support with once-daily application of progesterone gel is initiated in the same day of oocyte retrieval. Embryos that are not transferred are cryopreserved for future use.

Oocyte collection, micromanipulation of gametes, embryo culture, and the transfer of embryos to the uterine

cavity are carried out under ISO 6, ISO 5, and ISO 7 cleanroom environments, respectively (ANDROFERT-VECO, Brazil) [24, 25].

INTRACYTOPLASMIC SPERM INJECTION TECHNICAL ASPECTS

Collection of Ejaculated Specimens: Effect of Stress on Semen Quality

It has been shown that infertility-related stress is significantly increased in couples undergoing ART treatments [26]. Although men tend to report less sexual stress and anxiety than women, a strong linkage between anxiety and sexual stress may be observed in men with severe male factor infertility [27]. In order to examine the impact of anxiety on semen quality, we conducted a study involving 160 men with severe male factor infertility enrolled in our ART program [28]. Ejaculated semen specimens were obtained at two different occasions using identical ejaculatory abstinence intervals: i) at the time of male infertility workup, and ii) at the time of oocyte collection for sperm processing and ICSI. We noted that sperm concentration and progressive motility were significantly lower in semen samples collected on the day of oocyte retrieval as compared to specimens provided for previous seminal analysis. Moreover, approximately 20% of men with severe oligozoospermia on diagnostic semen analysis produced an azoospermic specimen at the time of ICSI, requiring subsequent collection attempts or testicular sperm retrieval. A qualitative questionnaire analysis showed that the proportion of men experiencing anxiety was significantly higher at the day of female partners' oocyte collection than on previous semen analysis for diagnostic purposes. Our results suggested that acute psychological stress can impair semen quality of men with severe male factor infertility enrolled in ART. We advise our patients with severe oligozoospermia to cryobank their specimens prior to ART to avoid the risk of obtaining very poor semen specimens at the time of the ICSI cycle.

Optimal Time Interval for Microinjection

In ART treatment cycles involving severe male factor infertility, particularly in non-obstructive azoospermia, the time frame between oocyte retrieval and microinjection may be long due to technical difficulties for testicular tissue processing and sperm procurement. The oocyte lifespan is, however, limited, and it has been suggested that the oocyte retains its optimal developmental capacity if insemination is kept within a window of 10 hours from hCG administration [29-31]. We evaluated the impact of the time interval between hCG administration and sperm microinjection on fertilization and embryo development in severe male factor infertility ART [32]. A total of 550 ICSI cycles involving 4,334 oocytes were reviewed. The sperm sources for microinjection were ejaculate (n=364), epididymis (n=30) and testicle (n=75). Injected oocytes were grouped according to the time elapsed between hCG administration and microinjections. The case assignment was random and based only by workload. Our findings revealed that oocytes microinjected either before 38 or after 43 hours, in relation to hCG administration, exhibited significant lower 2PN fertilization rates and embryo development than those injected within the time interval of 38-42 hours (Table **2**). A significant proportion of oocytes were injected with testicular spermatozoa in the group of >43-hour time interval from hCG to microinjections. We suspected that the use of testicular spermatozoa from non-obstructed azoospermic men justified our findings of decreased fertilization and embryo development in this subgroup. However, reduced fertilization and embryo development were still observed, at marginally significant levels, in the microinjection interval subgroup of longer than 43 hours after excluding the oocytes injected with testicular sperm. Oocytes matured *in vitro* and injected on the next day also showed significant lower fertilization and embryo development. We concluded that the optimal time interval for microinjection in cases of severe male factor infertility is 38-42 hours after hCG administration. One should expect lower fertilization and embryo development with microinjections performed >43h after hCG, particularly in the cases of non-obstructive azoospermia. It is advisable to time sperm retrieval procedures at early hours on the day of ICSI, or at the previous day, in cases involving testicular sperm collection from men with non-obstructive azoospermia. This strategy allows microinjections to take place at the optimal injection window, thus avoiding jeopardizing ART outcomes.

Optical Magnification for Sperm Selection and ICSI

In a prospective and randomized clinical study, we examined the effect of optical magnification for sperm selection and microinjection on ICSI outcomes of 120 infertile couples

Table 2. ICSI Outcomes According to the Time Elapsed between hCG Administration and Microinjections. (*Adapted from Fertil Steril. 86, Suppl, Schneider DT, et al. Optimal time interval for intracytoplasmic sperm injection after human chorionic gonadotropin administration in severe male factor infertility, page S155, Copyright 2006, with permission from Elsevier*)

Time between hCG administration and microinjection	Oocytes injected (n)	% Normal 2PN fertilization	% Abnormal fertilization	% Top quality embryos on Day 3*	% Oocytes injected with testicular sperm (%)
Group 1 (<38h)	60	63 [43-86]	0	7 [0-28]	0
Group 2 (38-43h)	3014	73 [50-100]	0 [0-14]	50 [0-75]	26
Group 3 (>43h)	1079	56 [38-86]	0 [0-20]	33 [0-67]	37
Group 4 (day after)	181	44 [0-100]	0	NA	18
P-value	--	G2 *vs*. G3: p<0.01; G1-3 *vs*. G4: p<0.01	NS	G1 *vs*. G2: p<0.01; G2 *vs*. G3: p=0.02	G3 *vs*. G2/G4: p<0.01

*7-9 blastomeres of similar size, and grades I or II cytoplasm fragmentation on the day of embryo transfer (day 3). Values are expressed as median and 25%-75% percentiles. Kruskal-Wallis statistic was used for comparisons among groups. NS: not significant.

[21]. Patients were randomized according to the optical magnification power used for sperm selection and microinjection, i.e., 200X or 400X. Sperm with normal morphology were selected for injection, whenever possible, and sperm with large vacuoles and cytoplasmic droplets were excluded in the 400X magnification group. Microinjections were performed with the polar body at 6 or 7 o′clock position in the 200X group, and with the polar body at different positions in the 400X group to allow microinjections to take place at the vegetal oocyte pole. The normal 2PN fertilization rate was significantly increased in the 400X group (74.1% ± 23.8%) compared to the 200X group (57.4% ± 30.2%, p=0.001), while the percentage of abnormally fertilized oocytes was significantly reduced in the former (1.4% ± 4.7% *vs*. 11.4% ± 17.5%, p<0.0001). Our results also showed that the proportion of top quality embryos available for transfer and the clinical pregnancy rate were significantly higher in the 400X group (54.6% ± 34.9% and 38.8%) compared to the 200X group (27.8% ± 28.3% and 24.9% (p<0.001). Based on these data, it is our routine to use 400X magnification for sperm selection and microinjection in ICSI cycles. Although intracytoplasmic morphologically-selected sperm injection (IMSI) using high power magnification has been advocated to yield better ART outcomes [33], we found that it is very easy to exclude sperm with large vacuoles and/or with cytoplasmic droplets at 400X magnification, as shown by others [34]. At 400X magnification, it is also possible to identify and to perform microinjections at the oocyte vegetal pole to minimize the risk of damaging the oocyte meiotic spindle.

