Can telomere shortening be the main indicator of non-viable fetus elimination?

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Abstract

Background: Telomeres are transcriptionally inactive genomic areas, which, if shortened, are associated with pathological processes, unsuccessful fertilization, aging, and death. Telomere dysfunction has also been linked to chromosomal rearrangements and genomic instability. The role of telomeres in postnatal life has been extensively studied and discussed both in physiological as well as in pathological processes. However, the role of telomere length in prenatal development is still poorly understood, and mainly concerns the preimplantation stage. The aim of this study was to estimate relative telomere length in spontaneously eliminated human embryos between 5th and 12th week of gestation.

Results: Relative telomere length was measured from total genomic DNA using a real-time polymerase chain reaction approach. In this study, we examined relative telomere length in 80 spontaneously eliminated embryos and in 25 embryos eliminated due to induced abortions. Relative telomere length in spontaneous abortions was significantly lower (P = 0.000001) compared to the induced abortions. Spontaneous abortions with aneuploid anomalies (monosomy X, trisomy 21, trisomy 16 and triploidy) were characterized by shorter telomeres, compared to spontaneous abortions, subgroup with euploid (46,XX) karyotype.

Conclusion: Spontaneously lost pregnancies are characterized by shortened telomeres, especially in embryos with aneuploidies. We hypothesize that the shortening of telomeres is involved in the processes leading to spontaneous abortions.

Keywords: Relative telomere length, Spontaneous abortions, Aneuploidy, Euploidy

Background

Telomeres are transcriptionally inactive areas of the human genome, involved in maintenance of genomic integrity. Telomeric DNA consists of 10–15 kb long hexamer iterations (TTAGGG)n [1, 2], which end with a 3′-single-helixed area, creating a D-loop [3, 4]. Formation of telomeric loop takes place with support of the shelterin complex, which consists of 6 proteins (TRF1, TRF2, TIN2, RAP1, TPP1 and POT1). The main function of this complex is to prevent degradation of telomeres [5]. During each cellular division, telomeric DNA is shortened by 50–200 bp [6–8]. As soon as the length of telomeres becomes critically low, the cells stop dividing and enter apoptosis [9, 10]. Thus, telomeres are believed to be the so-called cellular clock, which controls division and cellular death. Moreover, telomere dysfunction is linked to chromosomal rearrangements, genomic instability, tumorigenesis and cellular senescence [11, 12].

Telomere length (TL) is proposed to be an indicator of biological age of an organism, and a significant correlation between age and TL has been shown [13]. Role of telomeres during postnatal development was intensely studied and discussed both for normal and pathological conditions. In particular, telomere length is decreased in diabetes mellitus [14], cardiovascular disease [15], liver disorders [16, 17], cancer [18], and severe premature aging phenotypes [19]. Decreased TL has also been linked to adiposity [20, 21], low social and economic status [22], chronic emotional stress [23], smoking [24], increased mortality [25] and others.

However, the role of telomere length in prenatal human development remains mostly unknown, both under pathological as well as under physiological conditions, and the available data is contradictory [26–29]. Few recent studies mainly focused on preimplantation stages of...
human embryonic development available due to preimplantation diagnostic procedures [30–33]. According to the recent data, aneuploid human polar bodies possess significantly shorter telomeres than euploid polar bodies from sibling oocytes, although, at the blastocyst stage, telomeres did not differ in euploid and aneuploid embryos [30, 31]. It is also likely that the chance of successful in vitro fertilization decreases along with the decrease in TL in oocytes. In particular, it has been shown by Keefe et al. [32, 33] that oocytes from women who did not conceive after in vitro fertilization had shorter telomeres compared to those who did. In addition, oocytes from cycles that produced fragmented embryos also had shorter telomeres.

In summary, shortened telomeres were already associated with pathological processes, unsuccessful fertilization, aging, and death. In this regard, here we estimated the relative telomere length in spontaneously aborted human embryos at 5–12 weeks of gestation (w.o.g). In addition, the results of this pioneering study were interpreted taking into account the presence of chromosomal anomalies.

**Methods**

In this work, we have studied relative telomere lengths (RTL) in 80 chorionic villi samples (CVS) from spontaneously eliminated product of conception at 5–12 weeks of gestational age (spontaneous abortions - SA) and from 25 induced abortions (IA) due to personal reasons at the same term of gestation. All studied pregnancies occurred naturally. Maternal age ranged from 20 to 34 years.

