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The Relationship between the Halosperm Assay and Semen Analysis Performed According to the 4th and the 5th Editions of the World Health Organization Guidelines

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Abstract

Background: As a standard reference to evaluate male factor infertility, the majority of fertility laboratories use the 4th or 5th Editions of the World Health Organization's semen analysis guidelines. Following the release of the 5th Edition, debate over its legitimacy has resulted in some laboratories using the 4th and others the 5th Edition. DNA integrity tests have been shown to be a valuable adjunct to semen analysis and have subsequently been adopted by many fertility laboratories. This study explored the prevalence of samples with high DNA fragmentation levels according to semen analysis categories using both the 4th and the 5th Edition reference ranges.

Materials and Methods: The study included 905 consecutive semen samples from 863 infertile couples attending a fertility clinic. A semen analysis was conducted according to both the 4th and 5th Edition guidelines published by the World Health Organization. DNA damage was assessed using the Halosperm G2 test kit and expressed as a percentage DNA fragmentation level.

Results: Alongside both the World Health Organization 4th and 5th Edition semen analysis criteria abnormal DNA fragmentation levels were more common in abnormal semen samples however elevated DNA fragmentation levels were also found in normal semen samples using the same criteria. Of the samples that were graded as normozoospermic according to the 5th Edition guidelines 16% were deemed to have elevated DNA fragmentation levels compared to 11.7% graded by the 4th Edition guidelines. The number of normozoospermic samples, graded according to the 5th Edition guidelines was significantly higher (n=697) than when the same samples were graded according to 4th Edition guidelines (n=385) (p=0.001). A significant proportion of samples with an abnormal DNA fragmentation level corresponding to the World Health Organisation 4th and 5th Edition criteria were evident in normozoospermic (p <0.05), normoteratozoospermic (p=<0.005) and normoasthenozoospermic (p<0.05) samples.

Conclusion: Our findings indicate that abnormal DNA fragmentation levels are proportionate to the World Health Organisation semen analysis criteria with fragmentation levels increasing according to the increasing number of semen analysis abnormalities. In some cases however, abnormal fragmentation levels were recorded when semen analysis was normal and normal fragmentation levels were recorded where the semen analysis was considered abnormal.

Keywords: DNA fragmentation; Halosperm; Semen analysis; WHO 4th Edition; WHO 5th Edition; Sperm; DNA damage

Introduction

Traditionally, male infertility is diagnosed using World Health Organization (WHO) standard parameters which are published in a laboratory manual providing guidelines to laboratories for processing human semen and cut-off values to determine normality or abnormality [1]. Since publication of the first manual in 1980 there have been a number of updates released with the latest two editions being the 4th Edition in 1999 and the 5th Edition in 2010 [2]. Substantial changes between the two editions have resulted in the same patient being diagnosed (under the 5th Edition guidelines) with a normal semen analysis when they would have been diagnosed with an abnormal semen analysis had the laboratory used the previous version cut-off values. However, the application and reference values presented in the 4th Edition resulted from vague reference populations and therefore lacked transparency, resulting in little consensus around the accuracy of these values [3,4]. Furthermore this was acknowledged by the WHO [2,5]. Although the 5th Edition has included clearly defined reference ranges, concerns have been raised over the studies that generated these values [2] and so debate has ensued over its legitimacy leaving no definitive agreement between laboratories as to which edition should be used [6].

While semen analysis is regarded as a key tool to evaluate male infertility [4] in spite of which reference values are used [7] it does not consider sperm DNA integrity. DNA fragmentation testing whilst considered useful, has not yet been universally accepted due to a lack of standardization of tests and protocols [8].

Since about 15% of infertile men undergoing a semen analysis will have semen within normal parameters [9,10], there has been a focus on sperm DNA fragmentation and its association with infertility. Elevated levels of DNA fragmentation have been linked with infertile

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men [10-14], poor fertilization rates [15,16], specific semen defects [17], miscarriage and poor artificial reproductive technology results [18]. Furthermore, infertile men have been shown to have substantially higher levels of DNA fragmentation than fertile men [13] and hence DNA fragmentation testing has been proposed as a valuable adjunct to routine semen analysis when considering the fertility potential of a man [16].

