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Sperm chromatin quality and DNA integrity in partial versus total globozoospermia

A.R. Talebi[1,2] | J. Ghasemzadeh[2,†] | M.A. Khalili[2] | I. Halvaei[3] | F. Fesahat[2]

[1]Recurrent Abortion Research Center, Shahid Sadoughi University of Medical Sciences, Yazd, Iran

[2]Research and Clinical Center for Infertility, Shahid Sadoughi University of Medical Sciences, Yazd, Iran

[3]Departments of Anatomical Sciences, Faculty of Medical Sciences, Tarbiat Modares University, Tehran, Iran

Correspondence
Farzaneh Fesahat, Research and Clinical Center for Infertility, Shahid Sadoughi University of Medical Sciences, Yazd, Iran.
Email: farzanehfesahat@ssu.ac.ir

[†]We pay our regards with deepest gratitude to the departed soul to rest in peace.

Summary

Globozoospermia is a severe form of teratozoospermia with low incidence in infertile patients, considered as one of the important causes of male infertility. The objective was to investigate the chromatin/DNA integrity as well as apoptosis in ejaculated spermatozoa of cases with partial or total globozoospermia. Fifty-seven semen samples were divided into three groups of partial globozoospermia (n = 17), total globozoospermia (n = 10) and normozoospermia (control; n = 30). Sperm chromatin condensation, DNA integrity and apoptosis were assessed using cytochemical assays. The results showed significant differences in sperm parameters of count and motility between two case groups versus controls. The percentages of spermatozoa with abnormal chromatin packaging and protamine deficiency were significantly higher in total and partial globozoospermic men compared to normozoospermic samples. Also, the rates of TUNEL-positive spermatozoa were significantly increased in both globozoospermic cases with respect to the control (18.3 ± 10.1 and 12.3 ± 9.2 versus 5.9 ± 3 respectively). However, no significant differences were noticed between two subgroups of patients with regard to sperm DNA denaturation, DNA fragmentation and apoptosis. Abnormal chromatin packaging, DNA damage and apoptosis were significantly higher in cases than controls. The sperm chromatin/DNA anomalies may be considered as one of the main aetiology of ART failure in globozoospermic patients.

KEYWORDS
DNA integrity, globozoospermia, male infertility, sperm chromatin

1 | INTRODUCTION

Globozoospermia, a rare and severe disorder with incidence of less than 0.1%, is known as one of the male infertility causes. Total globozoospermia is diagnosed by the presence of 100% round-headed spermatozoa lacking an acrosome, whereas partial globozoospermia ejaculates contain both normal and globozoospermic spermatozoa (Agarwal, 2003; Dam et al., 2007). It is shown that round head spermatozoa contain abnormal chromatin structure and or fragmented DNA which may cause pregnancy loss, birth malformations, cancers or genetic diseases in the infants (Potts, Newbury, Smith, Notarianni, & Jefferies, 1999). Thus, evaluation of sperm chromatin/DNA status should be important in globozoospermic work-up. There are several controversial issues on differences in semen parameters, sperm chromatin structure and apoptosis rates between globozoospermic and fertile men (Perrin et al., 2013; Vozdova et al., 2014). For example, Dam et al. (2011) evaluated the semen parameters of thirteen globozoospermic sperm samples in comparison with nine normal samples and showed that concentration and progressive motility were significantly reduced in globozoospermic patients whereas spermatozoa with abnormal morphology were increased significantly. Furthermore, a case report by Taylor et al. (2010) showed that there were normal characteristics in sperm parameters except the morphological criteria provided from globozoospermic patient according to WHO 1999 compared with the normal group. It was also reported that there was no significant increase in DNA denaturation

or fragmentation rates in globozoospermic spermatozoa when compared with fertile men. In other words, globozoospermia was not essentially related to the abnormalities in chromatin structure and DNA strands (Larson et al., 2001).

Deemeh, Tavalaee, Razave, and Nasr-Esfahani (2007) considered two patients with total and partial globozoospermia and reported high degree of sperm protamine deficiency in both patients (87% and 95% respectively). In addition, DNA fragmentation index (DFI) was increased in both patients (Deemeh et al., 2007). However, several studies showed discrepant results from DFI in DNA of globozoospermic patients compared with fertile men (Evenson, Larson, & Jost, 2002; Perrin et al., 2011; Sermondade et al., 2011).

