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GAMETE BIOLOGY

Efficacy of swim-up versus density gradient centrifugation in improving sperm deformity rate and DNA fragmentation index in semen samples from teratozoospermic patients

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Abstract
Purpose To compare the efficacy of swim-up and DGC in improving sperm deformity and DNA fragmentation and to determine which method is better in teratozoospermic patients requiring artificial reproduction.
Methods The present study compared the effects of swim-up and density gradient centrifugation (DGC), the two most commonly used semen preparation methods, on sperm deformity rate and DNA fragmentation index (DFI) in semen samples from teratozoospermic patients.
Results The results demonstrated that both swim-up and DGC yielded a significantly lower sperm deformity rate and DFI in comparison to unprocessed whole semen, with DGC having more favorable results. Sperm deformity rate in unprocessed whole semen samples was significantly lower in the 20–29 age group than in the 40-49 age group, but no significant difference was observed in DFI between different age groups. There was no significant correlation between sperm deformity rate and DFI.
Conclusions Our findings suggest that enrichment of sperm with normal morphology and intact DNA in teratozoospermic patients could be achieved by both DGC and swim-up procedures, and that DGC is a better method.

Keywords Teratozoospermia · Sperm preparation · Semen analysis · DNA fragmentation

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Introduction

Teratozoospermic patients often require the use of assisted reproductive technology (ART) to achieve pregnancy. Over the past few decades, several studies on sperm processing for ART have been performed in patients with oligozoospermia, oligoasthenozoospermia or asthenozoospermia,[1, 2] and many kinds of sperm preparation procedures have been developed, including density gradient centrifugation (DGC) and swim-up [3]. However, studies concentrating on teratozoospermia are limited in number, and there have been no consensus on which is the best sperm processing method employed for ART in teratozoospermic patients [4–6].

The main purpose of semen processing in teratozoospermia patients is to select sperm with good viability, and at the same time to reduce the proportion of morphologically abnormal sperm. An ideal semen processing method should be gentle, to minimize the sperm damage, and maximize the recovery of morphologically and functionally normal sperm [7]. As an important conventional semen quality parameter, the percentage of morphologically normal sperm has a significant role in determining and predicting male fertility. The higher the sperm deformity rate, the higher the difficulty of natural fertility.[8] In addition, functional evaluation of sperm has recently attracted increasing attention.[7] Some researchers reported that sperm DNA damage can result in reduced male fertility, poor embryo quality, low rate of pregnancy and increased abortion rate [9–11]. Thus, the assessment of semen parameters such as sperm density, vitality, motility rate and morphology is not sufficient when evaluating the selection effect of a sperm processing method on sperm quality, and comprehensive assessment using other parameters that reflect sperm function such as DNA maturity and integrity is required.

At present, swim-up and DGC remain the most commonly used semen preparation methods, and there have been few

reports on the efficacy of these processing methods in improving DNA integrity or sperm deformity rate in teratozoospermic patients. The aim of the present study was to compare the efficacy of swim-up and DGC in improving sperm deformity and DNA fragmentation and to determine which method is better in teratozoospermic patients requiring artificial reproduction. We also explored whether age has any influence on sperm morphology and DNA fragmentation, and analyzed the correlation between DNA integrity and sperm deformity rate in teratozoospermic patients.

Materials and methods

Patient selection

A total of 118 semen samples collected from 118 patients seeking assisted reproduction from January 2012 to September 2013 at Shaanxi Maternal and Child Health Care Hospital were used in this study. The criteria used to determine inclusion were based on the criteria for teratozoospermia recommended by the fifth edition of the World Health Organization (WHO) manual for semen analysis, including the rate of morphologically abnormal sperm≥96 %, with a normal sperm count and mobility [12]. The Ethics Committee of Maternal and Child Healthcare Hospital of Shaanxi Province approved the study protocol, and written informed consent was obtained from each patient.

Sample collection and preparation

Semen samples were obtained by masturbation after 3–5 days of ejaculatory abstinence and allowed to liquefy at room temperature for 30 min. Semen analysis was performed in accordance with the current WHO guidelines. [12] Swim-up and DGE were performed after teratozoospermia diagnosis. Each sample was aliquoted into three centrifuge tubes: one for a conventional wash and swim-up, one for DGC separation and one as a control without any treatment.

