Re-evaluation of the value of sperm morphology in classical in vitro fertilization in a Northeastern Chinese population

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Abstract
Objective: This study aimed to re-evaluate the clinical value of a 4% cut-off threshold of sperm morphology in in vitro fertilization (IVF) in a cohort of a Northeastern Chinese population.
Methods: A total of 375 IVF cycles that met strict inclusion criteria were included. These cycles were conducted with semen analysis and oocyte fertilization. A total of 188 embryo-transferred cycles proceeded. According to sperm morphology, 375 cycles were divided into group 1 (329 cycles, <4% normal sperm morphology rate [NSMR]) and group 2 (46 cycles, ≥4% NSMR), and 188 transferred cycles into group A (151 cycles, <4% NSMR) and group B (37 cycles, ≥4% NSMR).
Results: The fertilization and normal fertilization rates were significantly lower in group 1 than in group 2. The normal fertilization rate was significantly correlated with an NSMR < 4% or ≥4%, but the fertilization rate was not significantly correlated with the NSMR. No significant differences were found in pregnancy outcomes between groups A and B.
Conclusions: This study suggests that infertile patients with an NSMR < 4% are more likely to have a poor normal fertilization status in IVF.

Keywords
Male fertility, sperm morphology, in vitro fertilization (IVF), embryo, oocyte, semen

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Introduction
Sperm morphology analysis has been one of the most common tests used for providing informative evaluation of male fertility over the past few decades.\textsuperscript{1,2} However, only a 4% normal sperm morphology rate (NSMR) is stated as a cut-off point in the World Health Organization (WHO)-5 semen analysis manual.\textsuperscript{3} Because of the low normal reference value, a trend is developing in which many laboratories or clinicians no longer regard sperm morphology as relevant.\textsuperscript{4} Sperm morphological assessments show large inconsistencies in different countries or regions, in different laboratories, or in different technicians in the same laboratory.\textsuperscript{5,6} Additionally, methodological changes also affect the percentage of morphologically normal spermatozoa.\textsuperscript{7} These factors may have an effect on clinical application results.

In clinical applications, evaluation of sperm morphology for predicting successful pregnancy is controversial.\textsuperscript{8} Some studies with an emphasis on quality control have shown that sperm morphology is an important predictor for male fertility.\textsuperscript{9–11} Some researchers have objectively shown that the predictive value of sperm morphology exists in practice because most fertile men preferentially have a high NSMR.\textsuperscript{12–14} However, other studies have reported that strict sperm morphology failed to predict success in \textit{in vitro} fertilization (IVF).\textsuperscript{15–17} Therefore, reappraising the value of sperm morphology in IVF is necessary owing to inter-laboratory discrepancies on morphological sperm evaluation.

This study aimed to retrospectively analyze IVF results, including preimplantation embryo development, progress of pregnancy, and delivery outcomes, of a Northeastern Chinese population that was grouped by the 4% cut-off value of the NSMR. Hopefully, this study will provide useful information on the clinical value of the sperm morphology threshold in IVF.

Materials and methods
Study population
A retrospective study was performed on infertile patients who underwent IVF in the Center of Reproductive Medicine, First Hospital of Jilin University, Changchun, China, from May 2011 to April 2016. This study included two types of analysis. In the first analysis, 375 cycles were strictly screened from 1559 primary IVF cycles to examine the effect of sperm morphology on preimplantation embryo development. The relationship between fertilization and sperm morphology was analyzed. In the second analysis, 188 transfer cycles were selected from 375 inclusion cycles for investigating the effect of sperm morphology on clinical outcomes. In each analysis, the patients were divided into two subgroups according to sperm morphology as follows: group 1 was an NSMR $<4\%$ with 329 IVF cycles and group 2 was an NSMR $\geq4\%$ with 46 IVF cycles; and group A was an NSMR $<4\%$ with 151 IVF transfer cycles and group B was an NSMR $\geq4\%$ with 37 IVF transfer cycles. All couples included in the study met the following criteria: 1) men aged <40 years and women aged <38 years; 2) semen volume $\geq1.5$ mL, sperm concentrations $\geq15 \times 10^6$ spermatozoa per mL, and total count $\geq39 \times 10^6$; 3) total motility (progressive motility and non-progressive motility) $\geq40\%$ and progressive motility $\geq32\%$; 4) female patients with no endometrial fibroids, endometriosis, or uterine adhesion due to previous uterine surgery; and 5) no chromosomal abnormalities. Informed consent was obtained from all couples. The study was approved by the ethics committee of the First Hospital of Jilin University.
**Sperm/semen analysis and manipulation**

Sperm samples were collected by masturbation for semen analysis after an abstinence of 3 to 5 days. Semen analysis was performed according to the WHO-5 recommendations. After semen samples were completely liquefied at 37°C, the samples were analyzed for sperm motility (graded: progressive motility, non-progressive motility, immotility). Total progressive motility was defined as progressive motility + non-progressive motility sperm motility. Semen volume, sperm concentrations, and total sperm count were also assessed.

