Impact of superovulation and in vitro fertilization on LINE-1 copy number and telomere length in C57BL/6 J mice blastocysts

Thalita S. Berteli1,2,3 · Fang Wang1 · Fabiana B. Kohlrausch1,4 · Caroline M. Da Luz2,3 · Fernanda V. Oliveira2 · David L. Keefe1 · Paula A. Navarro2,3

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Abstract

Objective

Millions of babies have been conceived by IVF, yet debate about its safety to offspring continues. We hypothesized that superovulation and in vitro fertilization (IVF) promote genomic changes, including altered telomere length (TL) and activation of the retrotransposon LINE-1 (L1), and tested this hypothesis in a mouse model.

Material and methods

Experimental study analyzing TL and L1 copy number in C57BL/6 J mouse blastocysts in vivo produced from natural mating cycles (N), in vivo produced following superovulation (S), or in vitro produced following superovulation (IVF). We also examined the effects of prolonged culture on TL and L1 copy number in the IVF group comparing blastocysts cultured 96 h versus blastocysts cultured 120 h. TL and L1 copy number were measured by Real Time PCR.

Results

TL in S (n = 77; Mean: 1.50 ± 1.15; p = 0.0007) and IVF (n = 82; Mean: 1.72 ± 1.44; p < 0.0001) exceeded that in N (n = 16; Mean: 0.61 ± 0.27). TL of blastocysts cultured 120 h (n = 15, Mean: 2.14 ± 1.05) was significantly longer than that of embryos cultured for 96 h (n = 67, Mean: 1.63 ± 1.50; p = 0.0414). L1 copy number of blastocysts cultured for 120 h (n = 15, Mean: 1.71 ± 1.49) exceeded that of embryos cultured for 96 h (n = 67, Mean: 0.95 ± 1.03; p = 0.0162).

Conclusions

Intriguingly ovarian stimulation, alone or followed by IVF, produced embryos with significantly longer telomeres compared to in vivo, natural cycle-produced embryos. The significance of this enriched telomere endowment for the health and longevity of offspring born from IVF merit future studies.

Keywords

Mice blastocysts · L1 copy number · Telomeres · IVF · Superovulation

Introduction

Telomeres are non-coding, repetitive DNA sequences of TTAGGG located at the termini of linear chromosomes [1]. It protects the end of chromosomes by forming single strands that invade the telomeric double helix to create loops, called t-loops. Together with associated proteins, telomeres prevent chromosome ends from being recognized as double-stranded DNA breaks, thus maintaining chromosomal stability [2]. With each cell division, telomere length (TL) shortens due to the inability of the DNA polymerase enzyme to complete synthesis if the lagging strand at chromosomal ends [3]. When cells divide, they cannot replicate approximately 50 base pairs of the lagging DNA strand at chromosome ends [4]. In most cells, this limits the number of divisions that a cell can tolerate because when telomeres reach a critical size, senescence and programmed cell death occur [5].

After fertilization, TL is reset in mice and humans. Mice oocyte telomere length is surprisingly short but significantly lengthens between the zygote and 2-cell stages [6]; despite the minimal telomerase activity, the enzyme capable of reconstituting telomere repeats [6, 7]. Maximum telomere shortening occurs in utero and during the first year of life (presumably due to the rapid cell division), then slows in
of p53 creates a permissive environment in which critically
apoptotic responses to dysfunctional telomeres [26]. Loss
[26]. P53 plays a critical role in enforcing senescence and
response and activation of the tumor suppressor protein p53
[26]. Impaired telomere function triggers DNA damage
or oxidative damage compromises chromosome end protec-
tion [26]. At the molecular level, critically short telomeres
trigger a DNA damage response that leads to senescence or
aging and aging-associated diseases in mice and humans
[17], decreased chiasmata and synapsis [17], and infertil-
ity [18]. At the molecular level, critically short telomeres
serve as a substrate for the retrotransposition of L1, independent of the endonuclease. This pathway
appears to be linked to L1 endonuclease loss. Transposition
to telomeres is increased in cells that express a dominant
negative allele (TRF2) that disrupts TL [29].
Because both TL and L1 are exquisitely sensitive to
environmental perturbation and essential for early murine
embryogenesis [30, 31] we questioned whether superovu-
lization (IVF) could alter TL and/or activation of L1 in mouse blastocysts. We compared L1 copy number and TL among C57BL/6 J mice blastocysts
produced in vivo from natural mating cycles (N), in vivo following superovulation (S), or in vitro following superovulation (IVF). We also examined the effects of prolonged culture on TL and L1 in the IVF group.