Swim-up

Two milliliters of liquefied semen was pipetted into the bottom of a centrifuge tube and 2 mL of G-IVF^{TM} medium (Vitrolife Inc., Gothenburg, Sweden) supplemented with 10 % human serum albumin (HSA, Vitrolife) was layered over the semen. The tube was then inclined at an angle of 45° and incubated at 37 °C for 1 h in a carbon dioxide incubator. Following this, the supernatant was aspirated into a 14-mL tube and centrifuged for 5 min at 300 g. The supernatant was discarded and the pellet was washed with G-IVF^{TM}. Centrifugation was then performed for 5 min at 300 g, and the pellet was finally resuspended in G-IVF^{TM} medium.

DGC

DGC procedure was performed using PureCeption^{TM} (In-Vitro Fertilization Inc., USA). In brief, 1 mL of liquefied semen was loaded onto a 40 % and 80 % discontinuous gradient (each 1.0 mL) and centrifuged at 300 g for 20 min at room temperature. The sperm pellet was washed in 3–5 mL of G-IVF^{TM} medium and centrifuged for 5 min at 300 g, and the resulting pellet was rewashed in 3–5 mL of G-IVF^{TM} medium. After further centrifugation for 5 min at 300 g, the final pellet was resuspended in G-IVF^{TM} medium.

Sperm deformity rate assessment

Sperm morphology was assessed using the Kruger/Tygerberg Strict Criteria as outlined by the WHO-5 manual [12]. Semen samples were placed on a previously cleaned slide and stained according to the conventional Papanicolaou staining protocol. Slides were observed under a microscope (Z2000-S, Germany). The number of abnormal sperm was counted under a microscope. Morphological abnormalities could occur in the body, tail, or head, such as amorphous head, double head, double tail, and short tail (Fig. 1). After assessing at least 200 sperm cells per sample, sperm deformity rate was calculated as a percentage by dividing the number of sperm with abnormal morphology by the total number of sperm observed.

Measurement of DNA fragmentation

Sperm DNA fragmentation was evaluated by sperm chromatin dispersion (SCD) test using the Halosperm® kit (Halotech Dna, Madrid, Spain). Briefly, semen samples were diluted with phosphate buffered saline (PBS) to a concentration of 5–10 million sperm cells per milliliter, and 60 μl was added to melted agarose and evenly mixed. Then a 20-μl aliquot of the cell/agarose suspension was placed on a super-coated slide and covered with a glass coverslip (22×22 mm). The slide was placed in a horizontal position throughout the entire process. After the slide was placed on a cold surface at 4 °C for 5 min in the fridge, the coverslip was removed and the slide was immersed in acid denaturant for 7 min at room temperature. Subsequently, the slide was incubated in lysis solution at room temperature for 25 min. After a 5-min wash in distilled water, the slide was dehydrated in a graded ethanol series (70 %, 90 %, and 100 %) for 2 min each and subsequently air dried. The dehydrated slide was stained with Wright-Giemsa stain and observed under a bright field microscope (Z2000-S, Germany) for halos. Spermatozoa with fragmented DNA had little or

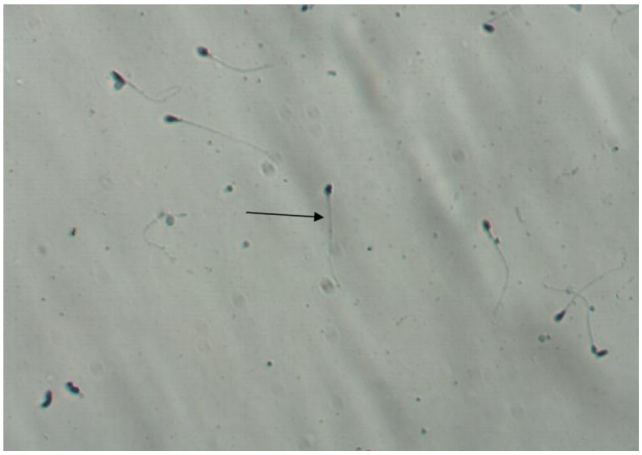

Fig. 1 Observation of morphologically abnormal sperm in semen samples from teratozoospermic patients. Sperm morphology assessment was performed using the Kruger/Tygerberg Strict Criteria. The black arrow indicates a morphologically abnormal sperm cell from a teratozoospermic patient. (Magnification, 400×)

no halo (Fig. 2). The degree of DNA dispersion was assessed by observing the relative halo size under bright field. DNA fragmentation index (DFI) was calculated as the percentage of fragmented sperm cells in a semen sample by assessing at least 500 sperm cells per slide.

