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Correlation between intra-uterine insemination outcome and Kruger strict criteria using two staining methods to detect the sperm morphology of infertile men

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Abstract:

Background

Since 1999 , the World Health Organization (WHO) recommended the assessment of sperm morphology by Kruger strict criteria(KSC). The manual of WHO contains the cut off values of sperm morphology measured by KSC for normal subject and patients treated by in vitro fertilization (IVF) and intra-cytoplasmic sperm injection(ICSI)without any information regarding intrauterine insemination(IUI) .

Objectives

The objective of this study is to achieve two major goals that are :1- Assessment of sperm morphology by applying KSC , using two different biological stains, named commercially Testsimplets (TS)pre-stained slides and Sperm-O-Scan (SOS)stain 2- To have a cut off value for morphologically normal sperm that resulting into a successful IUI outcome.

Materials and Methods:

Fifty five unselected couples attending the Institute of Embryo Researches and Infertility Treatment were included in this prospective study from December 2007 till August 2008 .The mean age of male partner was 31.7±1.87years with an average duration of 6.25±1.65years of infertility. The women were prepared for IUI. All the males were subjected to semen analysis with in vitro sperm activation using layering swim-up technique. Assessments of the morphologically normal sperm(MNS) of the samples were performed by using both, High power field and KSC by using TS and SOS stains before and after the activation. Correlation of the positive IUI results and MNS by KSC waw recorded denoting the cut off value for successful results.

Results

The mean of morphologically normal sperm applying KSC with the use of SOS stain before in vitro sperm activation was 16.10% and after activation was 37.85% and by using TS stain before in vitro sperm activation was 15.14% and after activation was 37.09% .No significant (P>0.05) differences was detected between the results of MNS of the two stains. The result of IUI was 11 positive pregnancies out of the 55 cases. The mean of MNS using KSC for these positive cases was 11.58% and 12.84% for SOS and TS stains, respectively.

Conclusions

The cut off value of MNS by applying KSC and using two different biological stains for a successful IUI program was 11.58% by SOS stain and 12.84% by TS pre-stained slides. Although the study found no statistical differences between the two stains, TS found to be easier, simpler and less time consuming technically than SOS stain.

Key Words: IUI, Kruger strict criteria, Testsimplets (pre-stained slides) , and Sperm-O-Scan stain

Introduction

Analysis of male fertility problems has been based on conventional semen profile with information analysis on the volume of the ejaculate, the concentration of spermatozoa, their motility and morphological appearance. Thus the diagnosis of male fertility relies solely upon the findings in a sperm analysis (1). Semen samples with reduced incidence of normal forms typically have reduced motility and capacity of penetration of cervical mucus, to penetrate zona-free hamster eggs and to fertilize the human oocyte (2). If fertilization of oocyte with morphologically abnormal sperm (MAS) occurs, a possibility of abnormal zygote formation followed by failure of implantation and subsequent early abortion could be expected, (3). Consequently, the major classification of sperm morphology assessment can be done either by staining techniques (4)or by employing strict criteria for normal shapes , which is developed by Kruger and co-workers (4).The Kruger system is morphometric measurement of the sperm compared with a fixed standard measurement of sperm head ,middle piece and tail ,so this method regarded as more sensitive way to assess morphology .In KSC 14% normally formed sperm considered as normal and it would be satisfied percentage for fertilization (5). Even that, the Kruger system is more time- consuming during staining and measurement of the sperm parts but the result of such assessment correlates better with other methods of assessing fertility of the sperm specimens, such as fertilization during IVF cycles (6). Although the studies carried out so many diagnostic tests for male infertility and the normal sperm morphology values, there is insufficient studies regarding the correlation between the results of IUI and sperm morphology using KSC .Therefore, the present study is performed to achieve the following goals:

1- The assessment of sperm morphology by applying KSC , using two different biological stains, named commercially TS (pre-stained slides) , and SOS 2- To have a cut off value for MNS that resulting into a successful IUI outcome.