Air Quality in the IVF Laboratory: Impact on ART Treatment Results

Airborne concentrations of toxics volatile organic compounds (VOC), chemical contaminants and particles may impair embryo development and pregnancy rates [35-37]. Volatile organic compounds are produced by industries, vehicles, heating exhausts, cleaning products, furniture and others. Considering these facts, some companies have designed filtration units to clean the air of the *in vitro* fertilization (IVF) laboratory and to decrease contamination with varying efficiency levels [38]. Cleanrooms are contaminated-free environments useful for certain critical applications in bioengineering, pharmaceutical and computer industries. We have studied the impact of high standard measures for air quality control inside the IVF laboratory and adjacent areas on ART outcomes [24, 25]. In one study, we evaluated 399 consecutive ICSI cycles in which the primary indication for treatment was severe male factor infertility. ART was performed inside two different *Cleanroom* IVF laboratories classified in accordance to the International Standard Organization (ISO) 14.644-1 [25]. IVF laboratory ISO type 6 was equipped with a positive pressure high efficiency particles air filtration (HEPA) system (VECO, Brazil) and a free-standing unit to filter and remove VOC (Coda Tower, GenX, USA). IVF lab ISO type 5 was built with proper engineering and material selection. It included a dedicated air handling system equipped with HEPA and carbon-impregnated filters, located in a separate room, to filter and remove particles and VOC from the IVF laboratory and adjacent areas such as the oocyte retrieval and embryo transfer rooms (ANDROFERT-VECO, Brazil). A positive pressure differential was maintained from the ISO 5 IVF laboratory to the oocyte retrieval (ISO 6 Cleanroom) and the embryo transfer rooms (ISO 7 Cleanroom). Incubators in both labs were equipped with in-line carbon impregnated HEPA filters for carbon dioxide from gas cylinders (GenX, USA). In this series, female age, duration of ovarian stimulation, amount of gonadotropin required per cycle, number of oocytes retrieved and proportion of ICSI cycles involving microinjection of epididymal or testicular spermatozoa were not statistically different between groups. We found that cleavage, embryo quality and clinical pregnancy rates were significantly increased, while miscarriage rates were reduced, in the group of ICSI cycles performed inside the ISO type 5 IVF laboratory as compared to the ISO type 6 laboratory (Table **3**). We concluded that air quality control in the IVF laboratory and critical areas may impact on embryo development and pregnancy results. Our observations are consistent with an association between the presence of air contaminants in the IVF laboratory and ART outcome.

ANTISPERM ANTIBODIES AND ICSI RESULTS

Antisperm antibodies (ASA) are found in 3% to 12% of men who undergo evaluation for infertility [12]. IgA and IgG immunoglobulins are of clinical importance because these are the subclasses that have been demonstrated in the ejacu-

Table 3. Comparison of ICSI outcomes between cycles performed in the ISO 6 and ISO 5 IVF laboratories. (*Adapted from Fertil Steril. 86, Suppl, Esteves SC, et al. Comparison between International Standard Organization (ISO) type 5 and type 6 cleanrooms combined with volatile organic compounds filtration system for micromanipulation and embryo culture in severe male factor infertility, pages 353-4, Copyright 2006, with permission from Elsevier*)

	ISO 6 IVF lab (n=187)	ISO 5 IVF lab (n=212)	p-value
Two pronucleate zygotes (% 2PN)	70.0 ± 25.4	69.7 ± 22.8	NS
Cleavage rate (%)	84.8 ± 28.2	94.1 ± 15.9	<0.001
Good quality embryos on day 3 (%)*	34.3 ± 28.7	49.4 ± 31.5	<0.001
Embryos transferred (n)	3.5 ± 1.8	3.4 ± 1.3	NS
Clinical pregnancy rate (%)	36.9	47.1	0.03
Miscarriage rate (%)	25.0	14.0	0.01

NS = Not significant. P< 0.05 considered significant. Values are expressed as Mean ± SD

*Good quality embryos: Embryos with 7-8 blastomeres on culture day 3, and cytoplasmic fragmentation ≤20%.

lates of men with antisperm autoimmunity. The formation of antisperm antibodies (ASA) may be secondary to the rupture of the blood-testis barrier. Obstruction, inflammation and trauma of the genital tract, as well as cryptorchidism, varicoceles and vasectomy have been associated with elevated levels of ASA [39]. ASA may cause sperm agglutination and/or immobilization, thus decreasing the fertilizing potential by impairing progression through the female genital tract and/or by interfering with the fertilization process. ASA may also impair sperm capacitation and acrosome reaction, thus affecting sperm-oocyte interaction [40, 41]. Decreased fertilization and cleavage rates are expected when ASA bound sperm are used in conventional *in vitro* fertilization (IVF) or in intrauterine insemination [42-44]. Fertilization rates tended to decrease as the amount of antibody increased in the direct immunobead test (IBT) [45], and very low fertilization rates have been observed when >70% of inseminated spermatozoa were coated with ASA. Once fertilization had occurred, the pregnancy rate was not affected by the severity of immunological factors [46].

Alternatives to overcome the deleterious effects of ASA, such as medication [47-49], sperm washing combined with intrauterine insemination [47, 50] and *in vitro* fertilization [42-45, 51-53] have been proposed. The effectiveness of these techniques in recovering antibodies-free spermatozoa are conflicting; most reports show limited success due to the difficulty of eluting the sperm cell surface by any washing method [54, 55]. In a previous report, we demonstrated that sperm processing may be helpful in eluting antibodies-bound to sperm. We studied 48 men with varying levels of ASA in the semen, as determined by the immunobeads binding test (IBT), and found that the population of antibodies-free spermatozoa was increased by 29% after discontinuous colloidal gradient centrifugation. We also noted that sperm washing was ineffective to remove ASA in approximately 30% of the cases, and advise that the potential benefit of this strategy has to be tested individually [56].

Microinjection of the compromised spermatozoa into the oocyte cytoplasm (ICSI) bypasses sperm-oocyte membrane interaction, and ICSI has been shown to increase fertilization when compared to conventional IVF in cases of male immunologic infertility. Nagy *et al.* analyzed the outcome of ICSI in 37 men with a proportion of antisperm antibody-bound spermatozoa of 80% or higher [57]. They concluded that fertilization, cleavage and pregnancy rates after ICSI were not influenced by the percentage of ASA-bound spermatozoa, by the dominant type of antibodies present, or by the location of ASA on the spermatozoa. However, embryo quality was lower in the ASA-positive group. In another study, similar results have been observed but a higher rate of first trimester pregnancy loss in the ASA-positive group has occurred [58]. Clarke *et al.* [59] and Check *et al.* [60] studied 39 patients with a strong positivity on IBT (>80%) and 93 patients with various degrees of autoantibodies, respectively. They found that fertilization and pregnancy rates were comparable between different levels of ASA on sperm. In order to re-examine data of ICSI in the light of the above mentioned reports, we analyzed ICSI outcome in large cohort of 351 patients at four different levels of ASA in the semen, as determined by direct immunobeads testing [10]. Our findings confirmed that fertilization, cleavage and pregnancy rates after ICSI were not influenced by the ASA levels on sperm (Table **4**). Additionally, we have neither observed the negative impact on embryo quality nor the increase in pregnancy loss in the sperm antibody-positive patients, as reported by others. We also analyzed whether cleavage velocity is altered by ASA levels, and our results indicated that, irrespective of ASA levels, cleavage velocity was not altered by antisperm antibody-bound to spermatozoa. In our study, we also compared ICSI outcomes between patients with ASA positivity and a group of patients in which ICSI was indicated for other reasons. Our findings demonstrated that fertilization, embryo development, pregnancy success and

Table 4. Comparative Analysis of the Outcomes of 351 ICSI Cycles Subdivided into 4 Groups According to the Percentage of ASA as Tested by Direct Immunobeads Assay, and 349 ICSI Cycles from Oligo/Asthenozoospermic Men in which ASA Testing has not been Performed (*Adapted from International Braz J Urol 34, Esteves SC, et al. Influence of Antisperm Antibodies in the Semen on Intracytoplasmic Sperm Injection, pages 795-802, Copyright 2007, with permission)*

