Int J Clin Exp Med 2018;11(5):5141-5146
www.ijcem.com /ISSN:1940-5901/IJCEM0063582

Original Article

Both the WHO 4th and 5th criteria for normal sperm morphology has no influence on fertilization and pregnancy outcomes in in vitro fertilization and intracytoplasmic sperm injection

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Received April 23, 2017; Accepted February 16, 2018; Epub May 15, 2018; Published May 30, 2018

Abstract: Objective To investigate the impact of normal sperm morphology (NSM) on fertilization and Pregnancy outcomes of in vitro fertilization (IVF) and intracytoplasmic sperm injection ICSI according to the WHO 4th or 5th criteria. Methods This retrospective cohort study is based on analysis of 202 IVF and 134 ICSI cases treated in our center from January to December 2015. IVF and ICSI cases were divided according to WHO 4th and 5th criteria for NSM. The outcomes of fertilization and pregnancy were compared for IVF and ICSI cases respectively. Results For both IVF and ICSI, there were no differences in the fertilization and pregnancy outcomes between two NSM groups for either WHO 4th or 5th criteria. Conclusion Neither WHO 4th nor 5th criteria for NSM has predictive value for the outcomes of fertilization and pregnancy in either IVF or ICSI.

Keywords: Normal sperm morphology, in vitro fertilization, intracytoplasmic sperm injection, fertilization rate, pregnancy rate

Introduction

Both in vitro fertilization embryo transfer (IVF-ET) and Intracytoplasmic Sperm Injection (ICSI) are important assistant reproductive technologies for infertility treatment. Many factors can affect the IVF/ICSI outcomes: for men, normal morphology sperm (NSM), density and sperm motility are of the vital parameters of semen analysis to reflect the male fertility [1], among which NSM is the most controversial indicator. The deformity of sperm head may signify the abnormity of genetic material and acrosomal reaction. Meanwhile, the abnormality occurring in tails may result in the reduction of the ability of sperm movement. It is supposed that NSM has a more powerful prediction for sperm-egg binding, embryonic development and pregnancies than sperm density and viability. In the LIFE study [2], NSM was significantly related to fertility rate. Menkveld et al. [3] suggest that NSM can be deemed as the indicator to predict IVF fertilization rates. Using the ROC curve analysis, the cut-off point values for sperm morphology was 4%. However, there is an opposite thought [4, 5] that the prediction of IVF fertilization outcomes by is limited. The influence of NSM on ICSI fertilization outcomes has also yet to be demonstrated. Moreover, the NSM criteria were changed from≥4% in the previous WHO manual to≥15% in the 5th WHO manual [6]. The extent to which the new standard will impact the semen evaluation, however, remains unclear to date. This research aims to retrospectively analyze the 336 cases treated with IVF or ICSI in our center to explore the possible effects of NSM on IVF and ICSI fertilization outcomes respectively.

Materials and methods

Source of materials

In this study 336 couples accepting the treatment for IVF or ICSI from Jan.-Dec. 2015 were selected: 202 cases with IVF and 134 cases

with ICSI. Eligible couples should live together for at least one year and have normal sexual life with no contraceptive measures but were still infertile. Eligible females should have normal menstrual cycle and have BMI ranging from 18 to 25 kg/m^2. Exclusion criteria included polycystic ovarian syndrome, endometrioma, or infertility due to asthenozoospermia, teratozoospermia, endometriosis or oviduct causes. Indications for IVF or ICSI included tubal, male, endometriosis. No IVF or ICSI were done for each couples before the one included in the study.

Methods

Reagents: Diff-Quik rapid staining kit (purchased from BRED Life Science Technology Inc. China) was adopted for sperm morphological analysis. In vitro fertilization and embryo culture reagent, such as G-MOPS, G-IVF, G1, G2, HSA-solution G-MOPS, G-IVF, G1, G2, HSA-solution, EmbryoGlue and Sperm Grad were all the chemical products from VitroLife Sweden AB.

Semen analysis: The sample was collected after a minimum of 2 days and a maximum of 7 days of sexual abstinence, which was obtained via masturbation into a clean specimen container. The analysis was performed in accordance with the standard of "Laboratory Manual for the Examination and Processing of Human Semen, 5th edition" [6], in which the sperm concentration, progressive motility (PR) percentage, total motility and volume were assessed. Two smears were made from the fresh semen sample. Once the smears had been air-dried, they were fixed and stained by the use of the Diff-Quik rapid staining. Slides were examined with oil immersion at ×1000 magnification by assessing 200 spermatozoa per replicate for sperm head, midpiece, principal piece, excess residual cytyoplasm, NSM percentage and teratozoospermia index (TZI), separately.