In our previous study, we compared the semen parameters, sperm protamine deficiency as well as apoptosis in ejaculated spermatozoa between total globozoospermic and normozoospermic men. The results showed that globozoospermic samples presented with higher proportion of spermatozoa with abnormal chromatin packaging, DNA fragmentation and significant reduction in sperm parameters than those from fertile men (Ghasemzadeh et al., 2015). Because of very low prevalence of globozoospermia, the above-mentioned reports showed different results. The majority of studies are in form of case reports, working with small samples size to assess the sperm parameter and chromatin integrity (Deemeh et al., 2007; Demir, Bozdağ, & Günalp, 2008; Larson et al., 2001; Sahu, Ozturk, & Serhal, 2010; Vozdova et al., 2014). Thus, in this study, we tried to consider different dimensions of sperm chromatin/DNA and apoptosis using standard cytochemical assays as important causes of low fertility rates in large population of globozoospermic men.

2 | MATERIALS AND METHODS

2.1 | Patient selection

In this case–control study, semen samples were collected from 57 men referred to our andrology laboratory. The ejaculates were divided into three groups of partial globozoospermia (n = 17), total globozoospermia (n = 10) and normozoospermia (n = 30) as control group. A comprehensive evaluation, such as physical examination, cytogenetic, immunological and reproductive hormonal assays, was performed for each man. Inclusion criteria for the globozoospermic patients were the presence of 50% to 100% of spermatozoa with round heads, small and without acrosome. The exclusion criteria were the presence of varicocele, age over 45, heavy smoking, diabetes, drug usage and alcoholism. This study was approved by the ethics committee at the Yazd Research and Clinical Center for Infertility, and informs consent forms were signed by all participants.

2.2 | Sperm collection and analysis

Semen samples were collected by masturbation after 2–5 days of sexual abstinence. Each specimen was allowed to undergo liquefaction and then evaluated for sperm concentration, motility and morphology according to the World Health Organization (WHO, 2010) criteria (Khalili, Mojibian, & Sultan, 2005). Briefly, sperm motility including progressive motility "grades a and b," nonprogressive motility "grade c" and immotile "grade d" was assessed manually by phase-contrast microscopy (Zeiss, Axiostar plus, Germany) at ×40 magnification. Papanicolaou staining was applied to evaluate morphological abnormalities, and at least 200 sperm cells were examined per sample. Sperm count was assessed by Makler counting chamber (Sefi Medical Co., Haifa, Israel; Talebi, Moein, Tabibnejad, & Ghasemzadeh, 2008). All analyses were performed twice by one experienced laboratory technician blinded to the study.

2.3 | Sperm DNA damage and apoptosis

2.3.1 | TUNEL assay

This method was performed according to our previous study (Ghasemzadeh et al., 2015). After fixation and washing with PBS, the samples were incubated with 0.3% H_2O_2 in methanol for 1 hr to quench endogenous peroxidase activity. Incubation with the TUNEL reaction mixture (50 μl) was performed in a humidified chamber and dark room at 37°C for 1 hr following cell permeability with 0.1% Triton X-100 (Sigma-Aldrich Co, St. Louis, USA) at 4°C for 5 min. After washing in PBS, they were stained with 50-μl converter-POD at 37°C for 1 hr and exposed to DAB (3,3-diaminobezidine tetrahydrochloride; Roche Applied Sci, Mannheim, Germany) substrate solution for colour development in a dark chamber at room temperature for 10 min. Finally, the percentage of apoptotic spermatozoa in each sample was determined by terminal deoxynucleotidyl transferase-mediated dUTP nick-end labelling (TUNEL) assay by In Situ Cell Death Detection Kit (Roche Diagnostics GmbH, Mannheim, Germany) using fluorescent microscopy. The normal DNA spermatozoa were detected as light green and damaged DNA spermatozoa as bright green (Larsen, 2000).

