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Analysis of sperm telomere length in men with idiopathic infertility

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Abstract
Purpose Telomeres are multifunctional nucleoprotein domains with hexanucleotide tandem repeat (5′ TTAGGG 3′) sequences, which cap the chromosome ends. However, the role of telomere and its length in sperm with regard to fertility remains unknown.
Methods In this pilot study, we analyzed 32 idiopathic infertile men and 25 controls for sperm telomere length by quantitative polymerase chain reaction (Q-PCR), and correlated it with sperm DNA fragmentation index (DFI) and reactive oxygen species (ROS) levels.
Results The relative sperm mean telomere length (T/S) of infertile men was found to be significantly lower ($p < 0.005$) when compared to controls (0.674 ± 0.028 vs. 0.699 ± 0.030). None of the sperm parameters such as sperm count, forward motility, morphology, ROS, and DFI were found to correlate with the sperm telomere length.
Conclusion Shorter telomeres in sperm may be one of the causative factors responsible for male infertility, but further detailed studies are needed to confirm these findings.

Keywords Sperm telomere length · Male infertility · DNA fragmentation index

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Abbreviations

DFI	DNA fragmentation index
ROS	Reactive oxygen species
SCSA	Sperm chromatin structure assay
Q-PCR	Quantitative PCR

Introduction

Male infertility is one of the major problems affecting married couples and the cause of infertility is classified as idiopathic in nearly half of the cases [1]. Though various causes such as abnormal sperm parameters, varicocele, and hypogonadism are reported to cause infertility, oxidative stress and sperm DNA damage have recently been reported to decrease the chance of conception and are implicated to be a major cause of idiopathic infertility and recurrent spontaneous abortions (RSA) [2, 3]. However, the role of telomere and its length on sperm function is not clearly known. Telomeres are evolutionary conserved hexameric tandem repeats that confer stability on chromosome, prevent end degradation, and maintain genomic integrity [4]. Not only their length but also their structure is critical for their function [5]. These hexameric guanine-rich repeats are multifunctional that provide chromosomal stability [6] and are the first site in sperm genome to respond to oocyte signal for pronucleus function and aid in synapsis, homologous recombination, and segregation [6, 7]. These nucleoprotein repeat sequences are located in the sperm nuclear periphery bound to histones and are more susceptible to oxidative injury [8]. As compared to other somatic cells where telomere length shortens with each division, telomere length in sperm, which is a terminally differentiated cell, is much longer than that of spermatogonia and

cells of other pre-meiotic stages [9]. Sperm telomere has been reported to play an important role in fertilization and embryo development as telomerase null ($TR^{-/-}$) sperm fertilized with wild type egg showed impaired oogenesis decreased quality of oocyte, increased rate of apoptosis, impaired chromosome synapsis and aneuploidy [10]. Moreover, telomere shortening results in DNA fragmentation [11], abnormal embryonic development, disruption of the normal telomere–telomere interaction resulting in loss of looped chromosome configuration [12]. The above-mentioned observations lead to the suspicion that telomeres might play an important role in chromosomal localization within the sperm nucleus [13]. Moreover, shorter telomeres are associated with various diseases and the effect of sperm telomere length on male fertility is not clear [14]. Hence, our aim was to analyze the sperm telomere length in both infertile men and controls and to correlate it with sperm DNA fragmentation and seminal ROS levels.

Materials and methods

A total of 32 men with idiopathic infertility having normal chromosomal complement and intact Y chromosome and 25 fertile controls were included in the study. All the infertile men (female partner found normal after gynecological and endocrinal examinations) were evaluated for cryptorchidism, varicocele, hypogonadism and hypospadias, and were found to be normal. Men seeking vasectomy and have recently fathered a child (not older than 2 years) were enrolled as fertile controls. This study was approved by the Institutional review board and written informed consent was obtained from all participants.