**Collection of chorionic villi samples**

A total of 105 chorionic villi samples (CVS) at 5–12 weeks of gestation were obtained from pregnant women, 80 of them from spontaneous abortions with 12 weeks of gestation were obtained from pregnant women, 80 of them from spontaneous abortions with 12 weeks of gestation (spontaneous abortions - SA) and from 25 samples from induced abortions (IA) due to personal reasons at the same term of gestation. All studied pregnancies occurred naturally. Maternal age ranged from 20 to 34 years.

All CVS samples were tested for absence of maternal decidua by histological analysis. Samples were washed twice with PBS and stored at −20 °C until DNA extraction.

**DNA extraction**

Genomic DNA was extracted using salting-out or phenol/chloroform method, and dissolved in Tris-EDTA pH 8.0. DNA concentration and purity was assessed using Qubit® 2.0 Fluorometer (Thermo Fisher Scientific). All DNA samples were stored at −20 °C until telomere length analysis.

**RTL measurement by real-time PCR**

Relative telomere length was measured from total genomic DNA using a real-time PCR assay [34]. PCR reactions were performed in the Eco Real-Time PCR System (Illumina, Inc), using 1X GoTaq® qPCR Master Mix (Promega) and specific primers (Table 1) in recommended concentrations [34].

Two reaction mixes of PCR reagents were prepared, one for amplification of telomere repeats (T), the other for gene 36B4 (S). Each PCR reaction contained 12 μl of the reaction mix (GoTaq® qPCR Master Mix and primers) and 7 μl (14 ng/ aliquot) DNA. Tel1 and tel2 primers for telomeres were added to the final concentrations 270 nM and 900 nM, respectively. The final 36B4 gene primer concentrations were: 36B4u - 300 nM and 36B4d - 500 nM. For each sample in whom T/S ratio was measured, tree identical aliquots of DNA were added to plate T (telomere) and another tree aliquots were added to the same well positions in plate SCG (single copy gene). Reference DNA was included into each PCR run.

The reference DNA for measurement standardization was obtained from whole blood of two healthy individuals (male and female). Standard curves for telomere length and for the single copy gene were generated by performing serial dilutions (dilution factor 1.68) from a reference DNA sample to produce concentrations of DNA ranging from 0.63 to 5 ng/μl in two parallel reactions – one for the telomeric sequences and the other for the reference gene (36B4) (Fig. 1). The data obtained from reference DNA measurement were used to setup calibration curves needed to estimate reaction efficiency and the average RTL. The relative ratio of telomere repeats copy number to 36B4 gene copy number (T/S ratio) in experimental samples were compared to the reference DNA sample. Telomere length was expressed as a relative T/S ratio, based on the calculation of the ΔCt [Ct(telomero)/Ct(single gene)] value, which was normalized to the average T/S ratio of the reference sample [2^(-ΔCt(sample)-ΔCt(control))].

Illumina Eco Software v4.1.11.2 was used to generate curves for the telomere signal (T) or the single copy

| Table 1 List of specific primers used for determination of telomere length |
|----------------------------------------|
| Primer name | Sequence (5’-3’) |
| Tel 1 | GGTTTTGGAGGGTGGGTAGGGTTTTGAGGGGTGGT |
| Tel 2 | TCCCCATATCCCTATCCCTATCCCTATCCCTAT |
| 36B4u | CAGCAAGTGGGAAGGTGTAATCC |
| 36B4d | CCCATTCTATCATCAACGGGTACAA |
gene signal (S) and to determine quantity of DNA for our research.

The coefficient of variation (standard deviation/mean) was calculated to be 0.89% for within plate measurements and 0.77% for measurement between plates.

Statistical analysis
The data acquired from RLT measurement was normally distributed (Shapiro-Wilk test, \( p = 0.00001 \)). Statistical differences between means were tested with ANOVA and t-test using Statistica 12 (StatSoft, Inc., USA).

Results
The average RTL in the combined group was 0.60 and was characterized by inter-individual variations within the range of 0.03 to 2.94. In the IA group, the average RTL was 1.17 with inter-individual variations ranging from 0.14 to 2.94. In the main SA investigated group, the mean of RTL was 0.43 with inter-individual variations ranging from 0.03 to 2.40 (Table 2).

