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Can the Prediction of Intrauterine Insemination Results by Used Aniline Blue Stain (ABS) and Sperm Chromatin Dispersion (SCD) Levels?

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Abstract

Introduction: This study aimed to perform routine seminal fluid analysis, sperm DNA fragmentation, and sperm function tests at the chromatin maturation level and evaluate pregnancy in the patients passing intrauterine insemination before starting Intrauterine Insemination (IUI) method. **Materials and Methods:** In this prospective study, 111 couples who underwent Intrauterine Insemination (IUI) in unexplained infertility patients were admitted to Al-Farah IVF and assisted reproductive center in Baghdad, Iraq between November 2020 and February 2021 were evaluated. Semen fluid analysis was performed based on (WHO 4th) guiding rules. In addition, Sperm Chromatin Dispersion (halo test) and sperm maturation were performed with Aniline Blue Stain (ABS). **Results:** Sperm Chromatin Dispersion (SCD) groups were compared in terms of pregnancy outcome; the positive pregnancy rate was found to be above in the normal SCD groups ($p = 0.0005$). In addition, Aniline Blue Stain (ABS) groups were compared in the terms of pregnancy outcome; the positive pregnancy rate was found to be higher in the normal ABS group ($p = 0.017$). **Conclusion:** Our study showed that the use of DNA fragmentation (SCD) and sperm maturation tests (ABS) together with routine semen analysis in intrauterine insemination cases will make a significant contribution to the prediction of Intrauterine Insemination (IUI) increased results. So, these results indicate a defect in the effect of DNA fragmentation on the outcome of intrauterine insemination.

Keywords

Sperm Chromatin Dispersion, Aniline Blue Stain, Sperm DNA

Fragmentation, Intrauterine Insemination

1. Introduction

Intrauterine Insemination (IUI) is a simple, inexpensive, and effective method of conception [1]. The first article was published about IUI in 1962 [2], IUI evolved from sperm preparation to ovulation induction over the years. Male infertility is a common disease and Intrauterine Insemination (IUI) is used to treat mild to moderate forms [3]. Previous studies have confirmed the relationship between the success and failure of intrauterine fertilization and the DNA fragmentation rate of sperm. DNA Fragmentation Index (DFI) levels were found to be negatively associated with the overall pregnancy rate in patients who underwent assisted reproductive types ($p < 0.0001$) [4]. Studies have shown that sperm chromatin condensation is generally abnormal in men participating in IUI, and most of them are normal sperm [5]. For infertile couples, a sperm analysis is routinely used to assess the malefactor. Semen analysis is regarded as the cornerstone of the male fertility work-up process. But the question of whether or not sperm analysis predicts natural conception is still controversial [6]. A traditional first step in evaluating male factor infertility is the semen analysis, which is believed to be insufficient for determining fertility in two types *in vivo* or *in vitro*. Seminal fluid quality is confirmed according to motility, morphology, and concentration of the spermatozoa [7]. Although sperm parameters cannot predict fertilization, sperm membrane or chromatin anomalies may exist. The number of protamines in chromatin has also been measured using indirect methods, such as different staining methods or fluorophores [8]. Any chemical change in the normal structure of the DNA in the sperm can be defined as a form of sperm DNA damage. Under these conditions, single-strand or double-strand breaks in the deoxyribonucleic acid of sperm are the most common type of deoxyribonucleic acid fragmentation [9]. For the correct transmission of paternal genetic information, normal sperm chromatin structure is essential, and DNA breaks in sperm are associated with decreased fertility [10]. DFI greater than 30% is statistically significant in sperm chromatin assays and is associated with poor fertilization rates in Intrauterine Insemination (IUI), IVF, and ICSI [11]. When the DFI is less than or equal to 27 percent, patients treated with IUI have a significantly higher chance of becoming pregnant [12]. In a study conducted by [13], couples with a DFI of 26 percent could conceive, the study concluded.

2. Materials and Methodology

We studied 111 couples with unexplained infertility due to both male and female factors between November 2020 and February 2021 at Al-Farah Specialist Fertility Center and IVF-Baghdad. Before undergoing IUI, all men with Normozoos-

permic infertility underwent an andrology evaluation. Aniline Blue Stain (ABS) was used for Sperm Chromatin Dispersion (halo test) and sperm maturation in a fertility assessment that included semen analysis, in accordance with (WHO 4th) recommendations, Table 1 shows Laboratory data for male cases. At the same time, examinations were conducted on the wife and included in this study was average women's age included between 20 to 40 years. Hormones assays such as serum Thyroid-Stimulating Hormone, Prolactin hormone, Follicular Stimulating Hormone, Luteinizing Hormone, and Estradiol Hormone were performed during the menstrual cycle on days 2 - 3, 11 - 12, and 21 - 22. Following that, serial vaginal Ultrasonography was used to evaluate ovulation status, uterine size, endometrial thickness, ovarian size, and the number and size of Antral follicles. Eventually, Hysterosalpingography and/or diagnostic laparoscopy were used to check tubal patency. Samples were collected according to informed consent for patients and the approval and ethical requirements of the infertility center (This study was approved by Al-Farah Specialist Fertility Center and IVF, Baghdad, ethics committee number 2/3/5157, 10/08/2020).