Semen analysis was performed in infertile men and controls as per WHO, 1999 guidelines. For morphology assessment, semen smear of 10 µL was prepared in a clean slide and fixed with 90 % ethanol and stained with Giemsa. At least 200 sperm per sample were evaluated for morphological defects. An aliquot of 100 µL of the raw semen was stored at −80 °C for DNA fragmentation analysis. Assessment of ROS was performed by chemiluminescence assay as described previously [15].

Sperm telomere length was determined from the sperm DNA by a quantitative real-time PCR-based method as described elsewhere [16]. Briefly, the relative mean telomere length was determined by comparing the value from absolute quantification of telomere DNA with a single copy reference gene, 36B4 (T/S ratio). These two assays were carried out as separate reactions on separate plates maintaining the sample positions between the two plates. Amplification signals were quantified by the standard curve method using a DNA template series (100 ng, 10 ng, 1 ng, 0.1 ng, 0.01 ng/µl) on every plate. All randomized DNA samples (20 ng) and standard dilutions were processed as triplicates on 96-well plates using Bio-Rad CFX 96 (Hercules, CA, USA). The purpose of the standard curve was to assess and compensate for inter-plate variations in PCR efficiency. Amplification of the telomeric repeat region was expressed relative to amplification of 36B4, a single copy gene (SCG) encoding acidic ribosomal phosphoprotein located on chromosome 12. Real time kinetic quantitative PCR determines, for each sample well, the C_t, i.e., the fractional cycle number at which the well's accumulating fluorescence crosses a set threshold that is several standard deviations above baseline fluorescence. A plot of C_t versus log (amount of input target DNA) is linear, allowing simple relative quantitation of unknowns in comparison to a standard curve derived from amplification, in the same plate, of serial dilutions of a known reference DNA sample. For this study, telomere (T) PCRs and SCG PCRs were always performed in separate 96-well plates. The primers for the telomere PCR are TeloF: 5′ CGGTTTGTTTG GGTTTGGGTTTGGGTTTGGGTTTGGGTT 3′ TeloR: 5′ GGCTTGCCTTACCCTTACCCTTACCCTTACCCTTAC CCT 3′. The primers for the SCG PCR are 36B4F: 5′ CAG CAAGTGGGAAGGTGTAATCC 3′, 36B4R: 5′ CCCATT CTATCATCAACGGGTACAA 3′.

Q-PCR profile

Two master mixes of PCR reagents were prepared, one with the telomere (T) primer pair, the other with the SCG primer pair. QPCR was performed as described by Cawthon [16] with slight modifications; amplifications were carried out in 10 µl reaction with 20 ng/µl of genomic DNA, and 1XSYBR Green (SsoFastTM Evagreen supermix, BioRad). The T PCR thermal cycling profile consisted of 10 min at 95 °C followed by 35 cycles of 95 °C for 30 s, 57.5 °C for 30 s, followed by a melt curve at 65 °C −95 °C 5 s. The SCG PCR thermal cycling profile consisted of 10 min at 95 °C followed by 40 cycles of 95 °C for 30 s, 57.5 °C for 30 s, followed by a melt curve 65 °C to 95 °C for 5 s. The T/S ratio (C_t (telomere assay)/C_t (SCG assay) was used to assess the relative length of telomere, while C_t is the fractional cycle number for a threshold fluorescence level to be reached during quantitative real-time PCR. All samples were run in triplicate along with a no-template control. Dissociation melting curves were run after each sample to ensure amplification of a single species. Replicates of each plate were done to ensure reliable values.

The telomere to SCG (T/S) ratio between infertile men and controls was compared by Student's t test. The correlation between the sperm T/S ratio and the other parameters were calculated by Pearson correlation co-efficient test. All the statistical analysis were performed using Medcalc trial version software. $p < 0.01$ was considered as significant.