**Material and methods**

**Mouse strain and animal handling**
C57BL/6 J female mice (N = 50) at 8 weeks of age and
C57BL/6 J males (N = 20) with confirmed fertility [32] were
maintained in the Animal Care Facility of the University
of São Paulo before experiments. During this period, the
animals were kept on a 12-h light–dark cycle, controlled
temperature, with water and feed ad libitum, following animal care guidelines.

**Superovulation protocol**
For superovulation, mice were injected intra-peritoneally
with 5 IU of equine chorionic gonadotropin (eCG; Novo-
ron—Syntex SA—Buenos Aires, Argentina) followed
48 h later by 5 IU of human chorionic gonadotropin (hCG;
Chorulon—Syntex SA, Buenos Aires, Argentina).
Experimental design

To investigate effects of superovulation and in vitro fertilization (IVF) on TL and L1 copy number of mice blastocysts, three groups were studied: (1) Natural (N) group: To eliminate the effect of ovarian stimulation and in vitro fertilization on outcomes, blastocysts were produced in vivo from females in the natural cycle (N); (2) Stimulated (S) group: Blastocysts produced in vivo after ovarian stimulation (S); and (3) IVF group: Blastocysts produced in vitro from stimulated cycles using fresh oocytes (IVF)—(Fig. 1, Table 1). All IVF procedures were performed in the Experimental Laboratory of the Human Reproduction Sector of the Clinical Hospital of the Ribeirao Preto Medical School, University of Sao Paulo (HCFMRP/USP) and the PCR experiments were performed at Department of Obstetrics and Gynecology, New York University, Langone Medical Center, New York, NY, United States.

Natural mating group

Male mice with confirmed fertility were mated overnight with one female (estrous positive) and vaginal plugs were recorded at 0.5-day post-coitum. Euthanasia was performed by cervical dislocation and blastocysts were collected by uterus flushing approximately 74 h later using Embryomax M2 medium (Millipore sigma). Blastocysts were washed 3 times in HTF homemade medium and fixed in 2ul PVP/PBS and kept at -80°C until analysis.

Ovarian stimulation and in vivo or in vitro fertilization

Female mice were injected intra-peritoneally with 5 IU of equine chorionic gonadotropin (eCG; Novormon—Syntex SA—Buenos Aires, Argentina) followed by 5 IU of human chorionic gonadotropin (hCG; Chrorulon—Syntex SA, Buenos Aires, Argentina), 48 h later. Male mice with confirmed fertility were mated overnight with one female (estrous positive) and vaginal plugs were recorded at 0.5-day post-coitum. Euthanasia was performed by cervical dislocation and blastocysts were collected by uterus flushing approximately 74 h later using Embryomax M2 medium (Millipore sigma). Blastocysts were washed 3 times in HTF homemade medium and fixed in 2ul PVP/PBS and kept at -80°C until analysis.

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Fig. 1 Experimental design: Three groups were defined related to their type of conception. Group N is the control, naturally conceived group. Group S represents the embryos that were derived from hormonally stimulated oocytes (eCG, hCG), and that had undergone natural fertilization and in vivo development. Group IVF is the in vitro fertilization group including superovulation plus collection of oocytes, IVF and embryo culture. Embryos were collected at blastocyst stage of development in all of three experimental groups. 74 h is an estimate of embryo development time and collection since we are not able to know precisely the mating time in the in vivo groups (N and S).
fertility were mated overnight with one female and euthanized for blastocysts collection and fixation were performed as described above in natural mating group. To investigate the effect of S plus in vitro fertilization (IVF group), epididymis were isolated, washed in M2 medium and punctured, and spermatozoa were capacitated for 1–2 h at 37°C, 5% CO2 in HTF homemade medium. Simultaneously, cumulus-oocyte cell complexes (COCs) were liberated from the oviducts of superovulated females using M2 medium and immediately transferred to other plate with pre-equilibrated HTF homemade medium covered by mineral oil (Irvine Scientific) at 37°C; 0.8 mM EDTA, 2% NP-40; 5 mM dithiothreitol (DTT)) to a PCR micro tube and heated at 75°C for 10 min in the “cool down step” protocol for room temperature for 5 min. A single-cell TL assay (SC-qPCR) [34] was used to measure TL and L1 copy number in mouse blastocysts. A key feature of this assay is a pre-amplification step, performed before quantitative polymerase chain reaction (qPCR). Primers were synthesized by Integrated DNA Technologies according to the assay described below.