Although DNA fragmentation testing can be conducted in a number of ways, the Halosperm assay has been described as a 'cheap and convenient' test [18] that has a relatively short and simple protocol with correlations having been observed with some of the most commonly recognised tests [11,19-21]. The aim of this study was to formally investigate the clinical significance of the Halosperm test and assess its relationship to the two most recent WHO semen analysis guidelines.

Materials and Methods

We studied 905 consecutive semen samples from 863 men who underwent semen analysis and DNA fragmentation testing using the Halosperm assay as part of their fertility treatment. The study was approved by the Edith Cowan University Human Research Ethics committee and the Joondalup Health Campus Human Ethics Committee.

Semen analysis

Men were instructed to have 2 to 5 days sexual abstinence before producing a semen sample for analysis. Semen samples were collected by masturbation into a clean 60 ml wide-mouthed universal container and processed within 1 hour of ejaculation and liquefaction. Semen analysis was performed according to the WHO 4th Edition guidelines [22] for volume, concentration, motility and morphology. Semen samples were then classified according to both WHO 4th and 5th Edition criteria [1] as shown in Table 1.

Following semen analysis, DNA fragmentation analysis was carried out using the Halosperm G2 test kit (Halotech DNA SL). Details of this procedure have been described elsewhere [23] but briefly, semen samples were mixed with a liquefied agarose gel and placed onto a pre-coated slide. The slides were refrigerated for 5 minutes before being treated with a denaturing agent, followed by a lysis solution and finally staining solutions. The criteria to determine fragmented and non-fragmented DNA was followed according to the manufacturer's instructions. In essence spermatozoa with a large or medium sized halo depict unfragmented DNA whilst those with either a small halo or without a halo and those with a weak or irregular stained core depict fragmented DNA. The DNA Fragmentation Levels (DFL) for each sample was assessed by counting a minimum of 300 sperm under the x100 objective of the microscope. The numbers of sperm judged to have fragmented DNA were expressed as a percentage of the total number. Samples with a reading of >30% were deemed abnormal.

Statistical Analysis

Pearson correlations and analysis by ANOVA were performed to study the relationship between sperm DNA fragmentation and semen analysis results with post-hoc testing by Tukey's HSD and proportions compared by a Chi-squared test. The minimum level of significance was set at $P < 0.05$. Microsoft Excel and StatistiXL (Nedlands, Western Australia) statistical packages were used to perform all statistical analyses.

Results

Semen quality according to different WHO criteria

Using the reference ranges in the WHO manual 4th Edition, 385/905 (42.5%) semen samples were classed as normozoospermic with the remainder having 1 or more abnormalities as shown in Table 2. However, classification of the same samples using the reference ranges provided in the WHO manual 5th Edition showed that 697/905 (77.0%) were said to be normozoospermic, simply as a consequence of lower limits of normality. Table 2 also shows that of the 520 samples with 1 or more abnormalities according to WHO 4th Edition, 78 (25%) had the same number when classisfied according to WHO 5th Edition and 442 (85%) had a reduction in the number of abnormalities.

Semen analysis and DNA fragmentation

A summary of the 905 semen analysis results and their correlation with the corresponding DNA fragmentation results is shown in Table 3. A significant positive correlation was seen between SDF and sexual abstinence (ie longer abstinence associated with higher SDF), whereas significant negative correlations (ie lower semen quality associated with higher SDF) were seen between SDF and sperm concentration, motility and morphology.

The mean DNA fragmentation results according to semen quality are shown in Table 4. The only statistically significant difference in DFLs between samples classified under the 4th and 5th Editions was for the normozoospermic samples. However, for both classification systems as shown in Table 4, there were significant increases in the DFLs as the severity of semen abnormalities increased.