	Group 1 (n=194) 0-10% ASA	Group 2 (n=107) 11-20% ASA	Group 3 (n=33) 21-50% ASA	Group 4 (n=17) 51-100% ASA	Group 5 (N=349) ASA not determined	p-value
Sperm count ($X10^6$/mL)	33.0 [10.7-75.7][a]	44.7 [9.2-65.0][b]	58.0 [24.0-95.4][c]	13.3 [12.9-92.0][d]	2.7 [0.5-12.0][e]	a,b,c,d *vs*. e: p< 0.01
% 2PN fertilization	80.0 [66.7-100.0]	75.0 [60.0-90.0]	75.0 [66.7-83.3]	82.4 [70.0-100.0]	71.4 [50.0-87.5]	NS
% Abnormal fertilization	0.0 [0.0-5.6]	0.0 [0.0-6.7]	0.0 [0.0-6.7]	3.3 [0.0-7.1]	0.0 [0.0-12.5]	NS
% Slow cleavage velocity	45.5 [25.0-66.7]	50.0 [16.7-63.6]	36.7 [0.0-65.5]	42.9 [16.7-71.4]	42.8 [16.6-66.6]	NS
% Good quality embryos*	50.0 [27.3-66.7]	50.0 [25.0-71.4]	39.2 [18.3-66.7]	57.1 [28.6-83.3]	40.0 [20.0-61.5]	NS
% Clinical pregnancy	53.5%	52.8%	52.0%	50.0%	53.5%	NS

* 7-9 blastomeres of similar size, and grades I or II cytoplasm fragmentation on the day of embryo transfer (day 3). Kruskal-Wallis statistic was used for comparisons among groups. Values are expressed as median [25-75 percentiles]. NS: not significant.

miscarriage rates after ICSI in men exhibiting varying levels of autoimmunity against spermatozoa were within the same range as our population of ICSI patients with severely abnormal seminal parameters. We speculate that ASA may become inactive within the ooplasm after microinjection, or that a segregation process may take place during the first cleavage divisions, similar to the inactivation and segregation processes that also occur with the acrosome and sperm tail after microinjection.

IMPACT OF SPERM DEFECT SEVERITY ON ART

Traditionally, male factor infertility is described in terms of abnormal sperm concentration (oligozoospermia), impaired sperm motility (asthenozoospermia) or morphology (teratozoospermia). In several cases, a combination of two or more of these sperm variables defects may be observed and are termed oligoasthenozoospermia, oligoteratozoospermia, asthenoteratozoospermia or oligoasthenoteratozoospermia. In some cases, total absence of spermatozoa in the ejaculate after centrifugation may occur, a condition that is named azoospermia. It is still debatable if spermatozoa from normal, mild/moderate or severe abnormal semen may show different fertilizing abilities after ICSI [61]. Most studies report ICSI results based on the sperm source rather than on sperm defect severity. Results derived from these studies are difficult to interpret because the influence of spermatic defects is not taken into consideration. Moreover, the sperm source criteria may include spermatozoa from different etiologies. For example, ejaculated semen may either contain slightly abnormal spermatozoa from a man with a moderate varicocele or severely defective sperm from men with genetic disorders, like the Klinefelter syndrome and AZFc Y-chromosome microdeletions. Besides, spermatogenesis is very distinct in men with obstructive and non-obstructive azoospermia. While sperm production is normal in the former, it is severely abnormal, if existing, in the latter, despite the fact that in both cases ICSI may be performed using testicular spermatozoa [62]. In a recent study, our group took into consideration a different point of view by subdividing sperm deficiencies, as seen on semen analyses results, by the degree of severity, and also by the type of azoospermia [20]. We studied 313 ICSI cycles that were divided into two major groups according to the source of spermatozoa used for ICSI, i.e., ejaculated or retrieved from the epididymis/testicle. In the group of patients in which ICSI was performed with ejaculated sperm, cases were subdivided into four subgroups according to the results of the semen analysis: single defect (oligo-[O] or astheno-[A] or teratozoospermia-[T], double defect (a combination of two single defects and triple defect (OAT). In the group of azoospermic patients, cases were subdivided according to the type of azoospermia, i.e., obstructive and non-obstructive. Our results showed that significantly lower fertilization rates were obtained when either ejaculated sperm with triple defect or testicular sperm from non-obstructed azoospermic patients (63.4 ± 25.9% and 52.2 ± 29.3%, respectively) were used for ICSI as compared to other groups (71.3-73.6%). There was no difference in fertilization rates when sperm from the epididymis (74.7% ± 21.2%) or the testicle (69.1% ± 19.6%) of patients with obstructive azoospermia was used for ICSI, as compared with ejaculated sperm with mild (single sperm defect; 73.2 ± 22.1%) to moderate (double sperm defect; 72.1 ± 19.6%) alterations. We also noted that embryo quality, clinical pregnancy and miscarriage rates were not statistically different between ejaculated and obstructive azoospermia (OA) groups, independent of whether the spermatozoa from obstructive azoospermic patients were obtained from the epididymis or testicle. However, embryo quality and clinical pregnancy rates were significantly lower in the group of patients with non-obstructive azoospermia when compared to the other groups (Table **5**). Our results are in accordance with studies showing that severely defective sperm have a higher tendency to carry deficiencies, such as the ones related to the centrioles and genetic material, which affects the capability of the spermatozoa to activate the egg and start the formation and development of a viable embryo [63, 64]. The impaired fertilization rates after ICSI in men with non-obstructive azoospermia and oligoasthenoteratozoospermia may be explained by alterations in the early paternal effects, responsible for the initial stages of embryo development. Early paternal effects are dependent both on the spermatic cytosolic factor that dictates the completion of the oocyte meiotic division and the sperm centriole that participates in the formation of embryo mitotic fuses in early cellular

Table 5. ICSI Outcomes using Ejaculated, Epididymal and Testicular Spermatozoa (*Adapted from International Braz J Urol 34, Verza Jr S and Esteves SC. Sperm defect severity rather than sperm source is associated with lower fertilization rates after intracytoplasmic sperm injection, pages 49-56, Copyright 2008, with permission*)

	Group 1 – Ejaculated Sperm (n=220)				Group 2 – Testicular and Epididymal Sperm from Azoospermic Men (n = 93)		p-value
	Normal (n=86)	Single Defect (n=41)	Double Defect (n=45)	Triple Defect (n=48)	OA (n=39)	NOA (n=54)	
Fertilization (%2PN)	71.3±24.1[a]	73.2±22.1[b]	72.1±19.6c	63.4±26.9[d]	73.6±20.7[e]	52.2±29.3[f]	< 0.05 [d,f *versus* a,b,c,e]
Good embryo quality* (%)	48.4±34.8[a]	50.5±30.9[b]	46.9±31.1[c]	48.3±35.8[d]	46.3±30.0[e]	35.7±27.4[f]	< 0.05 [f *versus* a,b,c,d,e]
Clinical pregnancy (%)	40.9[a]	36.6[b]	44.4[c]	51.0[d]	51.3[e]	25.9[f]	0.01 [f *versus* a,b,c,d,e]
Miscarriage (%)	14.9	9.1	12.5	12.0	20.0	14.3	NS

One-way ANOVA was used to compare clinical and laboratorial parameters among groups. The chi-square test was used to compare pregnancy and miscarriage rates. P < 0.05 was considered significant; NS = not significant. * = 7-9 blastomeres of similar size, and grades I or II cytoplasmic fragmentation on the day of embryo transfer (day 3). OA = obstructive azoospermia; NOA = non-obstructive azoospermia. Values are mean ± standard deviation.

