CLINICS

REVIEW

The importance of semen analysis in the context of azoospermia

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Azoospermia is a descriptive term referring to ejaculates that lack spermatozoa without implying a specific underlying cause. The traditional definition of azoospermia is ambiguous, which has ramifications on the diagnostic criteria. This issue is further compounded by the apparent overlap between the definitions of oligospermia and azoospermia. The reliable diagnosis of the absence of spermatozoa in a semen sample is an important criterion not only for diagnosing male infertility but also for ascertaining the success of a vasectomy and for determining the efficacy of hormonal contraception. There appears to be different levels of rigor in diagnosing azoospermia in different clinical situations, which highlights the conflict between scientific research and clinical practice in defining azoospermia.

KEYWORDS: Azoospermia; Male; Infertility; Semen Analysis; Oligozoospermia.

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Azoospermia is a descriptive term referring to ejaculates that lack spermatozoa without implying a specific underlying cause. The condition is almost always an unforeseen finding when semen analysis is performed for any indication. Only in a few cases is azoospermia expected prior to semen analysis, such as in cystic fibrosis, Klinefelter's syndrome and previous vasectomy cases. Such azoospermic semen samples are found in up to 2% of the adult male population and 5-59% of infertile men (1). It is important for azoospermia to be distinguished from aspermia; specifically, the latter indicates the lack of semen formation or the lack of ejaculation, such as in the case of total retrograde ejaculation.

THE DEFINITION AND DIAGNOSIS OF AZOOSPERMIA

The appropriateness of the term azoospermia and the reliability of diagnosing the absence of spermatozoa have been the focus of debate over the past decade. The traditional definition of azoospermia is ambiguous, which has ramifications on the diagnostic criteria. The 5th edition of the World Health Organization (WHO) manual (2010) (2) adopted the following definition that was first proposed by Eliason in 1981: ''no spermatozoa are found in the sediment of a centrifuged sample'' (3). The American Urological Association offers the following, more detailed definition: ''no sperm after centrifugation at 3000 x *g* for 15 minutes and examination of the pellet'' (4). The aim of the examination of the pelleted semen is to exclude cryptospermia, which is the presence of a very small number of live sperm in a centrifuged pellet but not in a standard semen analysis. Thus, the accurate assessment of very low sperm counts is particularly important to avoid labeling severely oligospermic men as azoospermic. In one study, centrifuging semen at the low speed of 200 x *g* for 10 minutes revealed that 18.6% of men diagnosed with 'obstructive azoospermia' and 22.8% of men diagnosed with 'non-obstructive azoospermia' had motile and non-motile spermatozoa in the semen pellet (5). In addition to these laboratory considerations, the need for a change in the clinical definition of azoospermia to include its etiology, treatment, and prognosis has been repeatedly expressed (6,7).

THE ROUTINE ASSESSMENT OF SPERM COUNT

The total number of sperm in an ejaculate is influenced by testicular sperm production, the integrity of the conducting system, the presence of retrograde ejaculation (partial or total), and the duration of abstinence before the analysis. The WHO laboratory manual for the examination and processing of human semen includes standards to enhance the accuracy and precision of the sperm number estimates to make them reproducible. Special attention is required to control patient-related factors, such as the optimal abstinence duration of 2-7 days and the complete collection of ejaculate. Frequent ejaculation within a short period of time may deplete the epididymal stores, resulting in hardly any

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detectable sperm in the semen sample. Similarly, losing the first portion of the ejaculate, which is the sperm-rich portion, will significantly affect the accuracy of the assessment of sperm number. The last portion of the ejaculate is comprised mainly of seminal vesicular fluid (8). Thus, patients need to receive clear instructions, both verbal and written, on how to collect the entire ejaculate and to report the loss of any fraction of the sample.

Sound laboratory techniques are needed to reduce the amount of analytical error and enhance the precision of the sperm count. These techniques include the adequate mixing of the ejaculate, appropriate semen dilution if needed, and comparison of the replicate counts to determine if they are acceptably close (2). In addition to these technical details, there is a high biological variation in semen quality, including transient azoospermia, that may influence the clinical interpretation of seminal parameters (9). Transient azoospermia is also encountered secondary to toxic, environmental, infectious or iatrogenic conditions. Given this observation, repeating the examination of ejaculates on two to three occasions is helpful to obtain baseline data (9,10,11).

THE MACROSCOPIC FEATURES OF AZOOSPERMIC SEMEN SAMPLES

Opacity of the ejaculate

A normal liquefied semen sample with a normal cellular content will have a homogenous grey-opalescent appearance. Azoospermic samples and those with very low sperm counts appear less opaque.

