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Original Research Article

The association of male infertility with telomere length: A case control study

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ABSTRACT

Aim: To study the relevance of sperm telomere length and infertility in men.
Materials and Methods: Our case-control study included twenty-five males in couple, who were subfertile/infertil (test group) and twenty-five healthy males (control group) with proven paternity in the age group 21-35 years. The Absolute Sperm Telomere length (aSTL) was measured by real-time PCR. We investigated whether any significant difference in the aSTL value existed between the groups and analysed the relationship between aSTL and other sperm parameters.
Results: The mean (SE) aSTL recorded in the infertile cases was significantly shorter than for the control group; 140.60 (6.66) Kb/genome and 239.63 (12.32) Kb/genome respectively ($p < 0.001$) A moderate positive correlation was eminent between aSTL kb/genome and the total sperm count mil/ml (rho= 0.54, $p<0.001$), progressive sperm motility (rho= 0.56, $p=<0.001$) and sperm viability (rho= 0.51 $p=0.032$) in the infertile group.
Conclusion: The measurement of aSTL by real-time PCR is a simple and rapid method that offers further paramount information with respective to the quality of sperm. It is befitted for epidemiological studies, hence opening new perspectives in the evaluation of male infertility.
Limitations: Our study was confined to men aged between 21-35 years. Further comparative studies are needed to explore the significance of STL and infertility in older males. Additional studies will help illumine the significance of aSTL as a prognostic biomarker in assisted reproduction.

1. Introduction

In eukaryotes including human, the genetic material is majorly located in the cell nucleus; nDNA and a small fraction is in the mitochondria; mtDNA. The nuclear DNA is organised into linear units called ‘chromosomes’ while the mtDNA is circular and resembles the prokaryotic genome.[1] The genetic information carried by DNA is held in multiple sequences of nucleotides called ‘genes’. The protein coding genes constitute merely around one percent of the total DNA and the remaining sequences are non-coding.[2] The specialized heterochromatin region at the ends of each chromosome contains repeated non-coding DNA sequences that are designated as ‘telomeres’. The telomeric region, differs notably from the residual DNA in both structure and function. It represents an ‘evolutionary adaptation’ in eukaryotes to navigate the DNA end replication problem during cell-division.[3] The structural existence of these chromosomal end sequences was first invented in 1938 by Muller while studying terminal deletions in Drosophila chromosomes.[4] Their crucial role in preserving genomic integrity by preventing chromosomal end degradation and fusion was identified in the maize plant by McClintock.B in 1941.[5] Human telomeres are composed of repetitive hexa nucleotide sequence (TTAGGG)n that are associated with nucleoproteins oriented specifically

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towards the terminus of the chromosome 5'-TTAGGG-3'.[6] The 3'end, which is rich in guanine, is identified as the G strand. On the other hand, the complementary cytosine rich 5' end strand predominantly ends on an ATC-5' sequence and is called the C-strand.[7] A complex of six proteins called the 'Sheltrin complex' specifically wraps the TTAGGG repeat array. The sheltrin complex includes the Telomeric Repeat binding Factor 1 and 2 (TRF1 and TRF2), which recruit the other four proteins, namely TRF1and TRF2-Interacting Nuclear protein 2 (TIN2), Rap1, TPP1 and Protection of Telomeres1 (POT1).[8] The telomeres bestow chromosomal stability by shielding their ends from degradation during DNA replication. However these terminal arrays shorten progressively with each cell division. When the telomeres reach a critical length, the cells undergo senescence and subsequent apoptosis or programmed cell death. This process is called the 'mitotic clock' and is mediated through proteins such as BUB1, CENP-E, CENP-A, and Chk2.[9] In meiotic cells, telomeres tether chromosomes to the nuclear membrane to facilitate homologous pairing and initiate synapsis to form chiasmata in the early prophase.[10] Studies conducted in mice revealed that telomere attrition via genetic manipulation reduced synapsis and recombination.[11] Telomeres play a central role in the organization of the sperm nucleus as they anchor telomeres to the nuclear membrane. The spermatozoa telomeres are the first site in the sperm genome to respond to oocyte signals for the pro-nucleus formation and microtubule-guided movement after fertilization.[12,13]