Table 6. Mean Number of Injected Oocytes and Transferred Embryos, Live Birth Rates and Ratio of Injected Oocytes to Obtain a Live Birth after ICSI using Sperm from Men with Varying Degrees of Defective Spermatogenesis (*Adapted from Fertil Steril. 88, Suppl. 1, Verza Jr S, et al. Limiting the number of oocytes to be fertilized in vitro can dramatically decrease the ability of men with severe male factor infertility to have children, page S371, Copyright 2007, with permission from Elsevier*)

	Normal sperm; n = 168	Single defect; n = 124	Double defect; n = 105	Triple defect; n = 96	OA; n = 66	NOA; n = 67	*P*-value
Injected oocytes; n	7.7 ± 5.3	8.7 ± 5.5	8.5 ± 4.7	8.9 ± 6.3	9.3 ± 5.5	9.9 ± 5.7	NS
Embryo transfer; n	3.3 ± 1.5	3.5 ± 1.6	3.2 ± 1.4	3.2 ± 1.6	3.4 ± 1.3	3.1 ± 1.8	NS
Live birth rate; %	32.3	27.9*	26.5*	31.2	44.0*	26.8*	*<0.05
No. of injected oocytes/live birth	25.6	33.0	31.0	29.6	20.5	38.8	–
#Relative difference; %	34.0	14.9	20.1	23.7	47.1	–	–

NOA versus others groups. NOA: non-obstructive azoospermia; OA: obstructive azoospermia; ejaculated sperm with single defect (oligo- [O] or astheno [A] or teratozoospermia [T]; double defect (a combination of two single defects); triple defect (a combination of the three defects [OAT]); normal (ejaculated sperm without sperm defects).

divisions [65]. Although ICSI is a formidable therapy that trespasses obstacles faced by sperm in its function as a carrier, it cannot alter the message carried by the male gamete.

Our data suggest that the reproductive potential of men undergoing assisted conception is lower in the cases of severe male factor infertility, thus highlighting the importance of the male gamete in ART. In a recent study, we aimed to compare the live birth rates after ICSI using sperm from men with varying degrees of defective spermatogenesis, and to estimate the number of oocytes that is needed to be microinjected to obtain one live birth [67]. Our data included the outcome of 626 ICSI cycles that were divided into six groups according to the source and quality of spermatozoa (Table **6**). We observed that the live birth rates were significantly impaired when ICSI was performed using either testicular spermatozoa extracted from men with non-obstructed azoospermia or ejaculated sperm from men with defective spermatogenesis as compared to those with obstructive azoospermia (OA) and normal sperm. We found that 40-50% more oocytes are to be microinjected to result in a live birth, when spermatozoa from men with defective spermatogenesis is used as compared those obtained from normal or obstructed azoospermic males. Therefore, caution should be applied when regulatory laws on assisted reproduction are undertaken, such as the ones that came to force in Italy in 2004 [66] and in Brazil in 2011. Limiting the number of oocytes to be microinjected and the ability of embryo selection and cryopreservation may be particularly harmful to men with severe male factor infertility.

SURGICAL TREATMENT OF CLINICAL VARICOCELES PRIOR TO ART

Varicocele is the most prevalent condition affecting men with fertility-related problems, accounting for 35% of the cases [1]. Several studies have demonstrated that the surgical treatment of clinical varicoceles decreases seminal oxidative stress, increases seminal concentrations of antioxidants and improves sperm quality and the percentage of spermatozoa with intact DNA [68-70]. Recent meta-analyses on varicocelectomy also demonstrated that treatment of clinical varicoceles in infertile men with abnormal semen analyses significantly increased the chances of achieving spontaneous conception [9,71]. Even though spontaneous pregnancy remains the litmus test for evaluating varicocele treatment success, many patients with varicocele related-infertility will require ART due to the severity of sperm abnormalities and/or the presence of a significant problem affecting the female partner. Azoospermia and severe oligozoospermia in association with varicocele is reported to range from 4.3% to 13.3% [72]. The indication of varicocele repair prior to IVF/ICSI is unusual, but in certain circumstances varicocele treatment should be considered. In a recent study [73], we aimed to compare the clinical and laboratory outcomes of ICSI in a group of infertile men with treated and untreated clinical varicoceles. Our study group comprised 242 infertile men with a history of clinical varicoceles who underwent ICSI. Eighty men had a prior successful subinguinal microsurgical varicocelectomy and 162 men had any grade of clinical varicocele at the time of ICSI. Baseline semen parameters, hormone profile, testicular volume, duration of infertility, male and female partners' ages, distribution of varicocele grade and the proportion of female factor problems were not different in men with treated and untreated varicocele. Improvement in total number of motile sperm (6.7×10^6 pre, 15.4×10^6 post; $p<0.01$) and reduction in sperm defect score (2.2 pre, 1.9 post; p=0.01) were observed after varicocelectomy. The normal 2PN fertilization (78% *versus* 66%), clinical pregnancy (60.0% *versus* 45.0%) and live birth rates (46.2% *versus* 31.4%) after ICSI were significantly higher in men with treated as compared to those with untreated varicocele. Logistic regression analyses showed that the chances of achieving a clinical pregnancy (odds-ratio=1.82; 95% CI: 1.06-3.15) and a live birth (odds-ratio=1.87; 95% CI: 1.08-3.25) by ICSI were significantly increased while the chance of miscarriage occurrence was reduced (odds-ratio=0.433; 95% CI: 0.22-0.84) if the varicocele had been treated. In the group of patients who had varicocele repair, postoperative motile sperm counts and fertilization rates by ICSI were significantly higher in men who achieved a live birth compared to those who failed to impregnate their wives (Table **7**). Our data add new evidence by demonstrating that treatment of clinical varicoceles in men with a marked reduction in semen quality prior to

Table 7. Semen Analysis Results and ICSI Outcomes in 37 *vs.* 43 Infertile Couples with *vs.* without Live Birth in whom Male Partner Underwent Varicocele Repair (*Adapted from J Urol 184, Esteves SC, et al. Clinical outcomes of intracytoplasmic sperm injection in infertile men with treated and untreated clinical varicocele, pages 1442-1446, Copyright 2010, with permission from Elsevier*)

	Mean; SD Live Birth	Mean; SD No Live Birth	p-value (unpaired Student's t test or Pearson Chi-square test)
Male age (years)	35.9; 5.3	37.4; 5.3	0.21
Female age (years)	33.1; 4.4	33.2; 4.5	0.92
Male endocrine profile			
FSH (mIU/mL)	9.6; 7.7	9.8; 7.6	0.90
Testosterone (ng/dL)[a]	679.1; 793.8	502.4; 799.2	0.32
Testicular volume (right + left) (mL)	35.8; 7.7	36.5; 7.8	0.12
Varicocele side (n, %)			
Unilateral	17; 45.9	19; 44.2	0.87
Bilateral	20; 54.1	24; 55.8	0.88
Volume (mL)	2.9; 1.0	2.7; 1.1	0.40
Sperm count ($x10^6$)	13.1; 46.7	11.2; 39.7	0.84
Progressive motility (%)	46.7; 10.9	41.7; 15.9	0.11
Total motile sperm count ($x10^6$)	17.9; 4.7	12.6; 6.4	<0.01
% Strict morphology	5.7; 3.0	5.1; 2.9	0.36
Sperm defect score	1.8; 0.7	1.9; 0.7	0.52
No. oocytes:			
Retrieved	12.4; 7.4	12.3; 7.4	0.95
Metaphase II	10.3; 6.6	10.2; 6.5	0.94
% 2PN fertilization	79.7; 12.6	66.0; 21.0	0.03
% High-quality embryo	47.9; 25.1	48.2; 22.2	0.95
No. embryos transferred	3.3; 1.5	3.2; 1.4	0.75

IVF/ICSI increases the couple's ability to conceive. The reasons for these findings seem to be related to the overall improvement in sperm quality. A significant improvement in the total number of motile sperm and a reduction in sperm defects were observed. Higher fertilization rates were also obtained after ICSI, suggesting that the improvement in pregnancy rates following varicocelectomy may be also due to functional factors not tested during standard semen analysis such as seminal oxidative stress and sperm DNA integrity.