Groupings: According to the different ways of fertilization, IVF group and the ICSI group were operated severally on the basis of the NSM percentage. IVF can be divided into 2 groups: NSM percentage ≤4% and >4% (the WHO 4th criteria) or NSM percentage ≤15% and >15% (the WHO 5th criteria). Similarly, ICSI can be divided into 2 groups based on the WHO 4th and 5th criteria respectively. IVF fertilization rate = no. of oocytes fertilized/no. of oocytes obtained ×100%; ICSI fertilization rate = no. of oocytes fertilized/no. of mature oocytes ×100%; IVF 2 pronucleus (2pn) rate = no. of 2pn oocytes/no. of oocytes obtained ×100%; ICSI 2pn rate = no. of 2pn oocytes/no. of mature oocytes ×100%; cleavage rate = no. of zygote cleavaged/no. of normal fertilized oocytes ×100%; high-quality embryos rate = no. of high-quality embryos/no. of embryo ×100%; pregnancy rate = no. of pregnancies/no. of periodicities; miscarriage rate = no. of miscarriage/no. of periodicities, delivery rate= no. of childbirth/no. of periodicities [7].

In vitro fertilization and embryo culture: The woman's ovarian hyperstimulation was used by oral contraceptive long protocol. Final oocyte maturation was triggered with a single intramuscular injection of human chorionic gonadotropin (hCG) (10,000 IU). Transvaginal oocyte retrieval was scheduled 35 to 36 hours later. Individually cultured embryos were evaluated on the basis of blastomeres number, blastomeres size, fragment rate and presence of multinucleated blastomeres on day 2. A high-quality embryo was defined as a 4 regular blastomeres embryo and <25% fragment. A maximum of 2 embryos were transferred 3 days after oocyte retrieval. The luteal phase was supported by oral progesterone from the day of oocyte retrieval to the day of the pregnant test. Pregnancies were diagnosed by an increasing serum hCG levels that was tested 14 days after ET. A clinical recognized pregnancy was confirmed by the presence of a gestational sac on abdominal ultrasound examination during the 5th weeks.

Statistical analysis

Statistical calculations were performed using SPSS 17.0 statistical package for Windows. Continuous variables were described as mean ± standard deviation and categorical variables as proportion. The difference in fertilization and pregnancy outcomes between two NSM groups (based on WHO 4th and 5th criteria respectively) was tested using t test for continuous variables or χ^2 test for categorical variables. Multiple linear regression was used to assess the association of NSM with fertilization and pregnancy outcomes with adjustment for possible confounders including male age, female age, total dose of gonadoyropins, endometrial thickness on ET day, sperm concentration, progressive motility and TZI. *P* value of less than 0.05 was considered as statistical significance.

Table 1. Univariate analysis between different normal sperm morphology of with WHO 4th and WHO 5th criteria in IVF group