Sperm morphology was assessed in the initial semen analysis using the Tygerberg Strict Criteria as outlined by the WHO-5th manual. Briefly, 5 to 10 μL of semen (depending on sperm concentrations) was placed on a precleaned slide and stained using the Diff Quik staining protocol (Ankebio, Anhui, China). Two technicians, who had the national docimaster qualification and were trained for documenting teratozoospermia with the standard of the WHO-5, analyzed 200 sperm cells independently. Sperm cells were viewed under an oil immersion microscope with 1000× magnification and the values were averaged. Spermatozoa were determined to be normal if they met the following criteria.

1) The sperm head was smooth, regularly contoured, and generally oval in shape. There was a well-defined acrosomal region comprising 40% to 70% of the head area. The acrosomal region contained no large vacuoles. 2) The midpart of the sperm was slender, regular, and approximately the same length as the sperm head. 3) The principal part of the sperm had a uniform caliber along its length, was thinner than the midpart, and was approximately 45 μm long. The principal part sometimes looped back on itself if there was no sharp angle indicative of a flagellar break.

Quality control for morphological assessment included weekly calculation of the inter-observer coefficient of variation as obtained by concurrent evaluation of the same discarded semen sample. An inter-observer variation of <10% was considered to be acceptable. The dyes were checked daily for cross-contamination and were changed weekly.

**Ovarian stimulation**

Either the long gonadotropin-releasing hormone (GnRH)-agonist protocol or the short GnRH-antagonist regimen was used. Controlled ovarian stimulation (long or short) was decided by doctors on the basis of the physical condition of the patients. The procedure of ovarian stimulation was performed according to Revelli et al. Ovulation was triggered by a single injection of 5000 to 10,000 IU of subcutaneous human chorionic gonadotrophin (HCG; Livzon Pharmaceutical Group Co., Ltd., Zhuhai, China) when two or more ovarian follicles reached ≥18 mm in diameter. Oocyte pickup was then performed by transvaginal ultrasound-guided aspiration approximately 36 to 38 hours after HCG injection.

**Fertilization and embryo transfer**

In the cycles, the oocytes were inseminated with a sperm concentration of $2 \times 10^5$/mL 4 to 5 hours after oocyte aspiration. Fertilization of the oocytes was assessed at 18 to 24 hours after insemination. Fertilization was determined by observation of two clearly distinct pronuclei. Embryo cleavage and quality were further assessed 24 and 48 hours later. Embryos (range: 1–3) with acceptable developmental potential and quality were transferred on the third day of *in vitro* culture. Good quality embryos satisfied the following two criteria: 6 to 10 cells in the embryo
on day 3; and the appearance of the embryo under a high-power microscope reached grade 1 or grade 2 on day 3.\textsuperscript{19} Pregnancy was tested by a serum HCG assay 14 to 16 days after embryo transfer. Clinical pregnancy was confirmed by the presence of an intrauterine gestational sac(s) with a fetal heartbeat 4 weeks post-embryo transfer.

**Statistical analysis**

Analysis was performed using SPSS 19.0 software (IBM Corp., Armonk, NY, USA). The chi-squared test was used to compare discrete variables. The Wilcoxon rank sum test and Student’s \( t \)-test were used to compare continuous variables according to distribution and homogeneity of variance. Non-normally distributed variables are presented as median (quartile range), and normally distributed variables with homoscedasticity are shown as mean ± standard deviation. The relationship between sperm morphology or oocyte number with fertilization was evaluated by Spearman’s rank correlation analysis. All hypothesis testing was two-tailed and \( P \) values ≤ 0.05 were considered statistically significant.

**Results**

A total of 1559 IVF cycles were performed. Only 375 IVF cycles and 188 transfer cycles met our inclusion criteria in this study.

**Effect of the NSMR on fertilization and early embryo development**

In 375 IVF cycles, the effect of the NSMR on fertilization and early embryo development from oosperm to blastula was examined, including the fertilization rate (number of oosperms/number of oocytes retrieved \( \times 100\% \)), normal fertilization rate (number of two pronuclear [2PN] oosperms/number of oocytes retrieved \( \times 100\% \)), cleavage rate (number of cleavage embryos/number of oosperms \( \times 100\% \)), normal cleavage rate (number of 2PN cleavage embryos/number of 2PN oosperms \( \times 100\% \)), and high-quality embryo rate (number of high-quality embryos/number of 2PN cleavage embryos \( \times 100\% \)). There were no significant differences in general parameters, including male age, female age, years of infertility, basal follicle-stimulating hormone (bFSH) levels, antral follicular count, oocytes retrieved, progressive motility, sperm volume, sperm concentrations, and total sperm count between the first two patient subgroups (Table 1). No significant differences in early embryo development were found between the groups (Table 2). However, the fertilization rate and normal fertilization rate in group 1 were significantly lower than those in group 2 (\( P = 0.048 \) and \( P < 0.001 \), respectively, Table 2).