2.3.2 | Sperm chromatin dispersion (SCD) test

The SCD test was introduced by Fernández et al. (2003). It was adapted in this study according to the protocol from our recent work (Nabi, Khalili, Halvaei, & Roodbari, 2014). Briefly, after sperm preparation using direct swim-up, 30 μl of sperm suspension was mixed with 70 μl low melting agarose (Roche, Germany). The air-dried smears from 50 μl of this mixture were used for denaturation, lysing and dehydration steps respectively. At the end, the precoated slides were stained with the Wright stain solution (Sigma-Aldrich) in PBS (1:1). The sperm cells show different halo patterns as pursue: large halos considered as the halo size is more than the minor diameter of core width, small halo size was smaller than one-third of the minor diameter of core width and medium halo size was between large and small halos. Spermatozoa with no DNA fragmentation showed large or medium-sized halos; while, sperm cells with DNA fragmentation showed either a small or no halos. At least 200 spermatozoa were checked, and sperm DNA damage was calculated by dividing the abnormal to the total spermatozoa and reported as percentage (Gaur, Talekar, & Pathak, 2007).

2.3.3 | Acridine orange (AO) staining

Sperm DNA integrity was determined by AO staining. The smears were first air-dried and then fixed overnight in Carnoy's solution. Each sample was stained for 10 min in freshly prepared AO (0.19 mg/ml) in McIlvain phosphate citrate buffer (pH 4) for 10 min and was evaluated using fluorescent microscope with a 460-nm filter. The percentage of green (normal double-stranded DNA) and orange/red (abnormally denatured DNA) fluorescence spermatozoa per sample were calculated (Talebi et al., 2012).

2.4 | Sperm chromatin quality

2.4.1 | Chromomycin A3 (CMA3) staining

CMA3 (Sigma, St Louis, MO, USA) is a fluorochrome specific for guanosine cytosine-rich sequences, used for evaluation of the degree of protamination in sperm chromatin (Demir et al., 2008). Like AO staining, Carnoy's solution was applied for smear fixation. The slides stained by CMA3 solution (Sigma; 0.25 mg/ml in McIlvaine buffer; 7 ml citric acid, 0.1 M + 32.9 ml $Na_2HPO_4{\cdot}7H_2O$ 0.2 M, pH 7.0 containing 10 mM $MgCl_2$) for 10 min at room temperature. After mounting the slides by DPX, 200 spermatozoa were counted under florescent microscopy (BX51, Olympus, Tokyo, Japan) with a 460-nm filter and ×100 eyepiece magnification. The percentage of spermatozoa with bright yellow heads (CMA3+) and without brightness (CMA3−) were determined and reported as percentage (Agarwal, Bragais, & Sabanegh, 2008; Talebi et al., 2012).

2.4.2 | Aniline blue (AB) staining

Aniline blue (AB), as a cytochemically based dye, is used for detection of excessive histones in process of sperm chromatin condensation (Talebi et al., 2008). The air-dried smears were fixed in 3% buffered glutaraldehyde in 0.2 m phosphate buffer (pH 7.2) for 30 min at room temperature. Each smear was stained with 5% aqueous AB stain in 4% acetic acid (pH 3.5) for 5 min. Unstained or pale blue stained spermatozoa (AB-) were normal, and dark blue stained spermatozoa (AB+) were considered as abnormal. At least 200 sperm cells were evaluated in each slide, and the normal and abnormal spermatozoa were reported as percentage (Zavos & Goodpasture, 1989).

2.4.3 | Toluidine blue (TB) staining

TB binds to phosphate groups of DNA strands and reveals the sperm chromatin condensation status and DNA fragmentation (Rosenborg et al., 1990). Briefly, the air-dried smears were fixed in 96% ethanol-acetone (1:1) at 4°C. After hydrolysation of the slides in 0.1 NHCl at 4°C and washing with distilled water, they were stained with 0.05% TB for 10 min. Pale blue sperm cells were considered as normal, and dark blue or violet/purple spermatozoa were categorised to abnormal cells. At least 200 spermatozoa were checked in each slide, and the normal (TB-) and abnormal (TB+) spermatozoa were reported as percentage (Nallella, Sharma, Aziz, & Agarwal, 2006; Talebi et al., 2012).