Materials &Methods

A total number of fifty five unselected male patients who attended the Institute of Embryo Researches and Infertility Treatment, University of Al-Nahrain, during the period from November 2007 to October 2008 were included in this study. The patients were subjected to clinical assessment at Infertility Clinic which included full medical, surgical, social and familial history with physical examination which was performed by male infertility surgeon.

1-Investigations to assess the females reproductive status:

Physical examination was done following the case history .The investigations was performed to assess the normal menstruation and ovulation which include; serial vaginal ultrasound, mid luteal progesterone level and cervical mucus studies. Hormonal assay was done to the female namely; early follicular estradiol (E2), follicle stimulating hormone (FSH), luteinizing hormone (LH) level and mid cycle E2 level as part of the study. Their tubal patency was checked from the previous cycles by hystrosalpingoigraphy (HSG) and laparoscopy some times performed if indicated

2-Investigations to assess the males reproductive status:

All the males included in the study were examined by the male infertility consultant in the Institute. Complete history and clinical examination were done for them, and standard semen analysis was performed according to the WHO, (8) guidelines.

Semen Samples Collection:-

Every patient was asked to provide semen by masturbation after an abstinence period ranging between 3-5 days and not exceeding 7 days in a private and quite room adjacent to the semen analysis laboratory. Semen was collected in a clean, sterile, dry, plastic and warm disposable Petri dish, labeled with patient's name and the time of collection (9). Semen sample was then immediately transferred to the laboratory and placed in an incubator at 37C°, waiting for liquefaction.

Sperm Morphology Percentage:

Any morphological deviation from the normal structure of sperm was considered abnormal. Sperm morphology was performed in several methods, which involve:

I. High Power Field Method:

One drop (10mll) of liquefied thoroughly mixed semen was mounted between warmed slides and covered with a standard cover slip. At least two hundred spermatozoa were counted and percent of normal morphology was recorded (10).

$$Morpho\log ically\ Normal\ Sperm\% = \frac{Number\ of\ normal\ sperms}{Total\ number\ of\ sperms} X100$$

II. Modified Kruger Strict Criteria (MKSC):

In this study, a special pre-stained slide was used for examination of sperm morphology. The slide contains two dyes namely; 2.1 g/cm2 crystal violet acetate and 1.0 g/cm2 new methylene blue. One drop (10 l) of semen was mounted between warmed slides and standard cover slip and left for 5 minutes at room temperature then examined under light microscope (Olympus ,Japan) using oil-immersion power 1000X and graduated optic lens . Ten fields or 100 sperms were examined by measuring the length and width of the head axis for each sperm in the slide by the graduated optic and labeled in a table. The normal head axis length and width are 5-6 m and 2.5-3.5 m, respectively (7).The other stain used was Sperm O Scan stain. Sperm O scan stain is a special biological staining kit contains three different stains each in a separated plastic box. the translucent stain labeled number -1- the fixative .A second plastic box which is red color labeled number-2- Eosin stain .The third plastic box have the blue color labeled number-3- Blue stain (Giemza stain).The procedure of staining was performed as described by manufactured Company .

Round cells:

The measurement of round cells was achieved by visual field in the wet preparation. The sample considered normal when the round cells counts was less than 5 million/ml, while those suffering from infection showed more than 5 million per ml with clusters of phagocytic cells engulfed sperm cells. A normal ejaculate should not contain more than 5 106 round cells/ml (8).

3.2. Intra -Uterine Insemination

Ovarian stimulation program was started from the second day of the menstrual cycle, the usual drug used was pure FSH (Gonal-F® 75 IU) with a dose of 75-150 IU /day,

following up of the patient was conducted by repeated vaginal ultrasound to detect the size and number of ovarian follicles with the endometrial thickness .Serum E2 level was also measured to prevent ovarian hyperstimulation .When ovarian follicles reaches a good size usually 18 mm, human chorionic gonadotropin (HCG,Pregnyl® 5000IU) was given in a dose of 5000IU for one follicle and 10000 IU for more than one follicle. Then the insemination was done 24-36 hours after the HCG injection (11).