Varicocele treatment may be also considered in men with non-obstructive azoospermia of unknown origin and concomitant clinical varicocele. We have evaluated treatment outcome after subinguinal microsurgical varicocele repair in relation to testicular histopathology in a group of non-obstructed azoospermic men with clinical varicoceles [70]. Open diagnostic testicular biopsies were performed at the same time of varicocele repair. A single piece measuring approximately 3x3x3 mm was excised and the specimens were examined by histology and classified according to the predominant pattern, as Sertoli cell-only (SCO), maturation arrest (MA) and hypospermatogenesis (HYPO). Sertoli cell-only category indicated that germinative cells were absent. Maturation arrest (MA) category was defined as absence of mature spermatozoa, despite normal early stages of spermatogenesis. Hypospermatogenesis indicated that all stages of the spermatogenic cycle were present, including mature sperm, but there was a proportional reduction in the number of all germ cells at each level. Overall, the presence of spermatozoa in the ejaculates was achieved in 47% (8/17) of men after varicocele repair, but only 35% (6/17) had motile sperm in their ejaculates. Only men with testicular histology revealing HYPO (5/6) or maturation arrest (3/5) had improvement after surgery. Median motile sperm count for both groups were 0.9 X 10^6/mL and 0.7 X 10^6/mL, respectively. The mean time for appearance of spermatozoa in the ejaculates was 5 months. One (HYPO) of 8 men who improved after surgery contributed to an unassisted pregnancy. None of the patients who had sperm in the ejaculates after varicocele repair returned to azoospermic during the mean follow-up period of 18.9 months. However, appearance of spermatozoa in the ejaculate was not achieved in any men with testicular histology diagnosis of SCO. Our findings revealed that testicular histology diagnosis was the most important predictive factor for outcome. In our series, testicular volume and preoperative serum FSH levels were not predictive of treatment outcome. Despite the induction of spermatogenesis in men with hypospermatogenesis and maturation arrest, we found that semen parameters still remained severely abnormal after the surgery, with all individuals presenting severe oligozoospermia after repeated

routine semen analyses. In addition, 25% (2/8) of men who improved after surgery presented only immotile sperm in their ejaculates. Although sperm restoration is minimal and most nonobstructive azoospermic men who benefit from varicocele repair still require ICSI to conceive, it carries the possibility of ICSI to be performed using ejaculated sperm, which is technically easier and provides better results than using sperm harvested from testicular sperm extraction (TESE) [20]. Furthermore, it avoids the risk of ICSI cycle cancellation by an unsuccessful TESE or the use of donor backup.

Both studies presented in this section highlight the importance of the surgical treatment of clinical varicocele as a method to improve the couple's fertility potential by improving testicular function.

REPRODUCTIVE POTENTIAL OF AZOOSPERMIC MEN IN ART

Definition and Classification

Azoospermia is defined as an absence of spermatozoa in the ejaculate after centrifugation. This condition, which is found in 1-3% of the male population and approximately in 10% of infertile males, results in infertility but doesn't necessarily imply sterility [2]. In the case of azoospermia, two totally different clinical situations exist, i.e., obstructive and non-obstructive azoospermia. In obstructive azoospermia (OA), spermatogenesis is normal but a mechanical blockage exists in the genital tract, somewhere between the epididymis and the ejaculatory duct, or the epididymis and vas deferens are totally or partially absent. Causes of OA may be acquired or congenital. Acquired OA may be due to vasectomy, failure of vasectomy reversal, post-infectious diseases, surgical procedures in the scrotal, inguinal, pelvic or abdominal regions, and trauma. Congenital causes of OA include cystic fibrosis, congenital absence of the vas deferens (CAVD), ejaculatory duct or prostatic cysts and Young's syndrome [2]. Non-obstructive azoospermia (NOA) comprises a spectrum of testicular histopathology resulting from various causes that include environmental toxins, medications, genetic and congenital abnormalities, varicocele, trauma, endocrine disorders, and idiopathic. In both OA and NOA, pregnancy may be achieved through assisted reproductive techniques, i.e., *in vitro* fertilization associated to ICSI [2,70].

Sperm Retrieval Techniques for the Azoospermic Male

Several sperm retrieval methods have been developed to collect epididymal and testicular sperm for ICSI from men with azoospermia. Either percutaneous (PESA) or microsurgical epididymal sperm aspiration (MESA) can be used to retrieve sperm from the epididymis in men with obstructive azoospermia. Testicular sperm aspiration (TESA) or extraction (TESE) can be used to retrieve sperm from the testes either in men with OA who fail epididymal retrieval. In non-obstructive azoospermia, TESA or testicular sperm extraction (TESE) using single or multiple open biopsies or microsurgical testicular sperm extraction (micro-TESE) can be used to retrieve testicular spermatozoa.

In a recent study, we evaluated 145 ICSI cycles performed in couples whose male partner had OA over a 6-year period [74]. Cycles were divided according to the etiology of OA: congenital, vasectomy/failed reversal and post-infectious/trauma. Overall sperm retrieval rates (SRR) using PESA and/or TESA were 97.9% (Table **8**). Motile spermatozoa was obtained in 73.1% of the cases after the first or second PESA, and TESA was carried out after failed PESA in 29% of the cases. In our series, an adequate number of motile sperm for cryopreservation was obtained in approximately one third of the cases using percutaneous retrieval techniques. Our findings indicated that sperm retrieval is highly successful in men with OA, regardless of the cause of obstruction.

Conversely, individuals exhibiting NOA have historically been the most difficult to treat. In another study, we assessed

Table 8. Sperm Retrieval Rates and ICSI Outcomes in Obstructive Azoospermia. Retrieval Rates by Percutaneous Sperm Aspiration from the Epididymis (PESA) and Testicle (TESA) and ICSI Outcomes According to the Etiology of Obstruction (*Adapted from Fertil Steril. 94, Suppl., Esteves SC, et al. Success of percutaneous sperm retrieval and intracytoplasmic sperm injection (ICSI) in obstructive azoospermic (OA) men according to the cause of obstruction, page S233, Copyright 2010, with permission from Elsevier*)

	Congenital (N=32)	Vasectomy/Failed reversal (N=59)	Post-infectious/Trauma (N=54)
SRR using PESA; N (%)	21/32 (70.0)	37/59 (62.3)	44/54 (81.5)
Cumulative SRR using PESA + TESA; N (%)	32/32 (100.0)	56/59 (94.9)	54/54 (100.0)
Female Age in Years; Mean ± SD	31.0 ± 5.0	32.6 ± 6.2	32.9 ± 5.9
2PN Fertilization rate; Mean (%)	64.1	65.3	59.3
Top Quality Embryo for Transfer; Mean (%)	44.9	57.9	49.4
Number of Embryos Transferred; Mean	2.9	2.6	3.0
Clinical Pregnancy Rate per Transfer; N (%)	16/29 (55.2)	26/59 (44.0)	23/53 (43.4)
Miscarriage Rate; N (%)	5/16 (31.2)	7/26 (26.7)	3/23 (13.2)
Live Birth Rate per Transfer; N (%)	11/29 (37.8)	19/59 (32.2)	20/53 (37.7)