2.5 | Statistical analysis

The results were analysed using the SPSS software 20 for Windows (SPSS Inc., Chicago, IL, USA). The data are presented as mean ± *SD*. Kruskal–Wallis was used for comparison of different parameters between groups. The term "statistically significant" was used to denote a two-sided p value <.05.

3 | RESULTS

The results of total 57 samples regarding the age, duration of infertility and semen analysis are listed in Table 1. There were no significant differences in the men age, semen volume and duration of infertility between the groups. However, two types of progressive motility, percentage of pinhead spermatozoa as well as sperm concentration were statistically different between total and partial globozoospermic groups. Also, the findings showed significant reduction in both sperm count and progressive motility in partial globozoospermia compared with normozoospermia samples while there were no detectable changes in sperm counts between total globozoospermia

TABLE 1 Comparison of patients' characteristics and semen analysis between three groups

Variables	Partial globozoospermia	Total globozoospermia	Normozoospermia	*p*
Age (year)	35.2 ± 6.9	35.6 ± 5.9	34.2 ± 5.1	.7
Duration of infertility (year)	9 ± 7.1	9.5 ± 5.2	—	.4
Volume (ml)	2.6 ± 1.5	3.3 ± 0.9	2.8 ± 1.6	.1
Sperm count (×10^6/ml)	38.8 ± 26.7	86.9 ± 26.9	97.5 ± 48.1	.01[a] .00[b]
Progressive motility (%)	20 ± 18	34.5 ± 12.8	62.2 ± 7.5	.009[a] .00[b,c]
Pin head spermatozoa (%)	17.9 ± 13.8	8 ± 2.3	—	.01[a]

[a]Comparison between total globozoospermia and partial globozoospermia.
[b]Comparison between partial globozoospermia and normozoospermia.
[c]Comparison between total globozoospermia and normozoospermia.

TABLE 2 Comparison of chromatin/DNA status between three groups

Variables	Partial globozoospermia	Total globozoospermia	Normozoospermia	*p*
SCD(+)	61.7 ± 13.2	57.8 ± 11.4	11. 5 ± 6.2	.00[a,b]
TUNEL(+)	12.3 ± 9.2	18.3 ± 10.1	5.9 ± 3	.00[a].03[b]
AB(+)	81.3 ± 10.6	79.8 ± 12.9	24.2 ± 16.2	.00[a, b]
TB(+)	73.1 ± 16	86.3 ± 9.1	32.8 ± 18.6	.00[a, b]
CMA3(+)	60.1 ± 13.9	68.6 ± 11	26.1 ± 11.6	.00[a, b]
AO(+)	33.2 ± 26.4	30.1 ± 18.4	11.5 ± 7.5	.006[a] .00[b]

SCD, sperm chromatin dispersion; TUNEL, terminal deoxynucleotidyl transferase dUTP nick-end labelling; AB, aniline blue; TB, toluidine blue; CMA3, chromomycin A3; AO, acridine orange.
[a]Comparison between total globozoospermia and normozoospermia.
[b]Comparison between partial globozoospermia and normozoospermia.

and normozoospermic samples. Regarding sperm chromatin integrity, the rates of spermatozoa with AB+, TB+, CMA3+ showed a significant increase in total and partial globozoospermia groups when compared with normozoospermic samples (Table 2).

In addition, Table 2 indicates a significant increase in rates of TUNEL- and SCD-positive spermatozoa in total and partial globozoospermia groups compared to the normozoospermia group. However, there were no significant differences in sperm chromatin and DNA status between total and partial globozoospermic patients.

4 | DISCUSSION

Although previous studies have shown that globozoospermic men have high percentage of spermatozoa with chromatin/DNA anomalies, there are still some controversies in the results (Perrin et al., 2013; Sutovsky, Terada, & Schatten, 2001; Vozdova et al., 2014). Regarding these controversies, we should note two main points: first, many of researches are in the form of case report studies, evaluating limited number of globozoospermia cases (Larson et al., 2001; Vozdova et al., 2014). Second, the majority of these studies have used a limited number of assays; so, their results are limited to specific types of sperm anomalies. However, the present study evaluated the sperm parameters, chromatin condensation, DNA structure and apoptosis in a large group of patients using different standard assays to demonstrate different disorders of sperm chromatin and DNA integrity in two types of globozoospermia.