The telomeres are not replicated by DNA polymerase as the rest of the chromosome but by a specific enzyme called telomerase, which is a ribonucleoprotein complex that uses an RNA template for de novo synthesis of telomeric DNA. Telomerase activity is ascertained in developing human embryonic tissues, stem cells, germ cells, and most cancer cells but not in quiescent or terminally differentiated somatic cells.[14,15] Additionally to DNA replication during cell division, various factors like genetic predisposition, lifestyle, psychological stress, exogenous and endogenous genotoxic insults, and long-term exposure to reactive oxygen species (ROS) generated as by-products of normal cellular metabolic processes may deplete telomeres and accelerate telomere attrition in both dividing and non-dividing cells resulting in 'premature ageing'.[16] Despite the activation of the compensatory telomerase maintenance pathway(TMP) in telomerase positive germ cells, telomere erosion in sperm may occur promoting segregation errors, DNA fragmentation and apoptosis. This in turn may affect sperm counts and functional parameters and decrease the possibility of fertilization.[17,18]

The available literature stresses the significant impact of the telomere status on reproductive ageing in both men and women. However, these findings are discordant and wide studies are needed to clarify such correlation. We aim to establish whether telomere attrition to an extent can impair fertility in men.

2. Aim

We aim to study the relevance of sperm telomere length and infertility in men.

3. Objectives

1. To evaluate the sperm telomere length in infertile and fertile men.
2. To ascertain correlation between sperm telomere length and other sperm parameters.

4. Materials and Methods

4.1. Study setting

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4.2. Ethics approval

The current study is approved by the Institutes Ethics committee (Dr DY Patil University, Pune) Reference No DYPV/EC/342/2019 and has been performed in accordance with the ethical standards as laid down in the 1964 Declaration of Helsinki.

4.3. Study design

Case-Control study.

4.4. Study population

Our test group included human males in couple aged between 21-35 years with infertility, presenting to the Fertility clinic. Their female partner was found normal after gynecological examination and infertility evaluation.

4.5. Infertility criteria

The inability of a sexually active, non contracepting couple to achieve pregnancy in a duration of twelve months or more.' (WHO-ICMART glossary).

The control group included healthy male volunteers aged between 21-35 years who had fathered at least one child (full-term normal healthy child; conceived naturally).

4.6. Sample size

Our pilot study included a total of fifty human subjects; twenty five each in case and control groups.

4.7. Exclusion criteria

The subjects were excluded from the study if they suffered from the following: congenital (cryptorchidism and testicular dysgenesis or congenital absence of the vas deferens) or acquired (obstructions, testicular torsion, testicular tumour, orchitis, urogenital tract infections, varicocele) urogenital abnormalities, endocrine disturbances, genetic abnormalities, autoimmune diseases, co-morbid conditions (i.e., cardiovascular disease, diabetes mellitus, dyslipidemia or cancer).We also did not include males subjected to exogenous factors (i.e., medications, toxins, irradiation, and chemicals.) or with a history of smoking, alcohol consumption, and anti-oxidant drugs.

4.8. Methodology

All the subjects were informed about the significance, procedure, outcome, and risks of the study. We obtained their written informed consent. Their detailed family, medical, fertility, drug, and lifestyle history was noted in a pre-designed case record form. The test group subjects underwent thorough examination to rule out any pathologies of the urinary genital tract. We performed karyotyping to rule out any chromosomal alterations and Y chromosomal micro deletions.

