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Does the Cryogenic Freezing Process Cause Shorter Telomeres?

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Abstract

We have observed evidence of increased telomere shortening in short-term T-lymphocyte cultures following freezing and thawing of the original inoculum obtained by ficoll-paque gradient centrifugation, compared to T-lymphocytes that were cultured immediately without freezing and thawing from the same blood sample from 3 female and 3 male adults. Because freezing may have similar effects on other cell types, and because telomere shortening may only manifest its effects after many years or decades, we suggest there is a pressing need for evaluation of the effects of freezing on any cells envisioned for clinical applications, including embryo implantation.

Keywords

telomere shortening; cryogenics; FISH; PNA probe; telomere “loss”; T-lymphocytes; short-term cultures; embryos; stem cells

Telomeres, highly conserved TTAGGG repeats on the end of chromosomes, become shorter with each subsequent cell cycle, eventually leading to the inability to replicate [10]. Reduced telomere length has been associated with a number of specific conditions in previous studies, including replicative cellular senescence and apoptosis [1], tumorigenesis, *in vivo* cellular aging [9], heart disease, stress [5], dyskeratosis congenita, Alzheimer’s Disease, and dementia and mild cognitive impairment in Down Syndrome [7].

During the course of one of our previous studies [6], we made what we thought was a minor change in the cell culture protocol by freezing mononuclear leucocytes obtained via ficoll-paque gradient centrifugation until age- and sex-matched samples became available for comparison in parallel short-term PHA (phytohemagglutinin) cultures. When the frozen mononuclear leucocyte samples were thawed and cultured, we noticed a loss in magnitude

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Conflict of Interest Statement

The authors of this article have no conflicts of interest regarding this study, financial or otherwise.

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of signal following hybridization of metaphase chromosomes with a PNA telomere probe . This potentially indicated loss of telomere length. Based on these observations (data unpublished), we hypothesized that the freezing process may result in shorter telomeres.

We have now tested that hypothesis by obtaining whole blood samples (10 ml), under an IRB-approved protocol, from 3 female and 3 male healthy volunteers ranging in age from 39–68 years. Each sample was evenly divided, with half cultured immediately [mononuclear cells obtained via ficoll-paque gradient centrifugation in PHA-containing culture medium for 4 days at 37°C]. The remainder was cultured in exactly the same way following placement of the suspended cells in 2ml of cold freezing medium [10% DMSO (dimethylsulfoxide) and 90% fetal bovine serum] and frozen in 1 ml aliquots. The suspension was frozen at a rate of $-1^{\circ}\text{C}/\text{min}$ in the gaseous phase of liquid nitrogen using either a BF-5 Biological Freezer (Union Carbide) or a Handi-Freeze (Taylor Wharton), and placed in liquid nitrogen for approximately one week, thawed rapidly in a 37°C water bath, and rinsed several times before culturing in PHA-containing medium . Table 1 shows a striking increase [by a factor of about 3 times (t-test p values for all comparisons were $<10^{-6}$)] in signal loss, by tabulating the mean numbers of chromosome arms with no signal in 20 metaphases per sample (see Fig. 1). We replicated these findings by measuring fluorescence (using a Zeiss Axioskop 2 plus, fluorescence microscope) light intensity values in interphase preparations (also hybridized by the PNA telomere probe) using a MetaSystems image analyzer. These findings are also summarized in Table 1. Figs. 1a and 1b are metaphase and interphase cells obtained from short-term T lymphocyte cultures from the same individual both before (Fig. 1a) and after freezing (Fig. 1b).

We believe our observations of telomere shortening as a result of freezing may have wide-ranging implications across the depth and breadth of cryobiology, including the cryopreservation of: embryonic stem cells [2], human mesenchymal stem cells, hematopoietic stem cells, human induced pluripotent stem cells, amniotic fluid stem cells, oocytes [3], cartilage cell and tissue for biobanking, animal and human sperm [4, 11], and plant embryogenic cells [8]. Of course, our initial effort was focused exclusively on T-lymphocytes, and these findings may not generalize to other cell populations. However, given that telomere shortening would be expected to have delayed effects on cell functioning and cell line longevity, and given that freezing is broadly employed as a means of preserving cells for future use, including human embryos, the present findings suggest a pressing need for evaluation of the effects of freezing on any cells envisioned for post-freezing use including implantation.

Our paper raises many questions that need to be addressed by additional study including: the effect of repeat freezing on telomeres; the degree of telomerase activation after thawing; the effect of freezing on actual telomere length measured with more direct quantification; and the effect on maximum passage number.

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Fig. 1A

Fig. 1B

Figure 1.

Metaphase and Interphase FISH preparations from the same blood sample cultured before (Fig. 1a) and after (Fig. 1b) freezing. Fig. 1a. Metaphase (interphase nuclei also visible) obtained from short-term T-lymphocyte culture of mononuclear cells (from a male donor) obtained by ficoll-paque gradient centrifugation and immediately (no freezing) cultured at 200,000–400,000 cells/ml in PHA-containing medium at 37°C for four days. Metaphase and interphase preparations were hybridized with a DAKO telomere PNA probe, labeled by FITC (yellow-green) and DAPI (blue) counterstained. Five chromosome arms have no signal in this figure. The five telomeres with no PNA signal are marked by red “dots.” Fig. b. Similar to Fig. 1a except cells from the same sample were frozen in liquid nitrogen before short-term T lymphocyte culture and 15 chromosome arms had no signal versus 5 in Fig. 1a. The 15 telomeres with no PNA signal are marked by red “dots.”

Table 1

The mean number of chromosome arms with no signal (MNCANS) increased strikingly when leucocytes from the same sample were frozen before culture, in all cases (t-test p values < 10^{-6}). When interphase light intensity values from samples that were cultured immediately versus the same samples that were frozen before culturing were compared, strikingly reduced light intensities, reflecting telomere shortening, were observed in all cases (t-test p values < 10^{-6}).

Case No. and Sex	MNCANS (S.D.) ^I		Interphase x 10 ⁴ light intensity units(S.D.)	
	Unfrozen	Frozen	Unfrozen	Frozen
1F	5.5(1.8)	18.3(2.5)	12.6(3.0)	4.5 (0.9)
2 F	6.1(1.6)	17.9(2.8)	15.0(4.1)	4.1(1.0)
3 F	6.5(1.3)	18.9(1.8)	13.3(3.5)	3.7(0.6)
4 M	6.4(1.4)	17.9(2.4)	12.8(2.7)	3.05(1.0)
5 M	6.5(1.4)	19.8(2.6)	12.8(3.5)	4.3(1.4)
6 M	6.4(1.5)	19.7(2.1)	13.1(2.0)	4.2(0.8)

^I Mean number of chromosome arms with no signal from 20 metaphases per culture (S.D. = standard deviation)