Results and discussion

The quality control Q-PCR analysis showed that the slope of the standard curve for both the telomere and 36B4 reactions was −3.029 and −3.1, and acceptable linear correlation coefficient (R^2) value for both reactions was 0.97 and 0.99, respectively. The efficiency of Q-PCR for the telomere and SCG was 113.8 and 107.5 %, respectively (Figs. 1, 2). Interestingly, the relative sperm mean telomere length (T/S) of infertile men was found to be significantly lower ($p < 0.005$) when compared to controls (0.674 ± 0.028 vs. 0.699 ± 0.030). The box plot with median (minimum, maximum) T/S ratio of both infertile and control men was shown (Fig. 3). The lowest value of T/S ratio of infertile men and control was 0.5829 and 0.6517, respectively. However, the highest level of T/S ratio found in the infertile and control men was 0.7088 and 0.7546, respectively.

Semen parameters between infertile and control men are shown in the Table 1. Age and sperm telomere length showed no correlation in our analysis and none of the sperm parameters showed a significant correlation with the sperm telomere length (Table 2). Similarly, the two infertility markers, DFI and ROS levels, did not show any significant correlation with the sperm telomere length (Table 2).

Telomere length and structure play an important role in the maintenance of genome integrity and are also crucial for recombination and meiosis and the formation of pronucleus following entry of sperm into oocyte [10]. Though various diseases have been reported to be associated with telomere shortening [14], the role of sperm telomere length

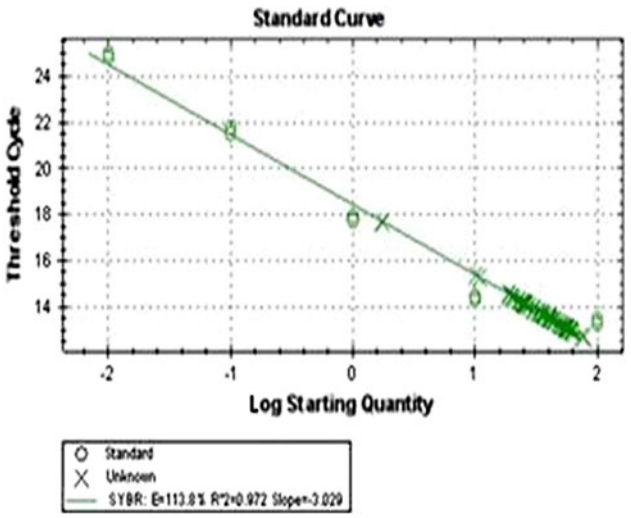

Fig. 1 Standard curve applied to estimate the relative DNA (log DNA) concentrations from the threshold value (C_t) of the real-time telomere length PCR products by serial dilution of known amounts of DNA

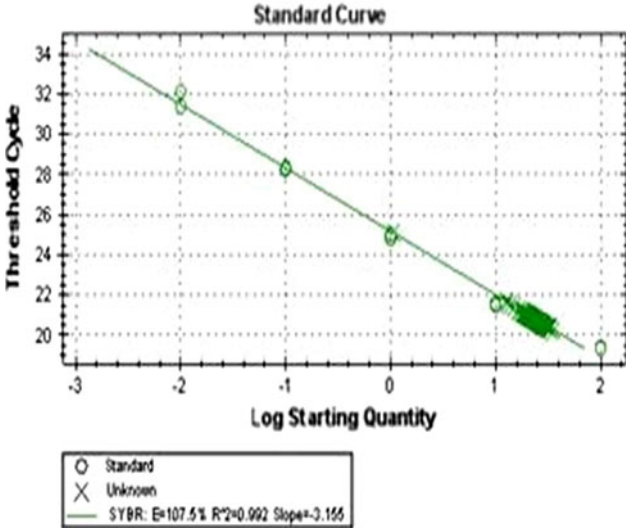

Fig. 2 Standard curve applied to estimate the relative DNA (log DNA) concentrations from the threshold value (C_t) of the 36B4 real-time PCR products by serial dilution of known amounts of DNA