SRR: sperm retrieval rate; One-way ANOVA and Chi-square test used to compare SRR and ICSI outcomes among groups. P-values were not statistically significant for all variables analyzed.

the success rates of sperm retrieval in a group of 176 men with NOA [75]. Retrievals were performed by TESA or micro-TESE and testicular histopathology results were available for 131 individuals. SRR were analyzed according to the histology patterns, i.e., hypospermatogenesis (hypo), maturation arrest (MA) and Sertoli cell-only (SCO), and to the etiology of NOA. Overall SRR rates by TESA and micro-TESE were similar (55.7%). However, SRR by TESA was significantly lower in cases of maturation arrest (MA) and Sertoli-cell only (SCO) combined (22.8%) compared to micro-TESE (39.2%). Both methods yielded similar SRR (100%) in cases of testicular histology showing hypospermatogenesis. Successful sperm retrieval rates were in the range of 50-70% in most etiology-specific causes of NOA. Testicular spermatozoa were obtained in all etiology-specific causes of NOA, such as varicocele, cryptorchidism, orchitis, genetic, endocrine and gonadotoxic-induced, and results were not different in etiology-specific subgroups of NOA (Table **9**). According to our data, micro-TESE performed better than TESA in MA and SCO histology subgroups, where focus of spermatogenesis were identified using the operating microscope. Testicular spermatozoa were obtained in all etiology-specific causes of NOA, and SRR seemed to be independent of its cause. Testicular histology results, if available, are useful to predict the chances to retrieve sperm in men with NOA. However, sperm can be obtained even in the worst scenario of Sertoli-cell only.

Table 9. Sperm Retrieval Rates in Non-Obstructive Azoospermia. Percutaneous Testicular Sperm Aspiration (TESA) and Microsurgical Testicular Sperm Extraction (micro-TESE) According to the Testicular Histopathology Results and the Etiology of Azoospermia (*Adapted from Fertil Steril. 94, Suppl., Esteves SC, et al. Sperm retrieval rates (SRR) in nonobstructive azoospermia (NOA) are related to testicular histopathology results but not to the etiology of azoospermia, page S132, Copyright 2010, with permission from Elsevier*)

	Presence of Testicular Spermatozoa; N (%)
By Method	
TESA (N=61); overall SRR, N (%)	34/61 (55.7)
Hypo	26/26 (100.0)
MA	2/6 (33.3)
SCO	6/29 (20.7)
Micro-TESE (N=70); overall SRR, N (%)	39/70 (55.7)
Hypo	19/19 (100.0)
MA	7/12 (58.3)
SCO	13/39 (33.3)
By Cause of NOA	
Varicocele (N=66)	45/66 (68.2)
Genetic (N=12)	6/12 (50.0)
Cryptorchidism (N=19)	12/19 (63.1)
Idiopathic (N=63)	33/63 (52.4)
Radio/chemotherapy (N=6)	3/6 (50)
Orchitis/Gonadotoxin/Endocrine (N=10)	10/10 (100.0)
Overall SRR; N (%)	109/176 (61.9)

NOA: non-obstructive azoospermia; SRR: sperm retrieval rate; Hypo; hypospermatogenesis; MA: maturation arrest; SCO: Sertoli cell-only.

In our experience involving 314 individuals with obstructive and non-obstructive azoospermia, the overall sperm retrieval rates (SRR), defined as successful surgical collection of spermatozoa for ICSI, were significantly higher in the group of OA (SRR=97.9%; N=142/145) compared to NOA (SRR= 55.2%; N=95/172) [76]. Logistic regression analysis revealed that the chances of retrieving spermatozoa were markedly increased in couples whose male partner had obstructive rather than non-obstructive azoospermia (odds ratio=43.0; 95% confidence interval 10.3-179.5). Our data clearly indicate that sperm retrieval rates are dependent on the azoospermia category, i.e., obstructive or non-obstructive.

Clinical Outcome of ICSI in the Azoospermic Male

In recent studies, we evaluated the reproductive outcomes by ICSI using surgically-retrieved sperm in men with OA and NOA [20,74-76]. PESA/TESA or TESA/micro-TESE was used in the attempt to retrieve spermatozoa in OA and in NOA cases, respectively. ICSI was performed using surgically-retrieved epididymal or testicular spermatozoa, as appropriate. Out of 2,136 couples undergoing ICSI from 2002 and 2008, 145 (6.8%) and 172 (8.0%) male partners had obstructive and non-obstructive azoospermia, respectively. We found that ICSI outcomes in men with OA were independent of the cause of obstruction [74]. Similar fertilization rates after ICSI using epididymal/testicular spermatozoa and embryo formation were obtained in different etiology categories of vasectomy/failed reversal, congenital or post-infectious/trauma (Table **8**). Moreover, clinical pregnancy, miscarriage and live birth rates were comparable, regardless of the cause of obstruction. In another study, we examined the reproductive potential of epididymal and testicular spermatozoa retrieved from men with OA. Laboratory and clinical ICSI outcomes did not differ between microinjections performed using either epididymal or testicular sperm (Table **10**) [20]. The clinical outcomes of ICSI using testicular or epididymal sperm aspirated from men with OA were similar than those obtained with ejaculated sperm from normal men and those presenting with mild/moderate semen abnormalities (Table **5**).

Comparison of ART outcome between men with obstructive and non-obstructive azoospermia, on the other hand, yelled different results. From our data involving 314 ICSI cycles, fertilization rates were significantly higher in the group of men with OA (60.5%) compared to NOA (51.1%; $P<0.01$) [76]. The overall pregnancy rates, defined as the live birth rate (LBR) per transfer, were 38.2% (50/131) and 25.0% (22/88) in the groups of men with OA and NOA, respectively ($P=0.03$). The chances of achieving a live birth by ICSI (odds ratio=1.86; 95% confidence interval 1.03-2.89) were significantly increased in couples whose male partner had obstructive rather than non-obstructive azoospermia.

Table 10. ICSI Outcomes Using Spermatozoa Retrieved from the Epididymis or the Testicle of Men with Obstructive (AO) (*Adapted from International Braz J Urol 34, Verza Jr S and Esteves SC. Sperm defect severity rather than sperm source is associated with lower fertilization rates after intracytoplasmic sperm injection, pages 49-56, Copyright 2008, with permission*)

Source of sperm for ICSI	Obstructive Azoospermia	
	Epididymal	Testicular
Number of Cycles; N=107	93	14
Female Age in Years; Mean ± SD	32.6 ± 5.3	32.1 ± 5.4
2PN Fertilization Rate; %	66.0[a]	56.6[a]
Cleavage Rate; %	99.4	95.7
Top Quality Embryo Rate for Transfer*; %	51.9	48.9
Cycles with Embryo Transfer; N (%)	88 (94.6)	14 (100.0)
Number of Embryos Transferred; Mean	2.8	3.3
Clinical Pregnancy Rate per Transfer; N (%)	45/88 (51.1)	7/14 (50.0)
Miscarriage Rate; N (%)	11/45 (24.4)	1/7 (14.3)
Live Birth Rate per Transfer; N (%)	34/88 (38.6)	6/14 (42.8)

Values expressed as means for fertilization, cleavage and embryo quality rates.
* 7-9 blastomeres of similar size, and grades I or II cytoplasm fragmentation on the day of embryo transfer (day 3).
One-way ANOVA and Chi-square test used to compare laboratory and clinical ICSI parameters between groups. Statistically significant results were not obtained for any of the outcome measures. $P<0.05$ was considered significant.