Statistical analysis

The Statistical Package for the Social Sciences (SPSS) version 13.0 for Windows was used for statistical analyses. Values are expressed as mean±standard deviation (SD). One-way analysis of variance (ANOVA) was applied to compare means among different age groups. When comparing means between different treatment groups, the paired Student's *t*-test was applied. Pearson's correlation analysis was employed to determine the correlation between the DFI and sperm deformity rate before processing. *P* values<0.05 were considered statistically significant.

Results

Effect of different treatments on sperm deformity rate

The paired Student's *t*-test was used to compare the sperm deformity rate in swim-up and DGC-treated samples. Compared with unprocessed whole semen samples, the sperm deformity rate was significantly decreased after processing by swim-up or DGC ($P<0.05$ for both) (Table 1). The sperm deformity rate in the DGC group was significantly lower than that in the swim-up group ($P=0.003$) (Table 1).

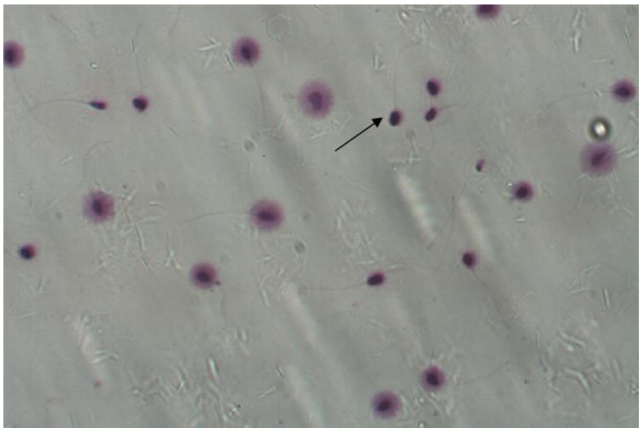

Fig. 2 Detection of sperm DNA fragmentation in semen samples from teratozoospermic patients. The black arrow indicates a spermatozoon with DNA fragmentation. (Magnification, 400×)

Sperm deformity rate between different age groups

The 118 patients recruited according to the WHO criteria were divided into three age groups: 20–29 ($n=32$), 30–39 ($n=63$) and 40–49 years ($n=23$). Semen analysis showed that, in unprocessed whole semen samples, the sperm deformity rate increased with age and the 20–29 age group had a significantly lower deformity rate than the 40–49 age group ($P<0.05$) (Table 2). However, in swim-up and DGC-treated samples, there was no significant difference in the sperm deformity rate between the different age groups (Table 2).

Effect of different treatments on DFI

The paired Student's *t*-test was applied to compare DFI in swim-up and DGC-treated samples. Compared with unprocessed whole semen samples, the DFI was significantly decreased after processing by DGC or swim-up ($P<0.05$ for both), and the DFI in the swim-up group was significantly higher than that in the DGC group ($P<0.05$) (Table 3).

DFI between different age groups

The mean sperm DFI in different age groups are presented in Table 4. The results showed that, in both unprocessed whole

Table 1 Effectiveness of different treatments in improving sperm deformity rate (%)

	Whole semen	Swim-up	DGC
Sperm deformity rate	97.659±0.966	95.165±5.422	94.136±6.721[#]
*t**		5.097	5.843
*P**		0.000	0.000

DGC, density gradient centrifugation. Data are presented as mean±SD. *Compared with preprocessed value; [#] $t=3.027$, $P=0.003$, compared with swim-up

Table 2 Sperm deformity rate (%) between different age groups

Age group	N	Whole semen	Swim-up	DGC
20–29	32	97.344±0.837	94.438±6.988	93.875±7.201
30–39	63	97.691±1.018	95.691±4.141	94.589±5.977
40–49	23	98.009±0.883*	94.739±6.122	93.261±8.063
F		3.378	0.651	0.358
P		0.038	0.524	0.700