Group	WHO 4th version				WHO 5th version			
	NSM<4%	NSM≥4%	t/χ^2	p	NSM<15%	NSM≥15%	t/χ^2	p
Fertility rate (%)	72.38±19.86	71.29±22.26	1.13	0.26	76.35±20.83	73.87±23.54	0.8	0.43
2pn rate (%)	63.99±19.43	59.76±21.97	1.06	0.29	61.77±20.19	58.41±23.57	1.1	0.27
Cleavage rate (%)	96.57±7.94	91.834±16.67	1.22	0.22	96.25±11.78	87.89±19.96	1.81	0.07
High-quality embryo rate (%)	71.12±27.38	64.06±27.72	1.38	0.17	67.14±27.68	62.31±27.68	1.23	0.22
Pregnancy rate (%)	46.7 (14/30)	53.5 (92/172)	0.48	0.49	53.6 (74/138)	50 (32/64)	0.23	0.63
Miscarriage rate (%)	20 (6/30)	13.9 (24/172)	0.74	0.39	15.9 (22/138)	12.5 (8/64)	0.41	0.52
Delivery rate (%)	40 (12/30)	46.5 (80/172)	0.44	0.51	45.6 (63/138)	45.3 (29/64)	0.002	0.96
Birth defects rate (%)	3.3 (1/30)	0.5 (1/172)	0.98	0.27	1.4 (2/138)	0 (0/64)	0.46	1
Male age at cycle start	35.59±6.07	33.27±4.91	2.52	0.01	33.69±5.37	33.64±4.94	0.08	0.94
Female age at cycle start	33.5±4.45	32.13±4.4	1.7	0.09	32.29±4.45	31.49±4.45	-0.29	0.77
Total dose of gonadoyropins (IU)	43.47±24.07	35.17±15.24	1.99	0.05	35.59±17.53	36.54±16.94	0.02	0.98
Total period of gonadoyropins (day)	14.61±14.96	12.36±9.59	1.15	0.25	12.82±11.31	12.61±9.67	0.14	0.88
Endometrial thickness on hCG day (mm)	11.06±2.39	11.45±2.62	-0.81	0.42	11.27±2.54	11.55±2.66	-0.76	0.45
Endometrial thickness on ET day (mm)	10.69±2.59	11.59±2.93	-0.65	0.1	11.26±2.96	11.75±2.74	-1.1	0.27
Sperm_concentration (×10^6/ml)	29.23±17.49	53.32±43.03	-2.65	0.008	42.89±39.82	61.47±41.91	-3.19	0.001
Progressive motility (PR, %)	38.89±19.07	32.82±12.03	2.08	0.035	33.31±13.20	33.79±13.45	-0.25	0.8
teratozoospermia index (TZI)	1.43±0.12	1.32±0.1	3.6	0.004	1.35±0.09	1.29±0.11	3.34	0.001
Percentage of oocytes aspirated (%)	81.61±25.57	78.56±20.77	0.76	0.44	78.39±22.22	80.12±20.68	-0.57	0.57

Table 2. Univariate analysis between different normal sperm morphology of with WHO 4th and WHO 5th criteria in ICSI group

Group	WHO 4th version				WHO 5th version			
	NSM<4%	NSM≥4%	t/χ^2	p	NSM<15%	NSM≥15%	t/χ^2	p
Fertility rate (%)	73.57±19.67	67.59±24.68	1.32	0.19	71.71±20.19	68.94±31.67	0.41	0.68
2pn rate (%)	66.41±20.54	61.63±25.91	1.01	0.32	64.69±21.27	64.36±32.01	0.05	0.96
Cleavage rate (%)	95.65±14.79	93.18±23.35	0.64	0.52	90.37±16.51	90.25±28.84	0.91	0.37
High-quality embryo rate (%)	62.46±29.68	75.67±34.81	-1.98	0.05	66.12±31.64	75.09±35.75	-0.99	0.33
Pregnancy rate (%)	55.3 (26/47)	42.5 (37/87)	2	0.16	48.1 (50/104)	43.3 (13/30)	0.21	0.65
Miscarriage rate (%)	14.8 (7/47)	11.5 (10/87)	0.32	0.57	13.5 (14/104)	10 (3/30)	0.25	0.62
Delivery rate (%)	51.1 (24/47)	35.6 (31/87)	3	0.08	40.3 (42/104)	43.3 (13/30)	0.08	0.77
Birth defects rate (%)	2.1 (1/47)	0 (0/87)	0.35	1	0.9 (1/104)	43.3 (0/30)	0.77	1
Male age at cycle start	33.23±6.08	36.49±6.01	-2.59	0.11	33.6±5.93	40.59±4.92	-3.38	0.002
Female age at cycle start	31.12±4.82	35.65±4.91	-4.5	0.001	32.17±5.17	37.42±3.88	-3.38	0.001
Total dose of gonadoyropins (IU)	32.29±14.43	44.75±20.93	-3.5	0.0007	35.36±16.95	48.5±22.47	-2.41	0.0175
Total period of gonadoyropins (d)	11.86±6.49	17.33±20.64	-1.94	0.056	12.98±10.24	20.58±28.54	-1.81	0.07
Endometrial thickness on hCG day (mm)	11.63±2.26	10.83±2.27	1.58	0.12	11.43±2.51	10.59±2.05	1.11	0.27
Endometrial thickness on ET day (mm)	11.46±2.13	10.83±2.91	1.16	0.25	11.34±2.47	11.32±2.39	1.29	0.2
Sperm_concentration (×10^6/ml)	10.92±14.48	48.62±58.94	-4.64	<0.001	20.48±34.4	69.04±56.23	-2.9	0.012
Progressive motility (PR, %)	38.89±19.07	32.81±12.23	1.49	0.14	30.91±13.2	30.85±13.45	-0.25	0.81
teratozoospermia index (TZI)	1.35±0.12	1.32±0.1	3.6	0.0004	1.35±0.09	1.29±0.11	3.34	0.001
Percentage of oocytes aspirated (%)	76.16±17.31	84.01±19.43	-0.94	0.35	80.44±18.32	91.59±13.34	-2.03	0.048