Table 1. Laboratory data for male parameters.

Parameters	Average	Minimum	Maximum	Standard deviation
Patient age (male)	35.144	23	44	4.58
BMI (Body Mass Index)	29.631	22.0	41.9	4.10
Sperm concentration (million/ml)	64.702	25	190	36.07
Sperm morphology (%)	23.00%	17%	30%	4.04%
Sperm motility (%)	37.00%	25%	69%	8.08%
Sperm chromatin condensation (%) (ABT)	42.00%	14%	67%	12.13%
Sperm Chromatin Dispersion (%) (SCD)	31.00%	9%	51%	11.43%

2.1. Sperm Chromatin Dispersion Assay

The test is based on the differential reaction from those that have DNA intact in the nuclei of sperm with fragmented DNAs. Regulated DNA denaturation, followed by nuclear protein extraction, leads to partially deproteins in which the DNA loops extend, forming chromatin-dispersion halos. However, either no dispersion halo or the halo does not produce any spermatozoa nucleoids whose sperm dioxide is fragmented.

As per the detailed method from the manufacturer (http://www.spermfunc.com/), Put the tube with the blue cap at a temperature of 80˚C for 20 minutes until the gel inside is completely dissolved. Then, transfer the tube to a temperature of

37˚C for 5 minutes. We took 100 µl of semen and mix it with normal saline until it reaches a concentration of 5 - 10 million per ml. We took 60 µl from the mixture and mix it with a tube of gel at a temperature of 37˚C, then directly take 30 µl and put it on the slides that have been cooled at a temperature of 2˚C - 8˚C and place over the sample the sliding cover with no bubbles and put the slides in the refrigerator for 5 minutes at a temperature 2˚C - 8˚C, then remove the slide cover horizontally and gently pull it. Place the slides in solution A (Denaturation solution) vertically for 7 minutes at room temperature. Place the slides in solution B (Lysis solution) vertically for 25 minutes at room temperature, then place the slides horizontally in distilled water for 5 minutes and repeat the process twice. Apply the slides at the concentrations of 70%, 90%, and 100% respectively for 2 minutes for each concentration. Then wash the slides with tap water, dry them on filter paper, add 15 drops of Wright's solution, add 30 drops of Wright's buffer to them, and leave the slides at room temperature for 15 minutes. Wash the slides with distilled water and let them dry. The counting is under a light microscope at 40× and in our study 500 sperms were counted for each sample. The DNA fragmented sperm appears without a halo and the sperm with the halo appears without the DNA fragment (**Figure 1**).

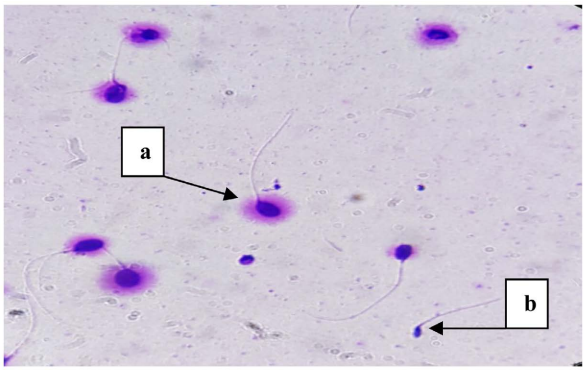

Figure 1. The picture shows (a) halo sperm, and (b) non-halo sperm.

2.2. Aniline Blue Staining Method

The mature sperm nucleoprotein is protamine that contains arginine and cysteine residues; the immature sperm nucleoprotein is a histone that contains lysine residue. The aniline blue binds to a lysine residue in the acidic condition and produces a dark blue compound which therefore indicates the presence of immature nuclear protein, while the sperm can be stained red by mature nucleoprotein.