As indicated in Table 3, the aneuploid SA was characterized by shorter telomeres in comparison to the euploid SA with interindividual variations from 0.07 to 1.66 and from 0.03 to 2.40, respectively. Statistical analysis showed a highly significant difference in RTL between SA with aneuploid and SA with euploid karyotype (aneuploid: 0.29 ± 0.03 vs euploid: 0.64 ± 0.12, \( P = 0.0015 \)) (Fig. 3).

Further, we compared RTL in SA with different types of aneuploidy - trisomy chromosome 21, trisomy chromosome 16, triploidy, and monosomy X (Table 4). The results showed no significant variation in RTL length among the various aneuploid groups (monosomy X: 0.36 ± 0.12; trisomy 21: 0.26 ± 0.03; trisomy 16: 0.28 ± 0.04; and triploidy: 0.27 ± 0.02, \( P > 0.05 \)) (Fig. 4). Consequently, the telomere

![Fig. 1 Standard curves used for calculation of relative DNA concentrations of telomeres (a) and 36B4 (b)](image)

![Fig. 2 Relative telomere length in chorionic villus samples. 1 – artificial abortions; 2 – spontaneous abortions](image)

Statistical analysis showed that RTL were significantly lower in SA group compared to IA group (SA: 0.43 ± 0.06 vs IA: 1.17 ± 0.14, \( P = 0.000001 \)) (Fig. 2).

The SA group was further divided into subgroups depending on the karyotype. Molecular cytogenetic (interphase mFISH with centromeric probe panels for chromosomes 13, 21, 14, 22, 15, 16, 17, 18, X and Y) and cytogenetic studies showed the following results in this group: 32 cases with euploid karyotype (46,XX or 46,XY) and 48 with aneuploid karyotype (autosomal trisomy 21 or 16, triploidy, monosomy X). Variability of the mean RTL for SA with or without chromosome number abnormalities are shown in Table 3.

### Table 2 Relative telomere length ratios in spontaneous and artificial abortions overall and within each group

| Overall | Spontaneous abortions | Artificial abortions |
|---------|------------------------|----------------------|
| Mean (max-min) | n | Mean (max-min) | n | Mean (max-min) | n |
| 0.60 (0.03–2.94) | 105 | 0.43 (0.03–2.40) | 80 | 1.17 (0.14–2.94) | 25 |

n- number of samples

### Table 3 Relative telomere length ratios in spontaneous abortions with euploid or aneuploid karyotype

| Overall | Euploid karyotype | Aneuploid karyotype |
|---------|-------------------|---------------------|
| Mean (max-min) | n | Mean (max-min) | n | Mean (max-min) | n |
| 0.43 (0.03–2.40) | 80 | 0.64 (0.03–2.40) | 32 | 0.29 (0.07–1.66) | 48 |

n- number of samples
length does not depend on the kind of aneuploidy in CVS of spontaneously lost pregnancies.

It should be noted that aneuploidy was not detected in IA group. In fact, euploid karyotype (46,XX or 46,XY) was found in all 25 samples. Therefore, we compared relative telomere length between euploid SAs and IAs and observed a highly significant difference in RTL (0.64 ± 0.12 and 1.17 ± 0.14 respectively, \( P = 0.006 \)). These results indicate that the shortening of telomeres seems to play a role in the early human intrauterine interruption of further growth and development (Fig. 3).

In addition, aware of the possibility of contamination of embryonic material with maternal cells, we compared the groups only with male karyotypes (Table 5, Fig. 5, Fig. 6). A total of 48 male embryos were examined: 14 - IA, 15 - euploid SA, 19 - aneuploid SA. Apparently, a significant difference is maintained when comparing the XY-euploid IA to XY-euploid SA embryos (\( P = 0.0012 \)) and even increased (\( P = 0.00002 \)) when comparing IA to aneuploid SA, further strengthening our hypothesis.