As previously discussed, the laboratory and clinical outcomes of ICSI using testicular sperm extracted from men with NOA were significantly lower than all other categories including ejaculated specimens obtained from men with varying degrees of defective spermatogenesis and epididymal/testicular sperm from men with OA (Table **5**) [20]. From these data, it is suggested that testicular spermatozoa of men with severely impaired spermatogenesis have decreased fertility potential, and may have a higher tendency 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 [20].

EXPERT COMMENTARY

The aim of this article is to present our 10-year experience with ART in the management of severe male factor infertility. Currently, ART is the only option for most men with severe male factor infertility to have biological offspring. Success has been achieved with this treatment modality in several male infertility conditions, including immunological infertility, severe oligozoospermia/other sperm deficiencies and azoospermia. It should be noted, however, that the reproductive potential of infertile men in ART varies according to severity of infertility. Therefore, strategies to optimize ART outcomes are encouraged. Many men with severe male factor infertility have lifestyle choices that include smoking, obesity, poor diet intake, use of gonadotoxic medication, and exposure to environmental toxins. These conditions are often associated with increased systemic or seminal oxidative stress and may have a negative synergistic effect on ART outcomes. We strongly advise patients enrolled in our ART program to improve their reproductive health by lifestyle modifications. Treatment of clinical varicocele prior to ART is another example of the impact of an intervention aiming to improve the overall male reproductive health.

When dealing with azoospermic males, the adoption of strict criteria to diagnose obstructive azoospermia is crucial for obtaining high success retrieval rate in the range of 90-100% using percutaneous techniques. Most cases of PESA failures are not necessarily technical failures since immotile spermatozoa can be retrieved. However, PESA may fail in certain cases of epididymal fibrosis due to multiple PESA attempts or post-infection. In these cases, TESA or PESA at the contralateral epididymis can be applied successfully. We routinely perform sperm retrieval under local anesthesia, with or without intravenous sedation. Percutaneous sperm retrieval techniques can be performed both for diagnostic and for therapeutic purposes. In the latter, sperm retrieval is often carried out at the same day of oocyte retrieval or at the day before. Patients are discharged one hour later and can return to normal activities in the same day. Oral analgesics are prescribed but pain complaint is minimal. The most common complication is fibrosis at the aspiration site. Some authors advocate that microsurgical epididymal sperm aspiration (MESA) allows the collection of larger and cleaner quantities of sperm than PESA, but this debate seems trivial. In our series, cumulative successful retrieval rate after PESA and/or TESA was higher than 95%, and an adequate number of motile sperm for cryopreservation was obtained in approximately one third of the cases. Although cryopreservation rate after PESA is not high, repeated aspirations can be carried out in men with OA with minimal morbidity and lower cost compared to MESA. When gradient centrifugation is chosen for PESA sperm processing, we spare part of the sample to be processed by simple washing. The reason is the unpredictability of gradient centrifugation to recover motile sperm in such cases. If recovery is less than desired, we can rely on the washed sample to select motile sperm for ICSI. Due to the relatively low sperm yields in PESA and TESA, it is important to use low volumes of media during sperm processing and wash the sample only once. Centrifugation force and time should be carefully controlled to avoid jeopardizing the often compromised sperm motility.

Although not absolute, we consider testicular histology as the best predictor for successful sperm retrieval in NOA men. The probability of obtaining sperm varies according to the testicular histopathology results. In NOA, our choice is to use micro-TESE rather than conventional TESE or TESA in cases of maturation arrest or SCO. Micro-TESE is also the safest technique regarding post-operative complications. Proper identification of testicular vessels under the tunica albuginea is made prior to the placement of an incision into the testis. The use of optical magnification and microsurgery technique allows the preservation of intratesticular blood supply, as well as the identification of tubules more likely to harbor sperm production. Therefore, efficacy of sperm retrieval is improved while the risks of large tissue removal are minimized. Nonetheless, our experience with TESA in NOA indicates that it may be highly successful in men with diagnostic testicular biopsy histopathology showing hypospermatogenesis or in those with a previous successful TESA attempt. However, if TESA fails, we neither perform a second aspiration in the same testis, at the same operative time, nor convert it to an open procedure, to avoid the risk of hematoma and testicular injury. From our clinical experience, it is very difficult to identify enlarged seminiferous tubules in such cases, even using the operating microscope, because extensive bleeding is often seen. In these occasions, we opt to perform TESA or TESE at the contralateral testis. For NOA patients without previous diagnostic testicular biopsy or TESA attempt, our choice is to perform sperm extraction using micro-TESE. Tissue removal in micro-TESE is often minimal as compared to conventional TESE; the small amount of tissue extracted facilitates sperm processing in the IVF lab. Selection of spermatozoa from a smaller population of contaminating testicular cells allows more ease and greater speed for sperm pick-up and injection process, as well as alleviates contamination and blockage of the injection needle with cells and debris. It is far less technically demanding and labor-intensive to extract spermatozoa from small volume specimens than large pieces of testicular tissue that must be dissected, red-blood cells lysed, and the rare spermatozoa searched for in a tedious fashion under an inverted microscope. TESE sperm processing may be incredibly labor-intensive and the searching process may miss the rare spermatozoa within a sea of seminiferous tubules and other cells. TESE/micro-TESE may be scheduled either for the day of oocyte collection and ICSI or the day before. In the latter, processed specimens are incubated in a closed HEPES-buffered culture system (microdrops under mineral oil) at room temperature, inside a laminar flow cabinet or in a cleanroom for a maximum of 48 hours, to avoid bacterial contamination. Culture of specimens at 37°C inside the incubator should be avoided since contamination with scrotum skin derived-bacteria is often seen. From our data, optimal fertilization by ICSI using surgically-retrieved sperm is obtained when the time frame from hCG administration to microinjection does not exceed 43h. Testicular tissue sperm processing, searching and selection of viable spermatozoa for ICSI may take several hours in NOA cases. Our laboratory takes approximately 11.6 minutes to handle a single testicular spermatozoon from processing to microinjection in NOA, but only 5.5 minutes in OA. In other words, the average time required to perform ICSI in a standard NOA treatment cycle involving 8-12 metaphase-II oocytes is approximately 2 hours. Therefore, we elect to perform micro-TESE the day before oocyte collection when a busy next day IVF laboratory workload is anticipated. Additionally, we recommend that two laboratory technicians work together during the initial processing steps (one mincing the tubules and the other searching for spermatozoa) to speed up the searching process and to allow a faster feedback to the surgeon who may decide to terminate the procedure if sperm is found or to continue dissecting the seminiferous tubules. Our laboratory performs the processing of testicular specimens by mincing and shredding the whole tissue instead of using enzymatic digestion. The mechanical preparation has the advantage of being fast, requiring about 15-30 minutes, while enzymatic digestion is more time consuming, requiring at least 4 hours for sperm preparation.