Semen Parameters	WHO 4th Edition	WHO 5th Edition
Volume (mL)	2	1.5
Sperm Concentration (10^6/mL)	20	15
Motility	50% (a + b)*	32% (a + b)*
Morphology (% normal)	14	4

*Grade a=rapid progressive motility (>25 μm/s), *Grade b=slow/sluggish progressive motility (5-25 μm/s).

Table 1: WHO 4th Edition and WHO 5th Edition semen analysis criteria cut off values. (Adapted from Esteves, et al. [28]).

Abnormalities		WHO 5th Edition				
		0	1	2	3	Total
WHO 4th Edition	0	385	0	0	0	385
	1	233	46	0	0	279
	2	72	81	19	0	172
	3	7	25	24	13	69
	Total	697	152	43	13	905

Table 2: The number of abnormalities (sperm concentration, motility or morphology) in 905 semen samples when classified according to the reference ranges of WHO 4th and 5th Edition manuals.

Variable	Value	Pearson's r	P value
Abstinence (days ± SEM)	4.1 ± 0.2	0.11	0.001*
Volume (ml ± SEM)	3.5 ± 0.1	0.05	0.159
Sperm concentration (x10^6/ml ± SEM)	68.6 ± 2.2	-0.17	0.000*
Sperm motility (% ± SEM)	59.0 ± 0.6	-0.30	0.000*
Sperm morphology (% ± SEM)	14.0 ± 0.2	-0.24	0.000*

Values are presented as mean ± SEM.

*Significant association.

Table 3: Pearson Correlation for DNA fragmentation level to corresponding semen parameters, 905 samples from 863 men were analysed.

Semen Quality	WHO 4th Edition		WHO 5th Edition		Significance between 4th and 5th Editions
	n	SDF (%)	n	SDF (%)	p
Oligozoospermia (O)	31	23.3 ± 2.8[th]	99	27.0 ± 1.7[l,o,p]	NS
Oligoteratozoospermia (OT)	77	27.6 ± 2.0[e,i]	18	31.0 ± 4.4	NS
Oligoasthenozoospermia (OA)	10	28.1 ± 6.4	21	33.4 ± 4.3[m]	NS
Oligoasthenoteratozoospermia (OAT)	69	37.0 ± 2.6[d,g,h,i]	13	46.6 ± 5.8[n,p]	NS
Normozoospermic (N)	385	17.5 ± 0.6[a,b,c,d,e,j]	697	20.3 ± 0.6[j,k,l,m,n]	0.001
Normoteratozoospermia (NT)	203	21.8 ± 1.2[c,f,g]	21	29.9 ± 4.9	NS
Normoasthenozoospermia (NA)	45	28.0 ± 3.2[a]	32	39.4 ± 4.6[k,o]	NS
Normoasthenoteratozoospermia (NAT)	85	30.7 ± 2.1[b,f]	4	36.1 ± 9.6	NS

Statistical differences within the same manual edition are represented with the same superscript and are significantly different ($p<0.05$). NS=Not significant.

Table 4: DNA Fragmentation levels (mean ± SEM) corresponding to semen parameters classified according to different WHO semen analysis editions (n=905).

Semen quality	Abnormal SDF/Total (%)		
	WHO 4th Edition	WHO 5th Edition	P value
Oligozoospermia (O)	6/31 (19.4%)	31/99 (31.3%)	0.100
Oligoteratozoospermia (OT)	22/77 (28.6%)	7/18 (38.9%)	0.195
Oligoasthenozoospermia (OA)	4/10 (40.0%)	10/21 (47.6%)	0.345
Oligoasthenoteratozoospermia (OAT)	37/69 (53.6%)	10/13 (76.9%)	0.060
Normozoospermic (N)	45/385 (11.7%)	112/697 (16.0%)	0.025*
Normoteratozoospermia (NT)	34/203 (16.8%)	9/21 (42.9%)	0.002*
Normoasthenozoospermia (NA)	13/45 (28.9%)	17/32 (53.1%)	0.016*
Normoasthenoteratozoospermia (NAT)	38/85 (44.7%)	3/4 (75.0%)	0.117

*Significant association.