Semen volume

The seminal vesicles contribute up to 70% of the normal ejaculate volume. The lower reference limit for semen volume is 1.5 ml (5th percentile, 95% confidence interval 1.4–1.7) (2). Although a low sperm volume is more likely to be due to the incomplete collection of the ejaculate, it may also be due to obstruction of the ejaculatory duct or congenital bilateral absence of the vas deferens (CBAVD) (12,13). In a study of 105 males diagnosed with CBAVD, the mean ejaculate volume was 0.7 ml (14). In CBAVD, there is dysplasia or absence of the seminal vesicles with the loss of its contribution to the semen volume. Retrograde ejaculation and frequent orgasms may lead to what is described in lay terms as 'dry ejaculation'. In these situations, there will be hardly any seminal fluid containing sperm.

Retrograde ejaculation should be suspected in any case of azoospermia and when the seminal fluid volume is <1 ml. The diagnosis is confirmed by finding spermatozoa in the post-ejaculatory urine. Spermatozoa found in the pellet after urinary centrifugation will mostly be dead due to the combined effects of osmotic stress, low pH and urea toxicity (15). The recovery of high-quality sperm after the induced modification of the urine composition and pH to facilitate its use in the intracytoplasmic sperm injection technique (ICSI) has been described (16,17).

Semen pH

The balance between the alkaline secretion of the seminal vesicles and the acidic prostatic secretion determines the semen pH. The importance of assessing the semen pH and its physiological reference range has been a matter of intense debate (18). The consensus lower reference value of the pH of liquefied semen is 7.2 (2). In CBAVD, the semen pH is characteristically low (<6.8) as a consequence of dysplasia or the absence of the seminal vesicles. When the fructose-rich alkaline secretion of the seminal vesicles is lost, the seminal plasma is formed mainly from the relative scanty and acidic prostatic secretion.

THE DIAGNOSTIC VALUE OF MACROSCOPIC FEATURES OF SEMEN IN AZOOSPERMIA

Semen volume and pH are important for determining the differential diagnosis of the cause of azoospermia. In patients with low-volume, acidic, azoospermic samples, the differential diagnosis is CBAVD or bilateral complete ejaculatory duct obstruction (EDO). A fructose assay is not needed because the volume coupled with the pH indicates no contribution from the seminal vesicles. Azoospermic ejaculates with a normal volume and alkaline pH indicate functional seminal vesicles and patent ejaculatory ducts. The differential diagnosis includes spermatogenic failure or an obstruction at the level of the more proximal vas deferens or epididymis but does not include CBAVD or bilateral EDO. In azoospermic cases with an alkaline, low-volume ejaculate, the seminal vesicles are present and functional, and at least one ejaculatory duct is open. Therefore, in cases of azoospermia, attention to the details of semen volume and pH may be quite helpful in establishing the diagnosis.

MICROSCOPIC EXAMINATION OF CENTRIFUGED SAMPLES TO DETECT SPERMATOZOA

When no spermatozoa are observed in replicate wet preparations, the semen sample can be centrifuged, and the pelleted semen can then be examined to determine if any spermatozoa are present. Whether spermatozoa are found in the pellet depends on the centrifugation time and speed and on how much of the pellet is examined (5,19).

Centrifugation time and speed

In the literature, there are different recommendations for the speed and time of centrifugation (Table 1). These recommendations appear not only inconsistent but also indecisive when terms such as 'at least' and 'less than' are used. In one study that attempted to resolve this confusion, 25 ejaculates from 'azoospermic men' were centrifuged at 600 x *g* for 10 minutes, and no sperm were found in the pellets (20). However, when supernatants resulting from the 600 x *g* centrifugation of the samples were centrifuged at 1000 x *g* for 15 minutes, spermatozoa were detected. Because no more sperm-containing pellets were detected by centrifuging the 1000 x *g* supernatant at 3000 x *g* for 15 minutes, the authors concluded that a minimum of 1000 x *g* for 15 minutes was adequate for the detection of azoospermia. The same study (20) demonstrated that centrifugation at 3000 x *g* for 15 minutes did not remove spermatozoa from the supernatant of 23 of 25 normozoospermic samples. Another study examined the interplay between the centrifugation speed and duration and demonstrated a dramatic increase in the appearance of spermatozoa in the pellet with both increasing time (10-15 minutes) and speed (600-3600 x *g*) of centrifugation (21). Thus, the accuracy of any centrifugation protocol of less than 3000 x *g* in pelleting all the spermatozoa in an ejaculate is uncertain. However, after high-speed centrifugation (3000 x *g*), motility may be lost

Table 1 - Different centrifugation speeds have been recommended to examine azoospermic ejaculates. Some of these recommendations appeared indecisive when terms such as 'at least' and 'less than' are used (italics are used to highlight such terms).