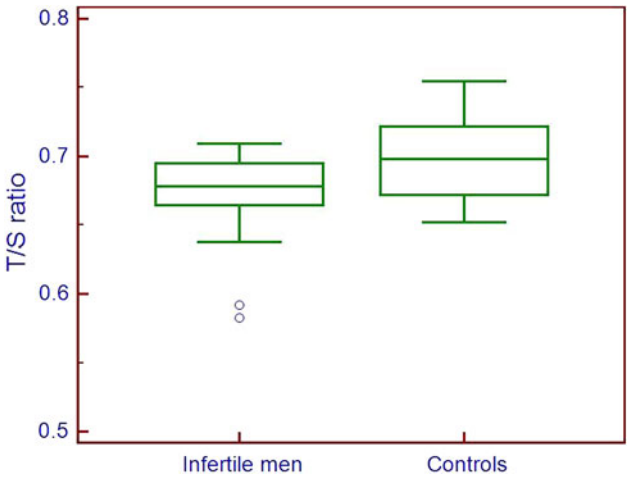

Fig. 3 Box plot distribution of percent T/S ratio (relative telomere length) of infertile men and controls. The *box plot* displays the 25th percentile, median, and 75th percentile. The *circles* represent outlier. The *horizontal lines* outside the box display minimum and maximum values

in the male fertility is not known. Sperm telomere plays a critical role in meiosis (synapsis, homologous recombination, and segregation) pronucleus formation at fertilization and microtubule-guided movement [10, 17].

Achi et al. [9] reported that sperm with longer telomeres help to maintain critical telomere length during cleavage, which aids in maintaining species-specific telomere length in the newborn. Therefore, our study showing compromised telomere length in the sperm may be one of the causative factors associated with impaired fertility. Telomere in germ cells is different from somatic cells and reorganization of telomere in sperm during spermatogenesis has been

Table 1 Semen parameters of infertile and control men

	pH	Sperm count ($\times 10^6$)	% Forward motility	% Normal sperm morphology
Infertile	7.93 ± 0.3	41.75 (21.4, 87.5)*	30 (12.5, 51.2)**	53 (34, 58.7)
Control	7.94 ± 0.3	86.6 (40.9, 190.2)	60 (50, 70)	62.5 (45, 70)

Values are represented as median (interquartile range). Values of pH are represented as mean ± SD

* $p < 0.01$, ** $p < 0.0005$ by Mann–Whitney test

Table 2 Correlation of sperm telomere length (T/S) with age and semen parameters

Semen parameters	*r* value	*p* value
Age	−0.2273	0.092
pH	0.0795	0.549
Volume	0.0462	0.732
Sperm count	0.0326	0.809
Sperm motility	−0.0697	0.590
Normal morphology	0.0735	0.586
DFI	0.0780	0.710
ROS	0.0885	0.629

demonstrated previously [17]. Studies have also shown that sperm with null telomerase ($TR^{-/-}$) fertilized with wild-type oocytes result in decreased fertilization and blastocyst formation, increased embryo fragmentation, and apoptosis [10]. Therefore, it may be possible that sperm with shortened telomere length may not respond to oocyte signals for pronucleus formation even if fertilization does occur. This may result in impaired cleavage, which will result in poor quality blastocyst, increased apoptosis, or failed implantation [18]. Telomeric DNA being guanine rich is more susceptible to oxidative stress, which is known to cause telomere shortening by oxidation of guanine bases to 8-oxoguanine, 8-OH 2-deoxyguanine, abasic sites, and single and double strand breaks. These are promutagenic and accumulate maximally in telomere and may induce more DNA damage. But we have not found any correlation between ROS levels and sperm DNA fragmentation with sperm telomere length. However, in an ongoing study with large sample size, the same correlation will be validated. Therefore, it is possible that shortened telomere may be the sole cause of impaired fertility, needs further investigations.

To our knowledge, this is the first prospective study addressing the potential contribution of relative telomere length in male infertility, though previously it has been demonstrated that sperm selected by swim-up have longer telomeres [19]. Also, it would be interesting to know if sperm selected for longer telomere length may improve IVF/ICSI carry-home live birth rate and may result in longer lifespan of the offspring. This may then form the basis of germ cell selection based on telomere length.

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Conflict of interest The authors declare that there are no conflicts of interest.

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