The diagnosis of pregnancy was done either by biochemical analysis (the detection of hCG in blood after 2 weeks of insemination) or by ultrasound examination, and continuously the patient was followed up by physical examination and by ultrasound examination to assess the pregnancy status.

Statistical analysis:

The results were statistically analyzed using the student's t-test and Chi - square depending on the nature of the data. When P-value reaches the 0.05, the result was considered significant (12).

Results

Table 1, reveals certain sperm function parameters before and after in vitro activation technique. The sperm concentration (million/ ml) after in vitro activation was significantly (P<0.001) lower (22.2±1.42) than that of before in vitro activation (42.78±1.85).The mean of active sperm motility (%) grade A (50.0 ±3.44) after activation was significantly (P<0.001) higher compared with before activation (7.0 ±1.41). Active sperm motility grade B was not significantly (P>0.05) different (37.01±2.67) following activation compared with before activation (33.76±1.43). The mean of MNS percentage after activation (68.04±2.93) was significantly (P<0.05) increase than that of before activation (42.05±2.40) as shown in table -1.

Table 2 reveals the sperm morphology using three different methods of staining used in this study. A highly significant (P<0.001) increase in the normal sperm morphology after activation by using HPF method (68.04%), KSC -S OS stain(37.85%) and KSC - TS stain(37.09%) compared to their corresponding values before activation(42.05%,16.11% and 15.15%, respectively).

Table 3 reveals comparison between mean MNS before and after in vitro sperm activation using SOS stain on a hand and TS stain on the other hand by KSC. The mean normal sperm morphology before in vitro sperm activation using SOS stain (16.10 ± 0.52)revealed non significant(P > 0.05) difference in comparison with that of TS stain (15.14 ± 0.47) applying KSC .At the same time no significant difference was obtained by using SOS stain (37.85 ±1.04) compared to that of TS stain (37.09 ± 0.090)applying KSC .

Table (1) Sperm function parameters before and after *in vitro* activation technique

Certain sperm parameters		In vitro activation	
		Before activation	After activation
Sperm concentration (million/ml)		42.78±1.85	22.2±1.42*
Sperm motility%	Grade-A	7.00±1.41	50. ±3.44*
	Grade-B	33.76±1.43	37.01±2.67
	Grade-C	23.30±1.03	8.72±1.434*
	Grade-D	36.25±1.336	3.90±0.86*
Morphologically normal sperm (%)		42.05±2.40	68.04±2.93

-Values are mean ± SEM
- No. Patients = 55.
-*P<0.001: significant differences from the other group.

Table (2) Comparison of sperm morphology before and after *in vitro* activation using different methods

In vitro activation	Methods of measuring the percentage of normal sperm morphology(%)								
	High power field method			Kruger strict criteria method					
				Sperm o scan stain			Testsimplet stain		
	Mean	SE.M.	Sig.	Mean	SE.M.	Sig.	Mean	SE.M.	Sig.
Before-activation	42.05	2.40	0.001**	16.11	0.53	0.001**	15.15	0.47	0.001**
After-activation	68.04	2.93		37.85	1.04		37.09	0.91	

Student's t test

Table (3) Comparison the Kruger strict criteria between sperm o scan and Testsimplet stains for measurement of sperm morphology.

Sperm morphology following *in vitro* activation	Methods of measuring normal sperm morphology	Mean ± SE M	Sig.
Sperm morphology before activation (%)	Kruger strict criteria SOS	16.10+0.52	0.176NS
	Kruger strict criteria TS	15.14+0.47	
Sperm morphology after activation (%)	Kruger strict criteria SOS	37.85+1.043	0.582NS
	Kruger strict criteria TS	37.09+0.90	

NS: P>0.05. No. Patients=55

In table 4, there was a significant correlation between the IUI outcome and the mean of MNS after *in vitro* activation and applying KSC by both SOS and TS. the mean of MNS for successful IUI before and after the activation *in vitro* by using HPF method (46.82% and 68.04%,respectively) was significantly (P<.001) higher than that of KSC using SOS stain (16.11% and 37.85%, table 2) or TS stain(15.15% and 37.09%,).