Results

Patients characteristics

A total number of 202 cases were included in IVF group and 134 cases in ICSI group. **Tables 1** and **2** show the average sperm concentration, PR percentage, and TZI index and for IVF and ICSI respectively. In IVF patients (**Table 1**), significant differences are noticed in male age, total dose of gonadoyropins, sperm concentration, PR percentage and TZI between two NSM groups according to WHO 4th criteria. Based on the 5th criteria, only sperm concentration and TZI were significantly different between the NSM groups. (P<0.05). Among ICSI patients

Table 3. Association of normal sperm morphology with fertilization outcomes in IVF patients*

Outcome	WHO 4th criteria			WHO 5th criteria		
	Estimate	SE	*P*-value	Estimate	SE	*P*-value
Fertility rate	2.75	6.88	0.69	-1.78	3.48	0.61
2pn rate	5.86	6.93	0.40	-3.1	3.39	0.36
Cleavage rate	2.01	2.86	0.48	-4.17	2.5	0.061
High-quality embryo rate	-6.76	8.77	0.44	-3.81	4.31	0.38

*With adjustment for male age, female age, total dose of gonadoyropins, endometrial thickness on ET day, sperm_concentration, progressive motility and TZI. For WHO 5th criteria, only sperm_concentration and TZI were adjusted in the model because colinearity occurred when all the other covariates were included.

Table 4. Association of normal sperm morphology with fertilization outcomes in ISIC patients*

Outcome	WHO 4th criteria			WHO 5th criteria		
	Estimate	SE	*P*-value	Estimate	SE	*P*-value
Fertility rate	-15.41	9.19	0.10	-3.88	10.17	0.70
2pn rate	-18.54	9.86	0.06	-2.78	10.99	0.81
Cleavage rate	-7.11	8.94	0.43	-7.04	9.65	0.47
High-quality embryo rate	-4.67	14.01	0.74	-4.92	14.56	0.73

*With adjustment for male age, female age, total dose of gonadoyropins, endometrial thickness on ET day, sperm_concentration, progressive motility and TZI.

(**Table 2**), there were significant difference in female age, total dose of gonadoyropins, sperm concentration and TZI between two NSM groups, regardless of the 4th or 5th criteria.

No association between normal sperm morphology and fertilization outcomes in IVF patients

There are no significant differences between fertility rate, 2pn rate, cleavage rate, high-quality embryo rate, pregnancy rate, miscarriage rate, delivery rate and birth defects in different NSM groups in IVF cases (**Table 1**) according to WHO 4th or 5th criteria. With adjustment for possible confounders, no evidence suggests NSM <4% or NSM<15% is associated with fertility rate, 2pn rate, cleavage rate, or high-quality embryo rate (**Table 3**).

No association between normal sperm morphology and fertilization outcomes in ICSI patients

The results from ICSI patients were similar to those from IVF patients. That is, no evidence suggests NSM<4% or NSM<15% is associated with fertility rate, 2pn rate, cleavage rate, or high-quality embryo rate, regardless of whether possible confounders were adjusted for (**Tables 2** and **4**).

Discussion

The examination of NSM percentage is now broadly considered to be a tool contributing to the clinic and to the fertility prognosis. The WHO criteria of the 4th and 5th version for NSM are 15% and 4% respectively. The clear viewpoints is that according to 4th ed. WHO manual the in vitro fertilization rate will decline with the NSM falling [8]. NSM was amended to 4% by 5th ed. WHO, which seems so low that it is unlikely for infertile men to achieve the criterion [3]. At present, the IVF technique greatly depends on the total number of male PR sperm count and ovum numbers to determine an appropriate fertilization way. ICSI is regarded as a crucial treatment for low IVF fertilization rate or avoiding complete fertilization failure, but it should be used with cautions because ICSI is costly and time-consuming and has potential genetic risks [7]. Further rescue-ICSI is needed for some IVF cases with low fertilization rate, which may influence the IVF outcomes [9]. IVF and ICSI have very different condition in semen and fertilization procedure compared with natural conception, which leads to the debate as to whether demarcation point (4% or 15%) is especially suitable for IVF and ICSI.