The correlations of individual fertilization status (individual fertilization rate = number of individual oosperms/number of individual retrieved oocytes \( \times 100\% \); individual normal fertilization rate = number of individual 2PN oosperms/number of individual retrieved oocytes \( \times 100\% \)) and sperm morphology with consideration of the oocyte number were then analyzed by Spearman correlation analysis. The number of oocytes had a significant negative effect on the individual fertilization rate (\( r = -0.121, \ P = 0.019 \), Table 3), but not the individual normal fertilization rate. An NSMR <4% or ≥4% (in the model, an NSMR <4% was assigned the rank of 0 and an NSMR ≥4% was assigned the rank of 1) was positively correlated with the individual fertilization rate (\( r = 0.116, \ P = 0.025 \)), but the specific NSMR value was not significantly correlated with these rates. Neither a specific NSMR nor NSMR <4% or ≥4% was correlated with the individual fertilization rate.
Effect of the NSMR on clinical outcomes

We then analyzed whether the NSMR affects the clinical outcomes of patients with male infertility in IVF. There were 188 IVF transfer cycles from 375 inclusion cycles. There were no significant differences in the basic parameters of male age, female

Table 1. General parameters of infertile couples included in in vitro fertilization cycles.

| Variables                     | Group 1 | Group 2 | Value | P    |
|-------------------------------|---------|---------|-------|------|
| Normal morphology (%)         | <4      | ≥4      | —     | —    |
| Number of cycles              | 329     | 46      | —     | —    |
| Female age (years)            | 30.00 (5.00) | 30.00 (6.00) | −0.597 | 0.551 |
| Infertility years             | 3.00 (3.00) | 4.00 (4.00) | −0.531 | 0.596 |
| bFSH (mIU/mL)                 | 6.36 (1.95) | 6.28 (3.33) | −0.118 | 0.906 |
| Antral follicular count       | 20.00 (11.00) | 17.50 (11.75) | −1.274 | 0.203 |
| Oocytes retrieved            | 11.00 (8.00) | 10.50 (8.25) | −0.783 | 0.434 |
| Male age (years)              | 32.00 (6.00) | 32.00 (7.00) | −0.350 | 0.726 |
| Progressive motility (%)      | 50.87 ± 11.12 | 53.76 ± 10.85 | −1.654 | 0.099 |
| Semen volume (mL)             | 3.00 (1.80) | 3.05 (1.48) | −1.452 | 0.146 |
| Sperm concentration (×10⁶/mL) | 74.50 (54.30) | 78.70 (48.82) | −0.287 | 0.774 |
| Total sperm count (×10⁹)      | 232.32 (185.52) | 231.46 (188.31) | −0.802 | 0.422 |

Values are mean ± standard deviation or median (interquartile range). ¹Non-normal distribution, analyzed by the Wilcoxon rank sum test; ²normal distribution and homoscedasticity, analyzed by the Student’s t test. bFSH: basal follicle-stimulating hormone.

Table 2. Effect of 4% sperm morphology on fertilization and early embryo development.

| Variables                          | Group 1 | Group 2 | Value | P    |
|------------------------------------|---------|---------|-------|------|
| Normal morphology (%)              | <4      | ≥4      | —     | —    |
| Fertilization rate (%)             | 80.44 (3053/3795) | 84.19 (410/487) | 3.905 | 0.048 |
| Normal fertilization rate (%)      | 61.77 (2344/3795) | 70.23 (342/487) | 13.214 | <0.001 |
| Cleavage rate (%)                  | 97.01 (2962/3053) | 98.54 (404/410) | 3.056 | 0.08  |
| Normal cleavage rate (%)           | 98.04 (2298/2344) | 98.83 (338/342) | 1.027 | 0.311 |
| High-quality embryo rate (%)       | 52.83 (1214/2298) | 53.25 (180/338) | 0.021 | 0.884 |

The chi-squared test was used for analysis.