Semen samples were collected in sterile jars by masturbation following a cohabitation abstinence period of three to seven days. The standard semen analysis was performed as per the WHO guidelines criteria of 2010 (Table 1) by a trained person. This included macroscopic and microscopic examination, sperm count, motility, vitality, and morphology assessment

We measured the spermatozoa count using a Neubauer sperm counting chamber after dilution with a fixative and evaluated the motility and morphology of 200 spermatozoa using optical microscopy. Eosin-Nigrosin technique was used to assess sperm vitality. Samples with leucocytospermia or semen bacteria were excluded from the study. A single test was sufficient, except for the cases with abnormal results. In these instances, the semen analysis was repeated after two weeks

4.9. DNA extraction

The semen sample was washed with phosphate-buffered saline (PBS) and genomic DNA was isolated using a previously published method (Darbandi et al. 2018).[19] The quality of the genomic DNA was assessed by Agarose gel Electrophoresis. We prepared 1% (w/v) agarose gels with a nuclear stain dye (1:65000x) in 0.5X TBE buffer. We mixed 5 μl of genomic DNA with 1 μl of 6X gel tracking dye. The DNA molecules were resolved at 5V/cm and detected under a UV trans-illuminator. The gel images were recorded using the BIO-RAD Quantity One- Gel Doc-XR gel documentation system.

4.10. Absolute STL measurement

Absolute telomere length (aTL) was measured using quantitative PCR using the method outlined by O'Callaghan and Fenech.[20] We generated a standard curve from the fluorescent signals given by serial concentrations of telomere oligomer DNA $(TTAGG)_{14}$. The concentration of each testing sample was predicted according to the standard curve. We used the single-copy gene (36B4) as a reference gene and measured its concentration in each sample using the same method. Telomere length was calculated as the ratio of the telomere DNA length to the 36B4 DNA length. Each sample was analysed in triplicate on a QuantStudio5 384-Well PCR Plate Real-Time PCR system (Thermo Fisher Scientific). The details of primers and standards used is as shown in Table 2.

The synthesized 36B4 and the telomerase oligomer standards being 75bp and 84bp respectively (i.e., TTAGGG sequence repeated 14 times). The stock standards were serially diluted to the optimum concentration required in PCR. We used the DNA from 1301 lymphoblastic cell lines as a long telomere control (70 kb) for initial validation of standard copy number. The stock primers were diluted (10 pm/μl) to an optimum concentration for real-time PCR. We quantified the DNA using a Qubit Fluorometer and used 20 ng of each DNA for qPCR. The PCR master mix was prepared as per the composition provided in Table 3. Ten μl of each master mix was dispensed into a 96 Real-time PCR well plate and 5 μl of genomic DNA was later added to each well. The PCR reactions were performed with two negative control reactions. The thermal cycling programs for the real-time PCR are found in Table 4. We used the Quantstudio-5, 384 well real-time PCR system (Thermo Fisher Scientific) and performed the analysis using the Quantstudio Design and Analysis software v1.51.

4.11. Statistical analyses

All of the statistical analyses were carried out using the Statistical Program for Social Sciences (SPSS Inc version 23) for windows. The data was reported as the standard error of the mean (mean±SEM). The participants' age was on the other hand reported as the standard deviation of the mean (mean±SDM). For the variables not normally distributed in the two subgroups, we used the non-parametric Wilcoxon-Mann-Whitney U and the Fisher extract tests. We applied parametric independent t-tests for the normally distributed variables to compare the groups. The association between STL and other parameters was analyzed with Spearman correlation and the results were reported as p-values and correlation coefficients (rho). The significance was defined as $P < 0.05$ at 95% CI.

5. Results

1. Comparison of Age, Sperm parameters among the Fertile and Infertile men is illustrated in (Table 5) and (Figure 1)
2. The comparative data of absolute sperm telomere length (aSTL in Kb/genome) values in the Fertile and Infertile groups; elucidated in (Table 6) (Figures 2 and 3)
3. The distribution of Primary and Secondary Infertility cases with their mean aSTL values as shown in (Table 7)
4. The association of mean absolute sperm telomere length (aSTL in Kb/genome) values with other sperm parameters in the Infertile group; shown in (Table 8) (Figure 4)