Briefly, pre-PCR was performed using DNA Polymerase Hot Start Version (TAKARA). The reactions were set up by aliquoting 21 μL of master mix into the PCR micro tubes containing 2 μL of mouse blastocyst genomic DNA. Each reaction was set up with 2.5 μL 10× PCR buffer, 2.5 μL 2.5 mM dNTP, 0.16 μL DNA polymerase, 0.5 μL each of telomere forward and reverse primer (10 μM, TeloF: 5′ CCGTTTGTGTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTG
Finally, the purified products from each blastocyst were aliquoted into each well of a 96-well plate to perform the quantitative real-time PCR. Each reaction included 10 μL 2× SYBR Green mix (Bio-Rad), 1 μL each of 10 μM forward and reverse primers used in the pre-amplification step, 4 μL molecular grade water and 5 μL genomic DNA to yield a 20-µL reaction. DNA samples were placed in a 96-well plate for telomere primers and reference gene primers, L1 primers and reference gene primers, respectively. A Bio-Rad thermocycler (CFX96) was used with reaction conditions of 95 °C for 10 min followed by 40 cycles of data collection at 95 °C for 15 s, 60 °C annealing and extension for 1 min). Each sample was run in triplicate along with a target-specific non-template control (NTC). Mouse embryonic stem cells were used as positive controls in each plate to assure concordance between plates. After thermal cycling and DNA amplification, CFX manager software was used to generate standard curves and Ct values for mouse telomere and reference gene (mB1) signals. For L1 copy number mouse 5srDNA was used as reference gene. To ensure high reproducibility of samples, only assays with real-time PCR efficiencies between 95 and 105% and intra-assay coefficient of variation (CoV) less than 1% were included in the analysis.

**Statistical analysis**

Following log transformation, analysis of variance with Tukey post-test compared TL and L1 among the 3 groups. Students t test compared TL and L1 between embryos cultured for 120 vs. 96 h in the IVF group. P-value < 0.05 was considered significant. Analyses were performed with SAS 9.4.

**Results**

**In vitro fertilization and development of embryos derived from fresh oocytes (IVF group)**

In the IVF group, 10 replicates produced a fertilization rate of 90.52% (95% CI 85.19–95.85), blastocyst formation rate at 96 h of 61.90% (95% CI 52.62–71.19) and cumulative blastocyst rate (96 plus 120 h) of 76.19% (CI 68.04–84.34).

**Comparison of telomere length in mouse blastocysts from N, S and IVF group**

TL of blastocysts from N group (n = 16; Mean: 0.61 ± 0.27) was significantly shorter than S group (n = 77; Mean: 1.50 ± 1.15; p = 0.0007) and IVF group (n = 82; Mean: 1.72 ± 1.44; p = 0.0001). TL in S group (n = 77; Mean: 1.50 ± 1.15) did not differ from the IVF group (n = 82; Mean: 1.72 ± 1.44; p = 0.3237; power of the test: 84%; Fig. 2).

**Comparison of L-1 copy number in mouse blastocysts from N, S and IVF group**

L1 copy number of blastocysts from N group (n = 16; Mean: 0.80 ± 0.31) did not differ from S (n = 77; Mean: 1.23 ± 0.75; p = 0.1386) and IVF group (n = 82; Mean: 1.09 ± 1.16; p = 0.6709, Fig. 3).