The percentage of normal sperm morphology before and following the activation in vitro by using KSC using SOS stain and TS stain was not revealed a significant (P>0.05) difference(Table 4).

Figure 1 revealed the cut off values of MNS by using different methods that resulted in a successful IUI outcome. There was a significant (P<0.05) different in the percentage of MNS between HPF method (46.82%) and KSC methods (11.58% for SOS and 12.84 % for TS).

Discussion

The present study showed that the mean sperm concentration was significantly decreased after preparation technique. This may resulted from the use of wash and spin or swim up *in vitro* activation techniques for the semen samples .These techniques were safely separation of spermatozoa based on their motility and morphology and gained significantly higher percentage of motility. At the same time, this activation techniques may decrease the numeric yield of spermatozoa due to inability of dead and abnormal sperm with poor motility to swim up and migrate from the sperm pellet to the upper layer of culture medium (13).

The sperm motility grade A and grade B were increased after activation .This was an expected result when using the activating media *in vitro*. The motility of the sperm can be improved using culture media containing pyruvate, inositole, glycerol and lactate. In addition, poorly motile sperm can often be stimulated by agent enhanced motility like ATP and pentoxifylline (14, 15).

The mean of MNS before *in vitro* activation 42.05 ± 2.40 which was increased to 68.04 ±2.93. This is the case with the use of the activating medium which separate the motile spermatozoa with normal morphology and intact DNA from total sperm population, leaving behind immature or senescent spermatozoa, morphologically abnormal one and those with damaged DNA (16).

Table (4) Correlation among different measuring methods of sperm morphology and IUI outcome.

Methods of measurement sperm morphology(%)	In vitro activation	IUI positive N=11		IUI negative N=44		Sig.
		MNS	(%)	MNS(%)		
		Mean	SEM	Mean	SEM	
HPF	Pre-activation	46.82	4.28	47.16	2.27	0.946 NS
	Post-activation	68.73	5.80	74.86	2.61	0.308 NS
KSC SOS stain+	Pre-activation	11.58	1.23	15.73	0.57	0.148 NS
	Post-activation	29.27	1.43	40.00	1.03	0.001*
KSC TS stain	Pre-activation	12.84	0.83	15.16	0.56	0.955 NS
	Post-activation	28.73	2.09	39.18	0.73	0.001*

*. Correlation is significant at 0.01 level (2-tailed).
+ P>0.05 between SOS and TS values pre and post -activation
Sperm o scan=SOS, Testsimplet=TS