Impaired sperm morphology is characterized by higher proportion of sperm head vacuoles, which in Fekonja's study related to lower fertilization rate only in IVF cycle but had no relation to pregnancy rate [10]. In Li's study [11], fertilization and pregnancy outcomes decreased significantly with decrease in NSM. Tavares [12] used Diff-Quik staining for NSM analysis, the quick and low-cost assay which can provide useful information regarding IVF success. We used the same method in this study but found no statistical differences in 2pn rate, cleavage

rate and high-quality embryo rate between 4 subgroups of IVF cases. These results are not consistent with the findings of the research by WHO [6]. Ghirelli-Filho et al. [13] adopting the WHO standards strictly made NSM analysis of 244 semen samples undergoing IVF cycles and suggested that there was no predictive value for NSM percentage on IVF outcomes. Similar results were found by Jiang et al. [14], who adopted the WHO standards and showed that NSM had no correlation with the amount/rate of 3pn zygotes, rate of 3pn embryo development, fertilisation rate or progesterone-induced AR rate in IVF. In Sun's opinion [15], under the condition of normal sperm concentration and vitality, comprehensive consideration for NSM and the woman's age were necessary to determine the intrauterine insemination or IVF treatment. There are possible reasons. First, sperm washing is required in IVF operation. While the low-vitality and poor-formed sperms are weeded out by this step, the rest of the them always have higher quality than before. Second, the number of sperm added to each ovum are similar but not the same, the fluctuations may compensate for the faultless of NSM. Third, for the development of the embryos, clinical pregnancy rate and abortion rate has no significant difference, which may be related to our practice of choosing better quality embryos to transfer.

For ICSI, studies for the effect of NSM on ICSI outcomes are rare. In this paper, no statistical differences were noticed in fertilization results. Similarly, no statistical differences were fouding in Demir et al. study [16], in which 655 ICSI cycles divided by NSM percentage≥4% and <4% and the fertilization rates in two groups were separately 70.8% and 72.0%, respectively. Sariibrahim [17] compared clinical results of ICSI for NSM based on Kruger's strict criteria. This study categorized 332 ICSI cycles into three groups (NSM<4%, between 4-14% and ≥ 14%) and the results are in line with our studies. By dividing the 103 cases of ICSI with the NSM levels of 0% and >1%, Pereira et al. [18] showed that the fertilization rate and pregnancy rate between the groups were not associated with NSM. This may be due to the fact that ICSI technique does not require sperm with normal zona pellucid-combination ability. In ICSI operation a single sperm is artificially selected for microinjection. Whether there is high or low NSM percentage in the ICSI patients is not important. However, the important point here is, those with round head sperms would be excluded, which is almost the only special case affecting ICSI outcomes in sperm morphology.

Apart from the differences in samples size and study designs between various studies, the above differences may be explained in following aspects: first, although WHO has specified the various parameters of NSM, there are different processes in assessment of morphology. For instance, different staining methods (Papanicolaou, Shorr or Diff-Quik stain) are recommended by WHO, which may cause uncertainty of the results. Even though the same standard is followed, there may remains large differences (including experimental error and random error) in the implementation of this standard in medical personnel's daily work in andrology laboratory. Second, human semen quality is influenced by many factors and fluctuates from time to time. The conclusions of 5th ed. WHO manual were based on the semen samples from the couples with natural pregnancy. The lack of data from 1.4 billion Chinese male semen sample makes it questionable for the revision to be applied to Chinese population. Third, IVF/ICSI technology is carried out in numerous different laboratories. As fertilization and pregnant outcomes may be affected by semen, ovum, endometrium, incubation system and operating personnel in embryo laboratory, the in vitro experiment results may not reliably reflect the real sperm function in physiological conditions. In summary, our research shows that the sperm morphology percentage according to either WHO 4th or 5th criteria has limited insight for preoperative assessment on the fertilization outcomes in either IVF or ICSI. Further research is needed for the effects of NSM on IVF/ICSI fertilization outcomes.

Acknowledgements

The study was supported by the Science and Technology Foundation of Tianjin Municipal Commission of Health and Family Planning (No. 2014KZ122) and Key project of Tianjin Healthcare Industry (No. 14KG138).

Disclosure of conflict of interest

None.

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