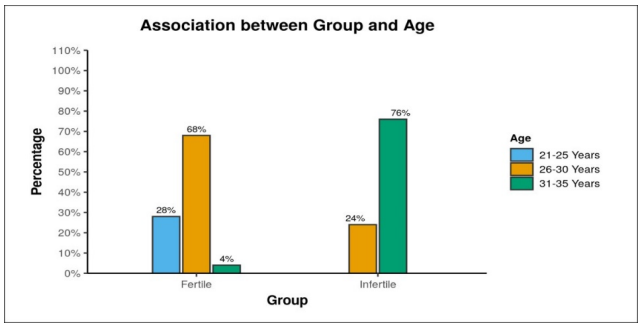

Fig. 1: Age distribution of subjects in the two groups

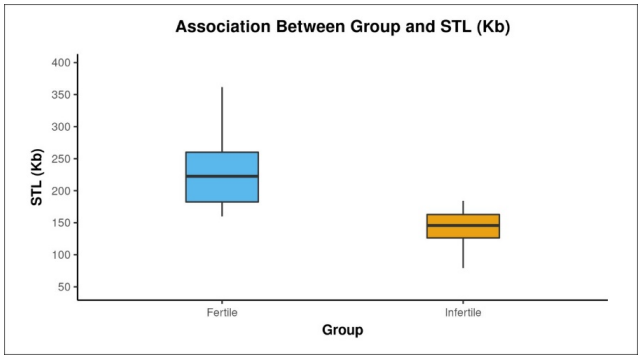

Fig. 2: Box plot: omparison of aSTL Kb/genome between Groups

The points represent individual cases. The blue trendline represents the general trend of correlation between the two variables, the shaded grey area represents the 95% confidence interval of this trendline.

6. Discussion

About 15% of couples worldwide are affected by primary or secondary infertility (i.e., inability to have any children or to conceive or carry a pregnancy to

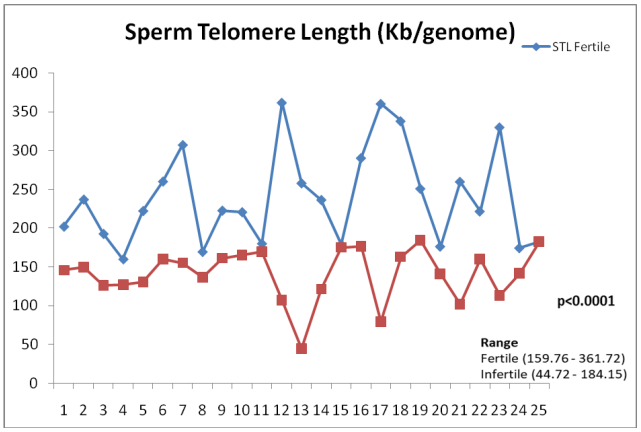

Fig. 3: Line Graph representation of aSTL Kb/genome values in the Fertile and Infertile subjects

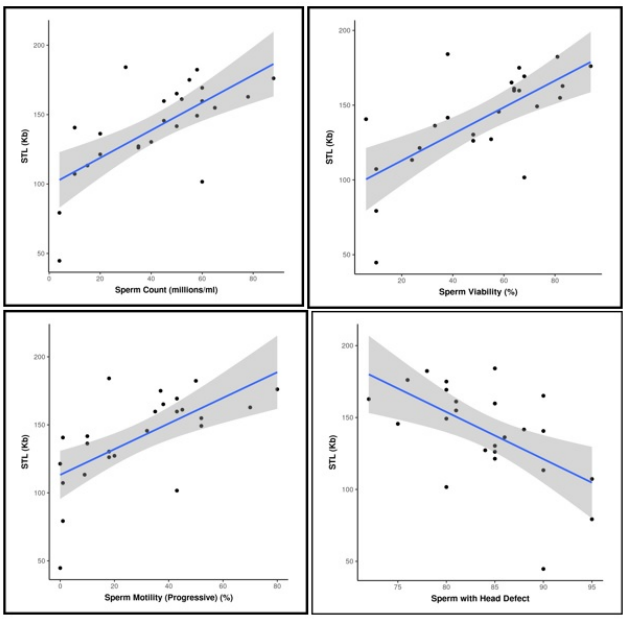

Fig. 4: Scatter-plot depicts the correlation between Sperm parameters and aSTL (Kb/genome)