**Comparison of telomere length and L-1 copy number in mouse in vitro produced blastocysts from 96 and 120 h of development**

TL of blastocysts recovered at 96 h (n = 67, Mean: 1.63 ± 1.50) was significantly shorter than that of embryos recovered at 120 h of embryo culture (n = 15, Mean: 2.14 ± 1.05, p = 0.0414, Fig. 4A). L1 copy number of blastocysts recovered at 96 h (n = 67, Mean: 0.95 ± 1.03) was significantly lower than that of embryos recovered at 120 h
Discussion

This study shows that ovarian hyperstimulation, alone or followed by IVF, produced blastocysts with significantly longer telomeres than in vivo, natural cycle-produced embryos. In addition, telomere length and L1 copy number of in vitro produced embryos cultured for 120 h exceeded those from embryos cultured for 96 h.

Mammalian oocytes have short telomeres [6]; however, following fertilization, at the two-cell stage, the length of the telomeres increases drastically [6], while telomerase activity remains undetectable in these cells. This increase did not come from the sperm, since telomeres elongate even in oocytes developing parthenogenetically. Besides, this effect was also observed in telomerase knockout mice, suggesting that an ALT-dependent mechanism plays an important role in mammalian preimplantation development [36]. TL in early-stage embryos is shorter than in the blastocyst stage. While telomerase-independent, recombination-based elongation of telomeres occurs during early pre-implantation development, the telomerase activity increases drastically in the blastocyst stage [36]. The most significant degree of telomere elongation occurs in the inner cell mass, the pluripotent cells that give rise to the embryo [37].

What is known about telomere dynamics during this period is largely limited to observational studies, both in mice and humans, especially due to the ethical limitations of using human embryos for research. However studies analyzing human embryos from assisted reproductive technologies show shorter telomeres in oocytes of women who do not conceive following IVF compared to those who conceived [38] and in oocytes from cycles generating fragmented embryos [16]. Besides, aneuploid blastomeres and polar bodies have shorter telomeres than euploid blastomeres from the same IVF patient and cycle [39].

To the best of our knowledge this is the first report of effects of ovarian hyperstimulation and IVF on genomic architecture during early development. Schaetzlein et al. reported that TL is determined at the morula to blastocyst transition by a telomerase-dependent mechanism. Telomere elongation is restricted to this stage of development and restores telomeres of fibroblast-derived cloned bovine embryos to normal length [40]. Analyzing bovine pre-implantation embryos they found a similar TL in morula derived from in vivo and in vitro fertilization suggesting that the in vitro culture per se had no significant effect on TL [40].

In contrast, in mice blastocysts, we report that ovarian hyperstimulation, alone or followed by IVF, produced embryos with significantly longer telomeres compared to in vivo, natural cycle-produced embryos. Ovarian hyperstimulation was the main factor responsible for these changes, since no statistical difference was found in TL between S and IVF group (p: 0.3237; power of the test: 84%). Telomerase activity is low during the fertilization stage, while methylation is highly active. Telomerase activity is high during the blastocyst stage, while methylation is low, which suggests inverse similarities of telomerase and the methylation—de-methylation cycle [41]. Previously superovulation was associated with abnormal methylation in mice embryos from superovulated compared to non-superovulated female mice [42]. And an extended embryo culture has been associated with imprinting disorders owing to abnormal methylation and de-methylation cycle [41]. Therefore, considering that

Fig. 4 Logarithmic distribution of TL and L1 copy number in mouse in vitro produced blastocysts recovered at 96 or 120 h of embryo culture in IVF group. The groups (120 h and 96 h) were compared statistically with each other *p < 0.05; n is the number of blastocysts included in each group
IVF steps could impact methylation and de-methylation process it may have similar effect in TL in early embryos when the methylation changes are marked and the methylation control over retrotransposons is reinstated. Different ART steps, such as superovulation [43], IVF and ICSI [44], embryo culture [45], components in the culture media [46] could affect the most important period of epigenetic reprogramming.

In our study, TL in blastocysts cultured for 120 h was significantly longer than that of embryos cultured for 96 h and L1 copy number of blastocyst cultured for 120 h exceeded that of embryos cultured for 96 h, demonstrating that extending in vitro embryo culture had an effect not only on TL but also on inducing an increase in L1 copy number.