As per the detailed method from the manufacturer (http://www.spermfunc.com/), After liquefying the sample in the incubation of 37˚C for 30 minutes, we took at least 200 µl of semen and put it in the Eppendorf and add 1 ml of Normal Saline and mix well, and then yes, centrifugation for 5 minutes 1000 g (repeat this step twice) and then we add 100 µl of solution A (Sperm wash medium) and mix it well. Take 5 µl of the mixture and put it on the slides and let it dry at room tem-

perature, then add 3 drops of solution B (Fixative solution (methanal)) on top of the slides and wait 3 minutes to dry and wash it with tap water and let the slides dry on filter paper at room temperature. We added solution C (Aniline blue stain) three drops and leave it for 5 minutes, and then wash it with tap water. We place the slides in solution D (Elution (HCL)) vertically for 5 minutes, and then wash and dry the slides on filter paper. Then added solution E (Xanthene stain) (xanthene and Sodium azide) two drops to the slides for 5 minutes, wash them with tap water, and let them dry on filter paper at room temperature. The count is under 100× by oil immersion and in the picture below (**Figure 2**) the immature blue-colored sperm and the mature red-colored sperm. In our study, 200 cells were counted for each sample.

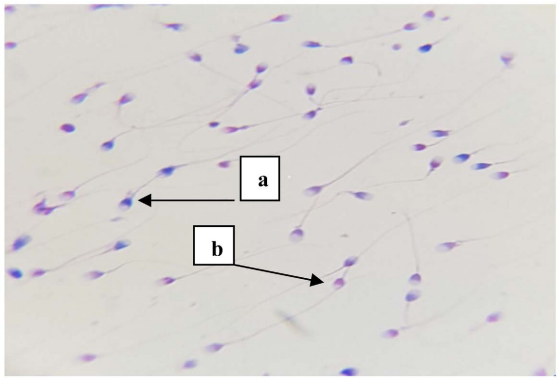

Figure 2. The picture shows (a) the immature sperm blue-colored, and (b) the mature sperm red-colored.

2.3. Intrauterine Insemination

All the women underwent IUI. letrozole 5 mg with or without Human Menopausal Gonadotropin (HMG) or HMG alone or Clomiphene citrate 50 - 100 mg were used in all-female inductions of ovulation at intrauterine insemination cycles [14]. In this study, 111 couples who were preparing for IUI were included. IUI was performed using approximately (0.3 - 0.5 ml) of the prepared semen, which was aspirated and connected to the endo-cervical catheter (Gynetics, Belgium) [15]. After more than 14 days, a female blood sample was taken to test for human chorionic gonadotropin (β-hCG), and the pregnancy results were reported. An indirect swim-up was used to prepare the sperm. According to our fertility center protocol, all study participants who underwent IUI followed our Fertility guidance center in the collection of samples.

2.4. Statistical Analysis

Arithmetical means, standard deviations, and percentage rates were used to present the data. The non-parametric (Spearman's rho) and (Chi-Square tests) correlation between SCD and ABS with Pregnancy results. And the Charts worked in Microsoft Word Excel 2007. The Statistical Package for the Social Sciences (SPSS) S.V. 26.0 was used for all statistical analyses (International Business

Marching, United State American). Statistical significance was defined as a $p \leq 0.05$.

3. Results

In our study, was examined the effect of two different methods on DNA fragmentation by Aniline Blue Staining and Sperm Chromatin Dispersion with pregnancy results for all samples. Before the intrauterine insemination process, ABS groups were compared in terms of pregnancy outcome; positive pregnancy rate founded to be a higher in the normal ABS groups ($p = 0.0001$) (Table 2) and (Figure 3) Clarification of the relationship between aniline blue staining and pregnancy outcome rate by artificial insemination.

In addition, SCD groups compared in terms of pregnancy outcome; positive pregnancy percentage founded to be higher in the normal SCD groups ($p = 0.008$) (Table 3) and (Figure 4) Clarification of the relationship between Halo Sperm and pregnancy outcome rate by intra uterine insemination.

Table 2. ABS levels with pregnancy results.

Pregnancy test	ABS	
	Normal ABS* < 30%	Abnormal ABS* > 30%
Positive result	24	17
Negative result	1	69
Total	25	86
Statistical analysis	$X^2 = 0.0001$ DF = 1 $p < 0.05$	

*Normal ABS < 30%, Abnormal ABS > 30% (according to the manufacturer's recommendations).

Table 3. SCD levels with pregnancy results.

Pregnancy test	SCD	
	Normal SCD* < 25%	Abnormal SCD* > 25%
Positive result	6	12
Negative result	30	63
Total	36	75
Statistical analysis	$X^2 = 0.008$ DF = 1 $p < 0.05$	

*Normal SCD < 25%, Abnormal SCD > 25% (according to the manufacturer's recommendations).