FIVE-YEAR REVIEW

Development in ART has been remarkable in the past few years. Over half a million ART treatment cycles are performed annually worldwide and the infertile male is a significant source for this increasing demand [77-79]. Several medical conditions affecting the male fertility status are now treated by ART, especially by the combination of *in vitro* fertilization and intracytoplasmic sperm injection. Nonetheless, the reproductive potential of sperm from men with defective spermatogenesis is decreased [20], while it is suggested that there is an increased risk of transmitting genetic and epigenetic defect for the embryo [80, 81]. Factors associated with higher numbers of aneuploid sperm are cigarette smoking, elevated follicle-stimulating hormone level, severe oligozoospermia and teratozoospermia [81]. Therefore, efforts should be made to improve the male health status prior to embarking on ART because current evidence suggests that fertility optimization may improve treatment outcomes [73, 82]. Men with varicocele related-infertility who require ART due to the severity of sperm abnormalities may benefit of varicocele repair prior to IVF/ICSI [73]. Even men with non-obstructive azoospermia with favorable testicular histopathology may restore sperm to the ejaculate after repair of clinical varicoceles [70, 83]. Sperm restoration, although minimal, yields the possibility of IVF/ICSI without the need of sperm retrieval techniques. In the group of patients who are still azoospermic after varicocelectomy, the chances of sperm extraction may be increased, and as a result the couple's chance for pregnancy, if the varicocele had been treated before sperm retrieval and assisted conception [82].

The laboratory management of male infertility cases involving very poor quality semen specimens requires special attention. After collecting ejaculates or epididymal fluid or testicular tissue, laboratory processing techniques are used to remove contaminants, cellular debris, noxious microorganisms and red blood cells. Processed spermatozoa may be either used for ICSI or cryopreserved. Spermatozoa collected from men with severely impaired spermatogenesis are often compromised and fragile. Therefore, adherence to state of the art laboratory techniques and quality control are recommended to avoid jeopardizing the sperm fertilizing potential in treatment cycles [21, 25, 28, 32].

Spermatozoa can be retrieved either from the epididymis or the testis, depending on the type of azoospermia, using different surgical methods such as PESA, TESA, TESE and micro-TESE. Meta-analysis results demonstrated no significant difference in any outcome measure between the use of epididymal or testicular sperm in men with obstructive azoospermia [84]. The etiology of the obstruction and the use of fresh or frozen-thawed epididymal/testicular sperm do not seem to affect ICSI outcomes in terms of fertilization, pregnancy, or miscarriage rates [74]. Sperm can be easily retrieved in cases of OA regardless of the surgical method while the best sperm retrieval technique in non-obstructive azoospermia is yet to be established. Current recommendations are based on cumulative evidence provided by descriptive, observational and controlled studies. Efficiency of TESA for retrieving spermatozoa in NOA varies from 10-30% [85], except in the favorable cases of men with previous successful TESA or testicular histopathology showing hypospermatogenesis. In such individuals, sperm retrieval rates by TESA are in the range of 70-100% [86-88]. In a recent systematic review the mean reported SRR for TESE was 49.5%. TESE with multiple biopsies resulted in higher SRR than fine-needle aspiration, a variation of TESA, especially in cases of Sertoli-cell-only (SCO) syndrome and maturation arrest [89]. In NOA, current evidence suggests that micro-TESE performs better than conventional TESE or TESA in cases of SCO, where tubules containing active focus of spermatogenesis can be identified [75]. Tissue removal in micro-TESE is often 50 to 70-fold less than conventional TESE [89, 90-92], and the small amount of tissue extracted facilitates sperm processing. Micro-TESE also appears to be the safest technique regarding post-operative complications [92-94]. The clinical outcomes of ICSI using testicular sperm extracted by TESA or micro-TESE in NOA are significantly lower than those obtained with either ejaculated or epididymal/testicular sperm from men with OA [20]. From the limited data available, it is suggested that the sperm retrieval technique itself has no impact on ICSI success rates [89]. However, frozen-thawed surgically-retrieved sperm from NOA men have significantly impaired reproductive potential than fresh ones [65, 84]. Meta-analysis results showed that fertilization rates by ICSI remained similar, but implantation was significantly higher (by 73%) with the use of fresh compared to frozen-thawed testicular sperm [84]. The concern that ICSI using sperm retrieved from men with either OA or NOA might be associated with increased risk for birth defects is still unresolved. In general, *in vitro* fertilization is associated with an increased risk of multiple pregnancies and congenital abnormalities (including hypospadias) [95]. ICSI in particular carries an increased risk of endocrine abnormalities, as well as epigenetic imprinting effects [95]. Although the absolute risk of any of these conditions remains low [95-98], current data is limited and study populations are heterogenic. It is therefore recommended that well-defined groups of ICSI with ejaculated sperm, ICSI with epididymal sperm and ICSI with testicular sperm, and a control group of naturally conceived children are closely followed up.

Despite the reduced ART success rates obtained with sperm from men with severely impaired spermatogenesis, some countries are either considering or have already implemented regulatory laws to restrict the production of embryos *in vitro* by limiting the number of oocytes to be fertilized. Recent studies critically evaluated the impact of these restrictions on ART outcome in couples affected by severe male factor infertility [66, 67, 99]. They concluded that limiting the number of treated oocytes to three per ICSI cycle significantly reduces the chance of transferring good quality embryos and thus achieving a live birth in cases of severe male factor infertility. Cryptozoospermic and NOA men are particularly affected by these restrictions.

KEY POINTS

- Assisted Reproductive Technology (ART) is a formidable treatment modality for most untreatable causes of male infertility, including immunologic infertility, severe oligozoospermia and azoospermia.
- Attention to some technical aspects during intracytoplasmic sperm injection (ICSI) may optimize treatment outcome in cases of severe male factor infertility: (i) men with severe oligozoospermia/ cryptozoospermia men should consider sperm cryobanking to avoid the risk of collecting very poor semen specimens at the time of the ICSI; (ii) the optimal time interval for microinjection is 38-42 hours after hCG administration; (iii) selection of sperm with adequate morphology for ICSI can be accomplished by using 400X optical magnification; it is also possible to identify and to perform microinjections at the oocyte vegetal pole to minimize the risk of damaging the oocyte meiotic spindle; (iv) air quality control in the IVF laboratory and critical areas may positively impact on embryo development and pregnancy results.
- Antisperm antibodies are not detrimental to ICSI outcome.
- The reproductive potential of infertile males undergoing ART is related to the severity of sperm abnormalities. Live birth rates are lower when ICSI is performed using either testicular spermatozoa extracted from men with non-obstructed azoospermia or ejaculated sperm from men with defective spermatogenesis as compared to those with obstructive azoospermia (OA) and normal sperm.
- Surgical repair of clinical varicocele should be considered in men with severely abnormal spermatogenesis or non-obstructed azoospermia prior to embarking on ART. Varicocelectomy may increase the chance of live birth by ICSI and restore spermatogenesis in non-obstructive azoospermic men (NOA) with testicular histopathology exhibiting maturation arrest or hypospermatogenesis.
- Sperm retrieval is simple and highly successful in men with OA, regardless of the cause of obstruction. In NOA, testicular spermatozoa can be obtained in all etiology-specific causes; efficiency rates are approximately 50% and do not differ according to the etiology-specific subgroups. Testicular histology results are predictive of sperm retrieval in NOA; micro-TESE performs better than TESA in maturation arrest and Sertoli cell-only histology subgroups. Sperm retrieval

rates are significantly higher in men with obstructive compared to non-obstructive azoospermia.

- The clinical outcome of ART in azoospermic men is dependent on the azoospermia being obstructive or non-obstructive. In OA, ICSI results are independent of the cause of obstruction. ICSI using testicular or epididymal sperm aspirated from men with OA are comparable. Conversely, the chances of achieving a live birth by ICSI are significantly reduced in couples whose male partner have non-obstructive azoospermia.

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CONFLICT OF INTEREST

None Declared.

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