term following the birth of one or more children).[21] Infertility is a complex condition due to varied causes, such as genetic, developmental, hormonal, environmental, and lifestyle factors. The pivotal role of telomeres in reproduction and infertility is still obscure. In this case-control study, we investigated the relationship between sperm telomere length, spermatogenic activity, and the ability to conceive naturally. We observed that the absolute STL was significantly shorter in men affected by primary and secondary cases of infertility than in healthy subjects of the same age (21-35 years). The mean (SE) aSTL was 140.60 (6.66) Kb/genome for the infertile participants and 239.63 (12.32) Kb/genome for the men with proven

Table 1: Semen analysis (WHO manual for 5th edition, 2010)

Parameter	Lower Reference Limit
Semen volume (ml)	1.5 (1.4 - 1.7)
Colour	translucent / whitish –grey
Ph	> 7.2
Total sperm number (106 per ejaculate)	39 (33 - 46)
Sperm concentration (106 per mL)	15 (12- 16)
Total motility (PR + NP %)	40 (38 -42)
Progressive motility (PR %)	32 (31-34)
Vitality (live spermatozoa %)	58 (55-63)
Sperm morphology (normal forms %)	4 (3.0- 4.0)

Source: Cooper TG, Noonan E, von Eckardstein S, Auger J, Baker HW, Behre HM et al. World Health Organisation reference values for human semen characteristics. Hum Reprod Update.2010;16(3):231-245[PubMed] [Google Scholar]

Table 2: Details of primers and standards used

	Oligomer Name	Oligomer Sequence	Amplicon size
Standards	Telomere Standard	(TTAGGG)14	84bp
	36B4 Standard	CAGCAAGTGGGAAGGTG TAATCCGTCTCCACAGAC AAGGCCAGGACTCGTTTGTACCCGTTGATGATAGAATGGG	75bp
PCR Primers	TeloF	CGGTTTGTTTGGGTTTGGGTTTGGGTTTGGGTTTGGGTT	> 76bp
	TeloR	GGCTTGCCTTACCCTTACCCTTACCCTTACCCTTACCCT	
	36B4F	CAGCAAGTGGGAAGGTGTAATCC	75bp
	36B4R	CCCATTCTATCATCAACGGGTACAA	

Source**:** O'Callaghan, N.J, Fenech, M. A quantitative PCR method for measuring absolute telomere length. Biol Proced 2011 Online 13, 3. https://doi.org /10.1186/1480-9222-13-3

Table 3: PCR master-mix composition

Components	Volume (micro-lit)
Genomic DNA	5.00
2X SYBR Green	6.25
Forward Primer	0.25
Reverse Primer	0.25
Nuclease free water	3.25
Total	15.0

Table 4: Real time PCR cycling conditions

Stage	Temperature (degree- celsius)	Time (minute:seconds)	Cycles
Initial denaturation.	95	1:00	
Denaturation.	95	0:15	
Annealing and Extension.	60	1:00	40 cycles
Data collection			
Melt Curve stage			

paternity (P <0.001). Additionally, the estimated mean (SE) aSTL was 137.25± 8.23Kb/genome for the participants with primary infertility and 143.70 ± 5.09 Kb/genome for the men afflicted with secondary infertility (P < 0.001). This suggests a possible contribution of altered STL in male infertility. Varied methods have been adapted by researchers to accurately measure telomere length and they are all encased with certain advantages and drawbacks. The Terminal Restriction Fragment (TRF) analysis using probes against telomere repeats was instrumental to unveil the first associations between telomere length and human diseases. TRF, which was established in the 80s, remains one of the most widely used methods to measure telomere length.[22,23]

Telomeres consist of 6-8 base-pair sequences, repeated hundreds or thousands of times. The actual sequence and number of repeats vary between species. The size of human telomeres ranges from 2-50 kilobases and consist of approximately 300-8,000 precise repeats of the sequence CCCTAA/TTAGGG.[24] The length of the spermatozoa telomeres is substantially longer compared to that of normal somatic cells. The human telomere sequence varies in length from 5 to 10 kb in somatic cells and 10 to 20