Table 5: Proportion of samples with abnormal DNA fragmentation levels (>30%) corresponding to WHO 4th Edition and WHO 5th Edition semen analysis criteria.

Samples classified as OAT by the 4th Edition semen analysis criteria had significantly higher DFLs than O ($p<0.01$) as did the samples classified by the 5th Edition ($p<0.001$). NA samples, classified under both 4th and 5th Edition criteria had significantly higher DFLs than N samples ($p<0.001$ and $p<0.0001$ respectively).

Comparing means of DFL for the various categories of semen quality is limited in that each group will have a mixture of samples with normal and clinically abnormal levels of DFL. Table 5 shows another way of looking at this by considering the proportion of semen samples that registered an abnormal sperm DFL (>30%) according to the corresponding semen analysis classification.

Samples recorded with sperm concentration, morphology and progressive motility levels outside of the WHO 4th and 5th Edition reference, revealed the highest proportion of samples with abnormal DFL (>30%), whilst the lowest proportion of samples with abnormal DFLs were shown to be within normal limits for all concentrations, morphology and progressive motility regardless of which manual's criteria were used.

Discussion

Semen analysis is intended as a screening test to identify potential sub-fertility in men. As such, the test must be conducted under standard conditions so that the results of one man may be compared directly with others. This includes a standard period of sexual abstinence prior to production of the sample, as well as the use of standardized analytical procedures. To this end, the WHO have produced a series of manuals which include guidelines on the performance of semen analysis, and a series of reference ranges against which the samples may be judged. These manuals have undergone periodic revision and the last two editions, the 4th and 5th, are the ones in most frequent use these days.

This study has evaluated the relationships between semen quality as determined using both the WHO 4th and 5th Edition semen analysis criteria, and DFLs as identified by the Halosperm G2 test kit. The results reveal significantly higher DFLs in samples that have been classified as being abnormal compared with those with a semen analysis within normal parameters regardless of which edition of the WHO guidelines are used. Oligoasthenoteratozoospermic samples had the highest DFLs when compared to oligozoospermic, teratozoospermic and asthenozoospermic samples and it was evident that the proportion of samples with abnormal DFLs was higher when the WHO 5th Edition semen analysis criteria were applied compared to the 4th Edition criteria. No statistical difference was observed however between the specifically categorised semen defects when the two different guidelines were applied. Only samples with both abnormal motility and morphology, as scored using the 4th Edition criteria, showed a mean abnormal DFL. In contrast, all samples with some abnormality scored using 5th Edition semen analysis criteria revealed a mean abnormal DFL. The general finding that there is an inverse relationship between DFL and sperm morphology and motility, i.e. higher DFL when the proportion of sperm with normal morphology and motility is reduced, concurs with other reports [17,24-27].

Semen samples showed an inverse relationship between sperm concentration with DFL. This is in accord with Irvine et al., [25] who used WHO 3rd Edition criteria which has the same sperm concentration criteria as the 4th Edition [28]. Nevertheless, the proportion of samples with normal parameters according to the WHO 4th and 5th Editions but with DFLs >30% were 11.7% and 16% respectively. This is aligned with others' findings whereby approximately 15% of infertile men tested have semen within normal parameters [9,10]. Table 5 shows that a higher proportion of semen samples with normal sperm concentrations according to the 5th Edition but abnormalities of sperm

morphology and/or motility have significantly elevated DFLs. The inclusion of a test of sperm DNA fragmentation is therefore important in providing additional information about the condition of the sperm. Men producing samples with abnormal DFLs may therefore approach assisted reproduction with a number of different strategies to help achieve a pregnancy, including the use of antioxidants [29], ICSI [16] or frequent ejaculation [30].

In summary, the present study has shown that there is an association between semen quality and DFL. The measurement of sperm DNA fragmentation does give further information on which to base decisions regarding future treatment. Care should also be taken as the reference range used in interpreting the semen analysis does influence the prevalence of abnormal DFL.

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