DGC, density gradient centrifugation; Data are presented as mean±SD. *$P<0.05$ vs. the 20–29 age group

Table 4 DFI (%) between different age groups

Age group	N	Whole semen	Swim-up	DGC
20–29	32	13.436±8.126	8.753±7.600	4.170±4.765
30–39	63	15.514±10.735	9.255±9.811	7.165±12.304
40-49	23	17.158±8.823	10.825±9.235	8.745±10.770
F		1.109	0.366	1.430
P		0.364	0.694	0.244

DFI, DNA fragmentation index; *DGC*, density gradient centrifugation. Data are presented as mean±SD

semen samples and swim-up- and DGC-treated samples, there was no significant difference in DFI between different age groups ($P>0.05$ for all).

Correlation between sperm deformity rate and DNA fragmentation index

The correlation between sperm deformity rate and DFI was determined using Pearson's correlation analysis. No significant correlation was observed between these parameters ($r=0.008$, $P=0.927$).

Discussion

Among currently available semen processing methods, swim-up and DGC are most commonly used preparation techniques in ART laboratories around the world [13, 14]. At present, semen processing methods are not optimized for teratozoospermia patients, and there has not been an optimal processing method for this kind of semen so far. In the present study, we investigated the effectiveness of swim-up and DGC in improving the sperm deformity rate and DFI in semen samples from teratozoospermic patients. We found that the sperm deformity rate was decreased after processing by both swim-up and DGC when compared to whole semen. This result is consistent with an earlier investigation by Hammadeh et al. who stated that the proportion of morphologically normal spermatozoa was higher after semen processing by swim-up or DGC compared to native semen samples [15]. This result was also demonstrated by other studies which showed that semen preparation by swim-up or Percoll density centrifugation yielded spermatozoa with better morphology [16, 17]. In addition, we found that swim-up produced a higher sperm deformity rate than DGC. An investigation by Jayaraman et al. showed that the percentage of morphologically normal sperm in teratozoospermic patients was significantly lower after semen processing by swim-up in comparison with DGC,[14] and Hammadeh et al. found that DGC yields a significantly higher percentage of morphologically normal sperm than swim-up in infertile patients [15]. These results are consistent with our finding. However, another study stated that there was no significant difference in the percentage of morphologically normal sperm recovered by the two methods in infertile patients [18]. This discrepancy may be explained by the difference in centrifugal media used, or the type of patients involved in the respective studies.

Table 3 Effectiveness of different sperm processing methods in improving DFI (%)

	Whole semen	Swim-up	DGC
DFI	15.271±9.739	9.425±9.103	6.661±10.523#
*t**		9.374	9.762
*P**		0.000	0.000

DFI, DNA fragmentation index; *DGC*, density gradient centrifugation. Data are presented as mean±SD. *Compared with preprocessed value; # $t=-3.773$, $P=0.000$, compared with swim-up

Our finding that the DFI was significantly lower in swim-up- and DGC-treated samples compared to the unprocessed whole semen samples suggests that both semen preparation methods could improve DNA integrity. However, DGC was found to be associated with a significantly lower DFI than swim-up. Consistent with our finding, Sakkas et al. found that DGC is more effective than swim-up in reducing the percentage of sperm with DNA damage [19]. In contrast, Zini et al. found that the percentage of spermatozoa with DNA damage decreased significantly after swim-up treatment compared with unprocessed whole semen in nonazoospermic patients, but a slight increase was seen after Percoll treatment [20]. Monqaut et al. observed that swim-up allows better selection of sperm with lower nuclear vacuolization and presumably lower DNA fragmentation than DGC [21]. These findings suggest that spermatozoa recovered by swim-up possessed higher DNA integrity than those by DGC. Jayaraman et al. pointed out that the percentage of TUNEL positive sperm after sperm processing by swim-up or DGC was significantly lower in teratozoospermic samples, and there was no difference in the incidence of TUNEL

positive sperm between the two techniques, suggesting that the two techniques are comparable in terms of recovering sperm with low DNA damage [14]. In addition, controversial results also exist concerning the selection ability and effect of different kinds semen preparation methods that could recover the maximal number of sperm with genetic competence [22–24]. These controversial results indicate that a wide variety of factors may affect sperm DNA integrity, including semen selection method, DNA integrity evaluation technique, and study population. Thus, there is an urgent need to carry out a systematic study on classical semen processing procedures to reveal the underlying causes of inconsistencies, optimize these methods and improve their use in clinical settings.