Table 5: Groups comparison -age and sperm parameters

Parameters	Group		P value
	Fertile(n = 25)	Infertile(n = 25)	
Age (Years)* ** mean ± SD	27.00 ± 2.14	33.20 ± 2.61	<0.001[1]
Age* **			<0.001[2]
21-25 Years	7 (28.0%)	0 (0.0%)	
26-30 Years	17 (68.0%)	6 (24.0%)	
31-35 Years	1 (4.0%)	19 (76.0%)	
Sperm count (millions/ml)* ** mean ± SE	53.96 ± 3.78	34.56 ± 3.66	0.001[1]
Total Sperm Motility (%)* ** mean ± SE	61.20 ± 2.08	30.32 ± 3.87	<0.001[1]
HSA Impression* **			<0.001[2]
TZ	1 (4.0%)	9 (36.0%)	
OATZ	None	7 (28.0%)	
NZ	24 (96.0%)	9 (36.0%)	

***Significant at p<0.05, 1: Wilcoxon-Mann-Whitney U Test, 2: Fisher's Exact Test.
NZ-Normozospermia, TZ-Teratozospermia, AZ -Asthenospermia, O- Oligozospermia
HSA- Human semen analysis

Table 6: Groups Comparison: variable aSTL (Kb) (n = 50)

aSTL (Kb) /genome	Group		Wilcoxon-Mann-Whitney U Test	
	Fertile	Infertile	W	P value
Mean (SE)	239.63 (12.32)	140.60 (6.66)		
Median (IQR)	222.55 (182.46 - 260.09)	145.62 (126.18-162.81)	600.000	<0.001
Range	159.76 - 361.72	44.72 - 184.15		

***Significant at p<0.05, Wilcoxon-Mann-Whitney U Test

Table 7: Distribution of type of infertility and their mean aSTL values (n=25)

Type of Infertility	Frequency	Percentage	STL Kb/genome Mean (SE)
Primary	13	52.0%	137.25 ± 8.23
Secondary	12	48.0%	143.70 ± 5.08
Total	25	100.0%	

Table 8: (Group: Infertile) Correlation between mean aSTL (Kb) and sperm parameters

Sperm parameters	aSTL Kb/genome Correlation	P-value
Sperm Count (millions/ml)	Correlation Coefficient (rho) = 0.54	<0.001
Total Sperm Number (millions/Ejaculate)	Correlation Coefficient (rho) = 0.47	0.054
Total Sperm Motility(%)	Correlation Coefficient (rho) = 0.48	<0.056
Sperm Motility (Progressive) (%)***	Correlation Coefficient (rho) = 0.56	<0.001
Sperm Motility (Non-Progressive) (%)	Correlation Coefficient (rho) = 0.36	0.077
Non-Motile Sperms (%)	Correlation Coefficient (rho) = -0.44	<0.062
Sperm Viability (%)***	Correlation Coefficient (rho) = 0.51	<0.032
Sperms with Abnormal morphology (%)	Correlation Coefficient (rho) = 0.49	0.051

*** Significant at p<0.05, Spearman Correlation.

kb in germ cells.[25] The TRF method requires many cells (~106) and provides a rough estimate of the average number of telomeric repeats per sample, as terminal restriction fragments tend to include variable amounts of sub-telomeric DNA sequences of 2.5-4 kb.[26]