Reference	Recommended centrifugation
Mortimer (1994) (23)	1000 x *g* for 15 minutes
the Nordic Association for Andrology (24)	*At least* 1000 x *g* for 15 minutes
WHO manual (1999) (25)	600 x *g* for 15 minutes to concentrate samples with low sperm counts (less than 2 sperm per 400x field) *Less than* 3000 x *g* for 15 minutes for all samples in which spermatozoa are not detected
Corea et al. (2005) (20)	*A minimum* of 1000 x *g* for 15 minutes was adequate for the detection of azoospermia
WHO manual (2010) (2)	3000 x *g* for 15 minutes for all samples in which no spermatozoa are detected

(23), and the concentration will therefore be underestimated (22). This literature survey indicates that the replication of results among laboratories using different centrifugal forces is unlikely to be consistent (22).

The most recent WHO manual (2010) suggested that when assessing an apparently azoospermic sample, consideration must be given to whether subjective data on the presence and motility of spermatozoa are sufficient or whether accurate spermatozoa counts are required. For example, when *motile* spermatozoa are sought in a post-vasectomy semen sample, the high-speed centrifugation of spermatozoa must be avoided, and only an aliquot of the undiluted sample can be assessed. The microscopic examination in this procedure can take longer (up to 10 minutes) than low-speed centrifugation because the sample will have a high cellular background. When no spermatozoa are observed in replicate assessments of pelleted semen, the WHO manual (2010) recommends reporting the sample as ''No spermatozoa were seen in the replicates, too few for accurate determination of concentration''. This guarded reporting takes into account the errors of counting (2,22) and the possibility that the absence of spermatozoa from the examined aliquot does not necessarily indicate their absence from the remainder of the sample. This approach is adopted when a case of apparent azoospermia is examined further to determine if there are enough spermatozoa to fertilize a limited number of eggs using the ICSI technique.

However, when the aim of pelleting semen samples is to obtain an accurate assessment of the sperm number, high-speed centrifugation should be used (3000 x g). This approach is necessary for male hormone contraception research and for diagnostic purposes, such as in CBAVD and the confirmation of sperm clearance after a vasectomy. In these situations, rendering spermatozoa immotile and promoting reactive oxygen species-induced sperm damage as a result of the high-speed centrifugation are irrelevant.

Alternatives to centrifugation

One alternative to centrifugation is the use of a low semen dilution (1+1 [1:2]) to evaluate larger volumes by either preparing more chambers or using chambers with an inherently larger volume, such as the improved Neubauer chamber (2,22). Because this technique utilizes a fixative to immobilize the sperm cells, it is not suitable when the semen sample is examined for the potential harvesting of sperm, if found, for ICSI treatment. The absence of spermatozoa from the examined aliquot does not necessarily indicate their absence from the remainder of the sample. If no spermatozoa are found in replicate assessments, the WHO manual (2010) recommends that the sample be reported as ''No spermatozoa were seen in the replicates, too few for accurate determination of concentration'' (2).

Another alternative is the use of fluorescence microscopy using an aliquot of semen diluted 1:2 with a fixative containing Hoechst 33342 bisbenzimide fluorochrome (1 mg/l) to label the sperm nuclei (2,22). Again, this method is not suitable when the identified sperm are used for ICSI treatment.

FUTURE DEVELOPMENTS

The standard method of semen analysis is to assess the macroscopic appearance and cellular content of the ejaculate. Investigation of the molecular composition of seminal plasma to explain the cause of sperm and seminal plasma abnormalities has thus far been limited to the evaluation of the fructose content and, in later years, the level of reactive oxygen species in the plasma (26,27,28)). More recently, the study of the seminal plasma proteome appears to offer the potential to identify biomarkers that may aid in the diagnosis of the causes of azoospermia. Many of the proteins in the seminal plasma are expressed in the testis and epididymis and are linked to fertility. Some of these proteins may be useful as noninvasive biomarkers to discriminate non-obstructive azoospermia from obstructive azoospermia (29).

The reliable diagnosis of the absence of spermatozoa in a semen sample is important for diagnosing male infertility, ascertaining the success of vasectomy, and determining the efficacy of hormonal contraception. From the laboratory point of view, inconsistent approaches to studying semen and imprecision can handicap both research and clinical practice (30). These technical issues are further compounded by the apparent overlap between the definitions of oligospermia and azoospermia. This overlap is present in the WHO manual (2010), and although appearing unjustified, it echoes the different levels of rigor in diagnosing azoospermia in different clinical situations and highlights the conflict between scientific research and clinical practice in defining azoospermia.

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