This study showed that the sperm deformity rate in whole semen samples in the 20–29 age group was significantly lower than that in the 40–49 age group, but there was no significant difference in the sperm deformity rate in swim-up- and DGC-treated samples between different age groups. A previous study by Siddighi et al. found that there were no age-related changes in strict normal morphology [25]. Plastira et al. found that the percentage of normally shaped spermatozoa in the 24–34 age group was significantly higher than that in the 35–54 age group in oligoasthenoteratozoospermic patients, but sperm morphology did not differ significantly between the two age groups in fertile subjects.[26] With regard to the association between age and DFI, in our study no significant difference was found in DFI between different age groups in both untreated teratozoospermic samples and those treated by swim-up or DGC. The influence of age on sperm DNA fragmentation is still unclear, and this is exemplified by the conflicting conclusions drawn from several related studies. Brahem et al. found no significant correlation between DNA fragmentation and age in teratozoospermic patients [4]. Dakouane also found no difference in DNA fragmentation rate between people of different ages [27]. However, some other investigators found a significant correlation between these parameters [28–30]. The above mentioned inconsistent results may stem from differences in study population composition, age span or group. It appears that there are unobvious relationships between age and DFI in the general population, while a more obvious one exists in infertile patients with morphologically abnormal sperm. Future studies based on large samples or mechanistic analysess are required to elucidate the exact associations between sperm morphology, DFI and patient age.

Studies on correlations between sperm morphology and DNA integrity in patients with abnormal sperm, especially teratozoospermic patients, are relatively rare. To our knowledge, the present study is one of the few studies which analyzed the relationships between sperm deformity rate and DNA integrity in teratozoospermic patients. Our study found that the DFI had no significant correlation with sperm deformity rate. This finding is not consistent with some other studies, [31, 32] which have demonstrated a positive correlation between abnormal sperm morphology and DNA fragmentation rate. Most of these studies are based on analyzing the relationship of specific morphological index such as abnormal sperm head or acrosome with DNA fragmentation, or comparing DNA integrity between fertile and infertile patients, while our study investigated the association between DFI and sperm deformity in teratozoospermic patients. This is perhaps one of the reasons for the inconsistent results. In view of this, the relationship between sperm deformity rate and the DNA integrity in different groups of infertile patients requires further exploration.

Currently, there are several kinds of semen preparation procedures, including the most used methods such as swim-up and DGC, and some other more advanced procedures such as electrophoretic separation, high-magnification sperm morphology selection, and hyaluronic acid binding [3]. Since each method has its own advantages and disadvantages, so far there has not been an ideal and reliable method to be used in ART in teratozoospermic patients. The classic semen preparation methods remain the standard procedures, and there is still not one advanced method that can entirely replace the classic ones. A combination of advanced and classic methods may represent an attractive new strategy that can overcome the shortcomings of advanced methods, such as complexity, effectiveness and safety. Therefore, it is essential to reevaluate the classic procedures by conducting in-depth studies with more sophisticated designs and larger sample sizes, so as to find a simple and efficient semen processing procedure to be used in ART.

The present study has a limitation. The incubation of sperm in contact with seminal plasma after liquefaction might initiate sperm DNA fragmentation. This may result in discrepancies in primary results between the two methods. This might be particularly obvious in case of teratozoospermic samples in which spermatozoa have undergone aberrant spermiogenesis and are clearly a source of ROS production [33]. Further studies that take into account these factors should be conducted in order to confirm our findings.

In conclusion, we have analyzed the efficacy of DGC and swim-up methods in improving sperm deformity rate and DFI in semen samples from teratozoospermic patients. Both processing methods can yield a lower DNA fragmentation rate and sperm deformity rate, and the results are more in favor of DGC. Although there is no significant difference in DFI between different age groups in both unprocessed and DGC or swim-up treated semen samples, a significant difference in sperm deformity rate was observed between the 20–29 and 40-49 age groups in whole semen samples, which was, however, not seen in the two treatment groups. Our results suggest that both DGC and swim-up are effective in recovering better

quality sperm, with DGC appearing to be better, although further studies are needed to confirm our findings.

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