Barbara McClintock’s Nobel Prizing winning research previously identified activation of retrotransposons as a response to stress [47]. A derivative hypothesis is that stress from ovarian hyperstimulation [48–50] and/or embryo culture activates transcription of L1. Our findings that prolonged embryo culture increased L1 copy number are consistent with Professor McClintock’s model. Intriguingly, however, embryo culture at earlier stages and ovarian hyperstimulation did not affect L1 copy number in mouse embryos, but did elongate telomeres. These two findings could be related. Recently we demonstrated a role for L1 in telomere elongation in preimplantation embryos [51]. Also, in cultured cells activation of retrotransposition leads to de novo L1 insertions at chromosome ends [52]. Activation of retrotransposition and integration of retroelements at telomeres has been reported in cells that survive mutations in telomere function [53, 54]. *Drosophila* use retrotransposons rather than telomeric repeats to protect chromosome ends [55]. *Drosophila* lack telomerase, but three telomere-specific retrotransposons maintain chromosome ends [56]. Future studies should elucidate the mechanisms underlying telomere elongation induced by ovarian superovulation and the potential role of L1 in this effect.

Most in vitro experiments suggest that oxidative stress accelerates telomere shortening [57–59], but telomere elongation also has been reported. The intensity of the stress may determine its effect on TL [60]. Mishra et al. reported disruption of normal telomere interactions, leading to loss of the looped chromosomal configuration [60]. In 112 infertile men, seminal ROS and 8-isoprostane levels were increased compared to controls. TL measured by real time PCR correlated positively with reactive oxygen species (ROS) levels, consistent with mild oxidative stress as a cause of telomere shortening. Severe oxidative stress, on the other hand, shortened sperm TL.

Superovulation and in vitro fertilization had no significant effect on L1 copy number in our study, but extended embryo culture duration increased L1 copy number. Carmignac et al., also found no effect of hyperstimulation on TE transcription around the preimplantation period, but in vitro culture increased TE expression at the blastocyst stage compared to in vivo development. By covering the transmission and mobilization of a transgenic L1 transposon, they also found that in vitro fertilization may alter the Mendelian rate of paternal heritage [61]. In vitro culture had an impact genome-wide on the embryo cultured in amino-acid-poor medium such as M16, showing an aggravet upregulation of TEs compared to amino acids rich medium (KSMOM) [61]. Changes in expression levels from ovarian hyperstimulation also were observed in some L1 subfamilies (*LI type f* and *LI type tf*) at the blastocyst stage [61].

While low-dose hormone did not affect the L1 methylation levels, superovulated mouse females treated with high-dose hormone showed decreased L1 methylation levels in blastocysts [62], suggesting that superovulation may disturb the balance between methylation and demethylation on L1. The mechanisms involved in this process need further evaluation.

Millions of babies have been born following IVF, yet debate continues about its safety to offspring. We found genomic effects of ovarian stimulation and prolonged in vitro embryo culture in mice, including telomere elongation and L1 activation. Since both TL and TEs can modulate gene expression, and show unique regulatory dynamics during the preimplantation period [61], the consequences of ovarian hyperstimulation and IVF for embryonic gene expression merit further study. Future studies should not only validate our findings using other methodologies to examine TL and L1 activation, but also examine the effects of these changes on TL and TE activity on longevity and cancer risk in IVF offspring.

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**Data availability** The data underlying this study will be shared on reasonable request to the corresponding author.

**Code availability** Not applicable.

**Declarations**

**Conflict of interest** The authors declared no conflict of interest.

**Ethical approval** This study was approved by the Faculty of Medicine of Ribeirão Preto Ethics Committee on the Use of Animals, under the number CEUA-FMRP/USP-107/2017.
Consent to participate Not applicable.

Consent for publication The author agree to its submission to the Molecular Biology Reports Journal and, if accepted, to its publication in this journal. We warrant that this article is original, does not infringe on any copyright or other proprietary right of any third party, is not under consideration by another journal and has not been previously published. Ethical approval has been sought and obtained as necessary and any conflicts of interest stated.

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