**Discussion**

Based on our and data obtained by others [35–46], it can be stated that telomere length is characterized by interindividual variability both in prenatal and in the postnatal human development. To date, very few studies focus on telomere length in the early stages of human prenatal development. Therefore, our study provides a unique example of studying RTL in early (5–12 w.o.g.) prenatal development and evaluate it according to anamnesis (SA and IA) and in association with aneuploid (autosomal trisomy, monosomy X and triploidy) and euploid karyotype.

Our results show, with a high degree of reliability (\( P = 0.000001 \)), that spontaneously lost pregnancies are characterized by short telomeres in comparison to induced abortions. Therefore, we report a strong correlation between telomere length and the viability of embryos. In particular chronic stress in pregnant women is associated with short telomeres in posterity [41, 43, 44]. In addition, a lower maternal folate concentration in early pregnancy is associated with shorter telomeres in the newborn [45]. Genomic instability is commonly found in newborns with short telomeres, which increases the risk of cancer and age-related diseases [46]. Our data shows significantly shorter RTL in spontaneously eliminated embryos with aneuploid (with trisomy of chromosome 21 or 16, triploidy and monosomy X) karyotype, regardless of the type of aneuploidy. Similar results were shown [30, 31] for aneuploid oocytes and embryos at the cleavage stage, although the telomere length was aligned at the blastocyst stage. The results of studies of telomere length in newborns with trisomy 21 (Down syndrome) are conflicting - from the claim of shortening [47] or lack of a likely difference in telomere length [48] to a

![Fig. 3](image-url) Relative telomere length in artificial and spontaneous abortions with or without aneuploidy. 1 – spontaneous abortions with aneuploidy; 2 – euploid spontaneous abortions; 3 – euploid artificial abortions

![Fig. 4](image-url) Relative telomere length in spontaneous abortions with different aneuploidies. 1 – monosomy X; 2 – trisomy 21; 3 – trisomy 16; 4 - triploidy

| Table 4 | Relative telomere length ratios in spontaneous abortions with different form of aneuploidy |
|---------|--------------------------------------------------|
| Monosomy X | Trisomy 21 | Trisomy 16 | Triploidy |
| Mean (max-min) | n | Mean (max-min) | n | Mean (max-min) | n | Mean (max-min) | n |
| 0.36 (0.08–1.66) | 12 | 0.26 (0.11–0.35) | 10 | 0.28 (0.07–0.49) | 13 | 0.27 (0.11–0.37) | 13 |

n - number of samples

| Table 5 | Relative telomere length ratios in chorionic villus samples with Y-chromosome |
|---------|--------------------------------------------------|
| Spontaneous abortions with euploid karyotype | Spontaneous abortions with aneuploid karyotype | Artificial abortions |
| Mean (max-min) | n | Mean (max-min) | n | Mean (max-min) | n |
| 0.37 (0.30–1.13) | 15 | 0.24 (0.07–0.49) | 19 | 1.36 (0.14–2.94) | 14 |

n - number of samples
probable elongation of telomeres in newborns with trisomy 21 versus newborns without chromosomal anomalies [49]. Similarly, ambiguous results are obtained in adults with monosomy X (Turner syndrome) [50], which indicate rather that there is no difference in the length of telomeres in Turner syndrome cells compared to individuals without chromosomal abnormalities.

**Conclusion**
Spontaneously lost pregnancies are characterized by shortened telomeres, especially in embryos with aneuploidies. We hypothesize that the shortening of telomeres is involved in the complex elimination machinery leading to early embryo death.

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**Abbreviations**

CVS: Chorionic villi samples; IA: Induced abortions; RTL: Relative telomere length; RT-PCR: Real-time polymerase chain reaction; S: Single copy gene; SA: Spontaneous abortions; T: Telomere repeat; TL: Telomere length; w.o.g: Weeks of gestation

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**Availability of data and materials**

The datasets analyzed during the current study are available from the corresponding author upon a reasonable request.

**Authors’ contributions**

NH, DZ - study concepts. IT, NH - collected of samples. NH, MT – performed measuring of RTL, data analyzes and interpretation. NH, IT, DZ, MT - manuscript preparation. All authors read and approved the final manuscript.

**Ethics approval and consent to participate**

The study was approved by the bioethics committee of the Institute of Hereditary Pathology NAMS of Ukraine. Informed consent was obtained from all the participants.

**Consent for publication**

Not applicable.

**Competing interests**

The authors declare that they have no competing interest.

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