Table 3. Correlations of individual fertilization with sperm morphology and the number of oocytes.

| Independent factors | Individual fertilization rate | Individual normal fertilization rate |
|---------------------|-------------------------------|-------------------------------------|
|                     | r     | P value | r     | P value |
| Normal sperm morphology (%) | 0.054 | 0.300 | 0.068 | 0.190 |
| Number of oocytes    | −0.121 | 0.019* | −0.078 | 0.132 |
| NSMR <4% or ≥4%      | 0.099 | 0.054 | 0.116 | 0.025* |

NSMR: normal sperm morphology rate. *Significant correlation. Correlations were analyzed using Spearman’s rank correlation coefficient.
age, infertility years, bFSH levels, antral follicular count, pre-ovulatory follicular count, follicular output rate, oocytes retrieved, endometrial thickness, progressive motility, sperm volume, sperm concentrations, total sperm count, and mean embryo transplant between groups A and B (Table 4). There were also no significant differences in clinical outcomes between these two groups (Table 5).

**Discussion**

We found the following findings in this retrospective study. 1) The NSMR was not an isolated predictor of individual male
fertility, but patients with an NSMR <4% had worse fertilization results and an NSMR <4% or ≥4% was positively correlated with fertilization. 2) There were no significant differences in progress of pregnancy and delivery outcomes, which reflect the limited effect of sperm morphology to embryo–fetal development after transplantation. 3) The normal fertilization rate was more sensitive to sperm morphology than the fertilization rate. Therefore, the normal fertilization rate could be a better endpoint for male fertility.

The effect of overall sperm morphological assessment on IVF outcomes has been the focus of many idiopathic infertility studies, but remains controversial. With in-depth development and constant research in IVF, especially after introduction of strict sperm morphology with the 4% cut-off value in the WHO-5th manual, an increasing number of studies have reported that strict sperm morphology is a poor predictor of cycle outcomes.15–17 Although some studies have shown the clinical value of sperm morphology, few studies have shown the importance of sperm morphology with strong statistical evidence.20 Li et al.10 and Zhu et al.21 showed that patients with isolated teratozoospermia had a significantly lower fertilization rate, but no difference in pregnancy rate, compared with patients with a normal semen profile. Similarly, the current study showed that only the fertilization and normal fertilization rates in patients with isolated teratozoospermia were significantly lower than those in patients with an NSMR ≥4%.

However, the present study showed that a specific NSMR does not show a positive correlation with the individual fertilization rate (Table 3). Zhu et al.21 reported that sperm morphology was positively correlated with the fertilization rate in IVF ($r = 0.057$, $P = 0.010$). They found that normal sperm morphology, as a confounding factor in IVF, only accounted for 3.3% of the variation in the fertilization rate ($r^2 = 0.0033$). This report indicated that the effect of the NSMR on individual fertilization success was small, which is consistent with our result. Therefore, the NSMR cannot be an isolated predictor for individual success of fertilization.

In clinical practice, embryologists usually choose the best quality embryo for transplantation. This diminishes the effect of the NSMR on embryo development and greatly offsets the initial developing discrepancy of implantation embryos in IVF. Therefore, evaluating progress of pregnancy and delivery outcomes concerning the NSMR can be regarded as appraising the effect of the NSMR on embryo–fetal development after transplantation. There were no significant differences in progress of pregnancy and delivery outcomes between groups A and B in the current study. This finding indicates that sperm morphology might have a limited latent effect on embryo–fetal development, rather than sperm morphology failing to predict male fecundity. Moreover, in the current study, there was no significant difference in early embryo development, including cleavage rate, normal cleavage rate, and high-quality embryo rate, between the groups. Therefore, sperm morphology has a limited effect on early embryo development in vitro. Li et al.10 showed that the high-quality embryo rate was the most important indicator for precisely assessing embryo quality. In the present study, the high-quality embryo rate was similar between the groups. This finding indicates that the NSMR does not affect embryo quality, as previously found by Terriou et al.22 This finding could also be useful for eliminating the discrepancy in high-quality embryo selection.

In this study, strict inclusion criteria were adopted to screen out patients. However, a requirement for oocyte
number was not included because patients with severe teratozoospermia were able to deliver a healthy neonate with only one available oocyte. The oocyte number was negatively correlated with the individual fertilization rate, but not with the individual normal fertilization rate. A possible reason for this lack of finding is that when calculating the relevant fertilization rate, the oocyte number, which is the denominator of the formula, has a negative effect on the value. However, normal fertilized oocyte development could counteract such an effect, as shown for the individual normal fertilization rate in Table 3. In conclusion, the normal fertilization rate, in a group as a whole or in individuals, is a better endpoint for male fertility than the fertilization rate because it could remove the negative effect of oocyte number. This speculation is consistent with Li et al. who found that the normal fertilization rate was more sensitive to sperm morphology than the fertilization rate.

The present study suggests that an NSMR of 4% is more useful than the NSMR for individual success of IVF. Infertile patients with an NSMR <4% are more likely to have a poor normal fertilization status.

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**Declaration of conflicting interest**

The authors declare that there is no conflict of interest.

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