Quantitative FISHis a more sensitive technique that uses digital fluorescence microscopy to determine telomere length after hybridization of metaphase spreads with a fluorescent PNA telomeric probe and quantification of the number of "signal-free ends" (<0.15 kb). Despite its ability to measure the telomere length of each chromosome accurately, this method is laborious, cumbersome, and expensive. Multiple controls are required to avoid inter/intra-session variability and only a few samples can be analysed at once. Hence, it is not well suited for high throughput studies,[27] Lafuente et al, demonstrated using the QFISH technique that STL was lower in males with primary infertility than fertile males and exposure

to oxidative stress (H2O2) lead to a severe shortening of the telomeres. They also observed a negative correlation between STL and sperm motility and concentration.[28] According to Biron-Shental et al., sub-fertile men (n=16) displayed a higher percentage of sperm telomere fluorescent aggregates and lower TERT levels than the fertile group (n=10).[29] However, Turner and Hartshrone who measured sperm telomere length in 45 men and oocyte telomere length in 32 women with Q-FISH reached a contradictory conclusion. They showed that the presence of long STL was not crucial for male fertility since oocyte-induced sperm telomere DNA modifications towards the blastocyst stage.[30]

Telomere length determination by Quantitative polymerase chain reaction (qPCR) assay measures telomere (T) and single-copy gene (S) signals in comparison to a reference DNA and yields relative T/S ratios that are proportional to the average telomere length. It is simple, fast, less expensive, and scalable for high throughput (HT) analysis. In the recent studies conducted by Thilagavathi et al and Liu et al, the relative mean sperm telomere length (T/S) was significantly lower ($p < 0.005$) in infertile men compared to the controls (0.674 ± 0.028 vs. 0.699 ± 0.030 and 2.894 ± 0.115Kb vs. 4.016 ± 0.603Kb, respectively).[31,32] The qPCR method can however, only provide the mean telomere length values per cell sample and not per cell or individual telomere. In comparison to the gold standard TRF analysis, qPCR measures relative telomere length and cannot provide absolute telomere length values in kilobases. However, this difficulty has been circumvented in our study by introducing an oligomer standard 84 bp in length with a TTAGGG sequence repeated 14 times (i.e., telomerase oligomer standard) to measure absolute telomere length by qPCR as described by,O'Callaghan and Fenech.[20] This is in agreement with Sahar Tahamtan et al. who evaluated sperm telomere length by qPCR using relative and absolute methods. The mean of the absolute (7.17 ± 1.18 vs. 13.44 ± 1.87; P= 0.006) and the relative (0.48 ± 0.12 vs. 1.14 ± 0.18; P= 0.004) telomere length was significantly lower in infertile men (n=18) with varicocele than fertile individuals (n=20).[33] The mean age of men in our study was significantly higher in the infertile group (fertile 27.00 ± 2.14 vs infertile 33.20 ± 2.61 years, $P < 0.001$). Several studies showed that age is a possible confounding factor that could affect telomere length. Therefore, we analysed telomere length data adjusted for age in spermatozoon using a regression model. The sperm counts and total sperm motility percentage were significantly lower in infertile men compared to fertile men (P< 0.001). Shorter STLs and their association with a lower sperm count, vitality, and protamination have been reported in patients with decreased sperm motility. It was also negatively correlated with DNA fragmentation in normozoospermic individuals.[34] In the current study, A moderate positive correlation was eminent between aSTL kb/genome and the total sperm count mil/ml (rho= 0.54, p=<0.001), progressive sperm motility (rho= 0.56, p<0.001) and sperm viability (rho= 0.51, p=0.032) in the infertile group. While aSTL was negatively correlated with Abnormal sperm morphology (rho= -0.49, p=0.051). (Table 8, Figure 4) Our findings are in concordance with Thilagavathi et al. who measured the relative STL of 25 fertile men and 32 men with idiopathic infertility and concluded infertile men displayed a shorter STL.[31]

7. Conclusion

We are among the few researchers who studied the relevance of telomere length in infertility by measuring absolute telomere length in male germ cells. Our method allowed a more direct comparison of results between experiments and provided an edge over the Relative TL qPCR assays. Based on our results, we conclude that sperm telomere dysfunction might affect spermatogenesis and fertilisation. The measure of aSTL unveiled new insights in the evaluation of infertility and could be considered as an additional diagnosis parameter. Further studies will clarify the significance of this parameter as a diagnostic marker in infertility and a prognostic biomarker in assisted reproduction.

8. Source of Funding

No external funding was either sought or obtained for this study.

9. Conflict of Interest

The authors of this study declare that they have no conflict of interest.

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