4.1 | Sperm parameters

We showed that although the volumes of semen samples were lower in case groups than control, but it was not significant. On the other hand, the sperm progressive motility and count showed a significant difference between groups (Table 1). It should be noted that the sperm count was also different between total and partial globozoospermic samples. In accordance with our results, Dam et al. (2011) assessed the semen from 13 globozoospermic and nine normal fertile men. They noticed a significant decrease in both sperm count and progressive motility in patients, when compared with normal group. Two studies reported similar results by working on only a few globozoospermic men (Demir et al., 2008; Larson et al., 2001). However, other studies showed that between semen parameters, sperm morphology is the most important factor in globozoospermic patients which predicts the success rate in assisted reproduction programme (Dam et al., 2007; Vicari, 2002). So, it is clear that total globozoospermia has lower rates of fertility than partial globozoospermia. It should be mentioned that round-headed sperm with no acrosomal cap is a defect in vesicular integration during acrosome formation in the process of spermiogenesis (Dam et al., 2011).

4.2 | Chromatin condensation

It is generally accepted that abnormal sperm parameters by itself do not reflect the fertility potential of spermatozoa and the integrity of chromatin and DNA is also an important issue to be investigated. For instance, nearly 8% of infertile men with normal sperm parameters show high degree of defective chromatin/DNA (Schulte, Ohl, Sigman, & Smith, 2010). Our study demonstrated that globozoospermic men had numerous sperm cells with excessive histone with protamine deficiency in their nuclei. We assessed chromatin abnormalities using cytochemical assays, such as AB, TB and CMA3. The findings represented a significant decrease in rates of spermatozoa with normal chromatin condensation in globozoospermic men.

In accordance with our results, two separate studies observed sperm chromatin abnormalities in form of low chromatin condensations by transmission electron microscopy (TEM) in globozoospermic samples (Nardo, Sinatra, Bartoloni, Zafarana, & Nardo, 2002; Taylor et al., 2010). Dam et al. (2011) also obtained similar results using TEM. Similarly, using TEM and indirect immunoflorescent method on three globozoospermic patients, it was concluded that there were vast abnormalities in sperm nuclei (Escalier, 1990). Similar to our results, Vozdova et al. (2014) concluded that rates of immature spermatozoa with abnormal histone and protamine were increased significantly in globozoospermic men. Parallel with our findings, high degree of protamine deficiency in two globozoospermic cases was reported using CMA3 (Deemeh et al., 2007). But, in contrast to our results, Larson

et al. (2001) by performing SCSA showed that globozoospermia was not related to sperm chromatin structure. However, according to the majority of studies, we can conclude that the process of sperm chromatin condensation is disturbed in globozoospermic spermatozoa, which may root in disorders in spermatogenesis.

4.3 | DNA integrity and apoptosis

One of the main aims of the present study was to investigate the DNA structure and apoptosis of sperm cells in a large group of globozoospermic men. We noticed a significant increase in sperm DNA fragmentation which is considered as the main sign of sperm apoptosis in these cases. To explain the above-mentioned results, it should be noted that abnormal replacement of histones by protamines can increase sperm DNA fragmentation and there is a clear correlation between sperm chromatin quality and DNA damage (Sakkas et al., 2002). In accordance to our findings, an increase in the rates of TUNEL-positive spermatozoa was observed in six globozoospermic men (Harbuz et al., 2011). On the other hand, while Vicari et al. (2002) reported similar results on DNA damage and apoptosis in globozoospermic men, others did not find any increase in the sperm apoptosis rates in similar patients (Sermondade et al., 2011). It should be considered that the most important factor for these differences is the sample size. For instance, one study worked on two globozoospermic patients and showed a significant elevation in sperm DNA fragmentation only in one of the cases (Deemeh et al., 2007). However, the evidence for the relationship between globozoospermia and sperm chromatin/DNA anomalies is numerous.

In conclusion, both partial and total globozoospermia are accompanied by abnormal sperm parameters, and abnormal chromatin packaging/DNA fragmentation. Thus, low rates of fertility in these men may be due to the abnormal sperm chromatin and DNA integrity in addition to lack of acrosome.

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CONFLICT OF INTEREST

There is no conflict of interest in this article.

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