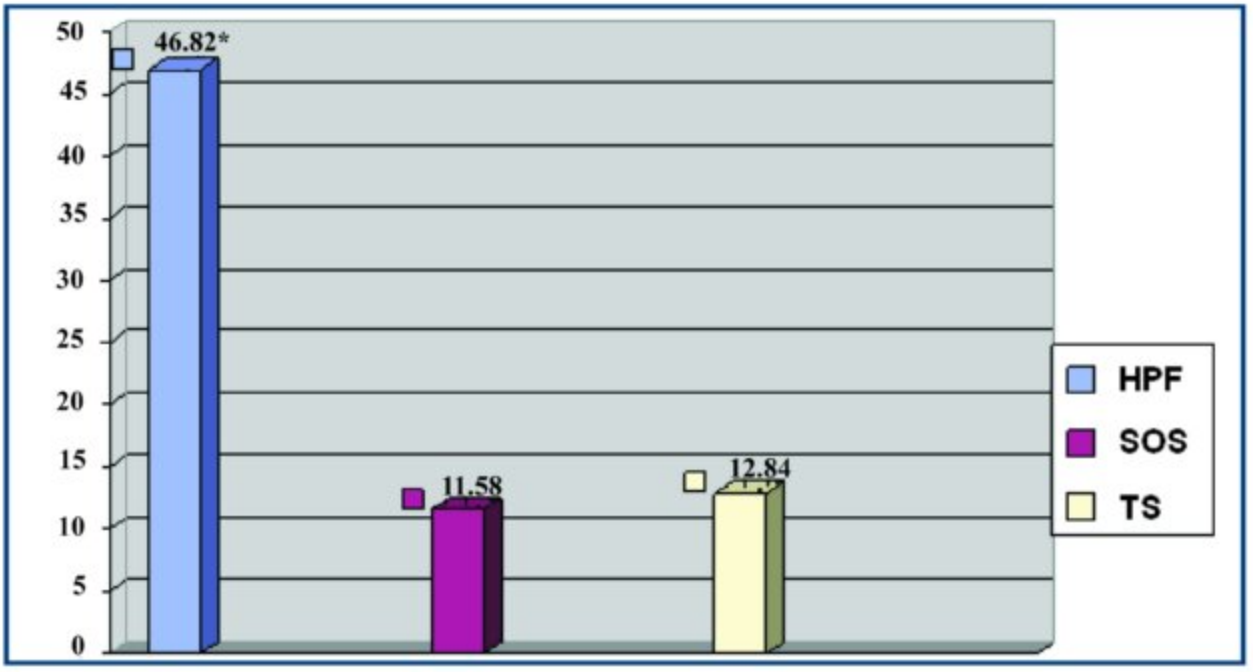

Figure 1: Cut off values for successful IUI program regarding different methods of measuring the percentage of morphologically normal sperm.

*P<0.05: significant
different from other staining methods
HPF : High power field , SOS : spermo-scanstain,TS : test simplet stain

Comparison of the mean of MNS before and after in vitro activation using the three methods i.e. HPF, SOS and TS, showed a significant differences, and that's reasonable observation because of the different methods of calculation between KSC and HPF (6). However, the mean of MNS before and after activation using the SOS revealed higher values of abnormality with no significant difference from TS which indicates that although the two stains has the same results by using KSC in assessing the MNS value , the use of TS is superior to SOS because the former is easier, faster, and even cleaner technically.

The mean of MNS of the 55 cases involved in this study before in vitro activation was below the normal value whether using HPF or by applying KSC using both S0S and Ts stains, which perhaps reflect one of the major causes of male infertility i.e. teratozoospermia . Semen samples with low percentage of sperm exhibiting normal morphology have greatly reduced prognosis for fertility despite normal sperm number and motility (5). After *in vitro* activation the mean of MNS was improved significantly to normal values, and that's because of the positive effect of *in vitro* preparation techniques that used in the present study . The development of *in vitro* sperm washing methods made it possible to deliver safely adequate numbers of spermatozoa directly to the uterine cavity .Sperm isolation techniques combining the removal of seminal plasma with the selection of highly motile sperm subpopulation were applied to artificial insemination leading to substantial improvement in success rates of pregnancy (17).

The result of IUI was 11 positive pregnancy out of the 55 cases. The present study offered a cut off value of KSC for a successful IUI program using different methods of measuring the percentage of MNS, where the cut off value using KSC by SOS stain was 11.58 %, whereas by Ts stain the KSC cut off value was 12.84 %. It has been reported that in normal subject the value of MNS by KSC method must exceed 14-15% to consider fertile, whereas men with >4 to < 14 must guided to IVF programs (8). There was no pregnancy in the rest 44 cases guided for IUI, in spite of normal value of MNS .This finding could be explained that another factors affecting the IUI result comes into play, (18). To optimize success, IUI is often used in conjunction with controlled ovarian hyper stimulation which increase the number of eggs available to be fertilized .The timing of insemination and the number of insemination per cycle may influence the ultimate pregnancy rate (11). Moreover MNS describes the external features of the individual sperm; it does not reflect the whole genetic material inside the sperm .It was noticed that the sperm genetic material play an important role in the existence of male infertility (19).

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