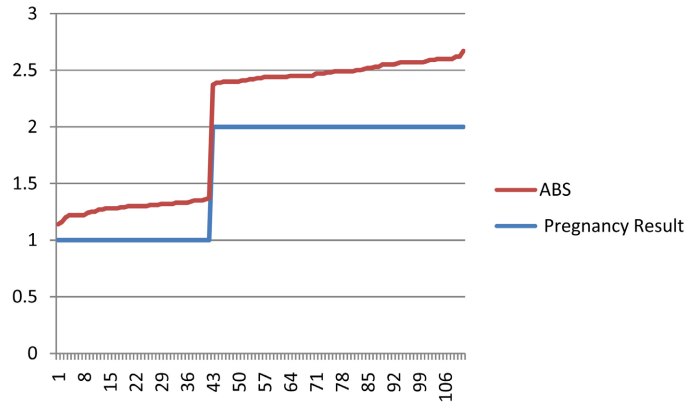

Figure 3. Explains the relationship between ABS and pregnancy results.

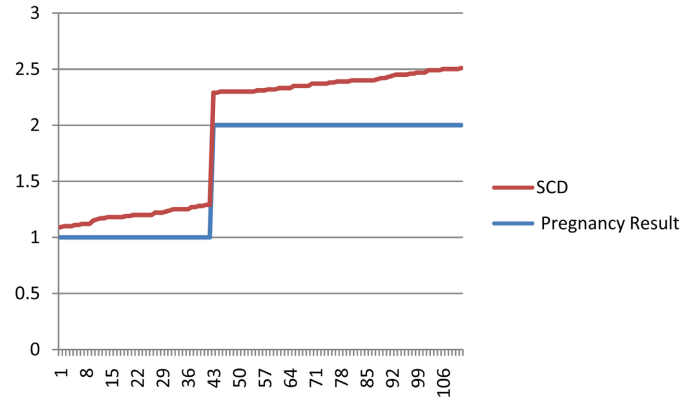

Figure 4. Explains the relationship between halo sperm and pregnancy results.

4. Discussion

Male factor infertility is a term used to describe couples in which infertility after one year, they have caused by a specific problem in the male and female partner [16]. Many types of research proved, that ovulation induction and Intrauterine Insemination (IUI) have both been shown to be effective treatments for male factor infertility unexplained and Infertility known causes [17]. Any procedure or treatment for fertility problems that involve handling eggs, sperm, or embryos outside the body is known as assisted reproductive technology [18]. Today, infertility clinics and IVF centers in Iraq and others Worldwide Countries face a huge problem in diagnosing the cause of male infertility, especially. The main criterion in the initial diagnosis of the cause of infertility in male couples is seminal fluid analysis [19]. And seminal fluid analysis depends on the criterion of sperm progressive motility, the total number of sperm, and the normal and abnormal form of the sperm [20]. In recent years, it has been believed that it is no longer sufficient to rely on routine semen analysis only in the case of healthy couples, so has begun on the analysis of sperm function including sperm DNA fragmentation [21]. Some are seeing an increase in the importance of sperm DNA fragmentation due to poor reproductive outcomes, lifestyle factors, and repeated pregnancy losses [22]. Some researchers believe that Sperm Chromatin Depres-

sion (SCD) is the result of an improperly packaged chromatin package during spermiogenesis, sperm apoptosis, or sperm transport through the male reproductive tract [23].

Defective chromatin packaging and DNA damage have a clear correlation [24]. In another study [25] showed that the sperm chromatin maturity, assessed by (ABS) dyeing, may portend the pregnancy in the couples with unexplained female infertility plus unexplained male infertility or mild male factors. The study concluded [26] Men with infertility can be distinguished from those who are fertile using sperm DNA fragmentation (TB and ABS staining). As well study [27] refereed IUI procedures should not be recommended when the DFI is >30%. Other research, further research is required to determine the correlation between IUI clinical studies and sperm chromatin tests [28]. As yet, there is no clinically relevant, standard DNA damage test for sperm with an appropriate cut-off level [29]. In our research, IUI results, SCD, and ABS levels were found to be positively correlated with pregnancy rate positive and negative results. And we concluded that aniline blue dye (ABS)-stained sperm chromatin analysis and Sperm Chromatin Dispersion (SCD) may be useful in predicting the success of intrauterine insemination.

5. Conclusion

Sperm Chromatin Dispersion and Aniline Blue Stain are simple methods, accurate, highly reproducible, and inexpensive for the analysis of semen and processed spermatozoa. Therefore, we believed that the Sperm Chromatin Dispersion and Aniline Blue Stain could potentially in males who unexplained infertility be used as a routine test in the andrology laboratory before starting Intrauterine Insemination treatment.

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Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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