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The Effects of Aspirin and N-3 Fatty Acids on Telomerase Activity in Adults with Diabetes Mellitus

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Abstract

Type 2 Diabetes mellitus is associated with aging and shortened telomere length. Telomerase replaces lost telomeric repeats at the ends of chromosomes and is necessary for the replicative immortality of cells. Aspirin and the n3 fatty acids eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) are commonly used therapies in people with type 2 diabetes for reducing cardiovascular disease events, though their relation to telomerase activity is not well studied. We explored the effects of aspirin, EPA+DHA, and the combined effects of aspirin and EPA+DHA treatment on telomerase activity in 30 adults with diabetes mellitus. EPA and DHA ingestion alone increased telomerase activity then a decrease occurred with the addition of aspirin consumption. Crude (F-stat=2.09, $p=0.13$) and adjusted (F-stat=2.20, $p=0.14$) analyses of this

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decrease showed signs of a trend. These results suggest that aspirin has an adverse effect on aging in diabetics who have relatively high EPA and DHA ingestion.

Keywords

Omega-3 fatty acids; eicosapentaenoic acid; docosahexaenoic acid; aspirin; telomerase; telomere

Background

Type 2 diabetes is a prevalent high cardiovascular risk condition¹ commonly associated with aging and shortened telomere length.² Telomeres, the termini of chromosomes, are composed of hexameric repeating units of DNA (TTAGGG) with associated proteins.^{3,4} Their purpose is to protect and maintain the integrity of genomic information. As cells undergo division, telomeres shorten, losing a small number of base pairs from the terminal sequence of chromosomes, but ultimately protecting essential information. This process continues throughout life until a critical limit is reached, at which point cell death occurs.⁵ It has been hypothesized that telomere shortening results in cellular aging, where the function of telomerase is to maintain the length of telomere, effectively enabling cells to continue to replicate, preventing aging effects. Telomerase is a complex ribonucleoprotein reverse transcriptase enzyme that functions to replace lost telomeric repeats at the ends of chromosomes^{3,6} by synthesizing and directing the telomeric repeats onto the end of existing telomeres using its RNA component.^{6,7} Thus, telomerase activity is necessary for replicative immortality of cells. Telomere length and telomerase activity have been of interest as potential biomarkers for age-related conditions such as cardiovascular conditions, though results have been mixed.^{8,9} Research suggests that telomere shortening may be accelerated by inflammation and oxidative stress.¹⁰ Further, shortened telomere length and increased oxidative stress have been described in patients with diabetes.¹¹ The relationship between telomere length and telomerase activity and the presence of type 2 diabetes has been inconclusive, though a pooled meta-analysis did find support for a relationship.^{2,12}

Anti-inflammatory and antioxidant agents, such as aspirin have demonstrated the ability to alter the rate of telomeric shortening¹³ and studies on factors which may influence oxidative stress such as physical activity, BMI, diet, smoking, and alcohol use have demonstrated associations with telomere length.² Aspirin ingestion may exert antioxidative effects, protecting cells from endothelial cell oxidative damage via the Nitric Oxide-cGMP Pathway.¹⁴ Research on telomere length and use of aspirin has found inconsistent associations between telomere length and aspirin use. It is possible that of the studies demonstrating an association, the mechanism may occur as a result of reducing inflammation, thereby reducing oxidative stress, specific to the patient population under study.

Like aspirin, EPA and DHA ingestion (often through marine oil intake), is also a commonly used cardio-protective measure and beneficial in metabolic syndrome with regards to insulin sensitivity and oxidative stress.^{15,16} The antioxidative effects of omega-3 fatty acids marine may influence telomerase activity and telomere length through their anti-inflammatory and antioxidative effects. Evidence suggests that omega-3 PUFA are able to inhibit inflammatory

mechanisms. Several studies have demonstrated increases in telomerase levels and telomere length following omega-3 supplementation.¹⁷ An additional study notes that omega-3 PUFA may reduce oxidative stress and inflammation, positively influencing telomere length.¹⁸

In short, oxidative stress potentially leads to accelerated telomere shortening, implicated in type 2 diabetes development.¹⁹ This may be particularly relevant to earlier stage metabolic conditions such as pre-diabetes, where interventions could aim to slow the progress of cell senescence, thereby influencing the severity and progression. The current study seeks to examine the effects of ingesting aspirin, EPA+DHA and the combined effects of both on telomerase activity in an attempt to provide future hypothesize pathways for future investigation.

Participants and Protocol

As previously described, this was an 8-week sequential-therapy unblinded clinical trial conducted at the University of Rochester's Clinical Research Center.²⁰ Participants were 30 adults aged 40 to 80 years with type 2 diabetes mellitus. All participants ingested non-enteric 81 mg aspirin and a dose of 4000 mg of over-the-counter fish oil with the mean concentrations of DHA and EPA in the study capsules of 406 ± 42 mg/ml and 330 ± 46 mg/ml, respectively. The clinical trial timeline and 6 phlebotomies for each subject is outlined in Table 1.²⁰ Each phlebotomy corresponds to acute and chronic dosing timepoints of aspirin, aspirin and fish oil, and just fish oil (following an aspirin wash-out). This study was approved by the Human Subjects Review Board at the University of Rochester.

Telomerase Activity

Relative telomerase activity (copies) was measured by using the TRAPeze Telomerase Detection Kit (Millipore S7710, EMD Millipore, Temecula, CA, USA)³. Telomerase activity is expressed as "copies" here, based on the linear standard curve generated by "TSR8" provided in the kit. TSR8 is an oligonucleotide with a sequence identical to the TS primer extended with 8 telomeric repeats, permitting the calculation of the amount of TS primers with telomeric repeats extended by telomerase in the sample. Data generated for the TSR8 standard curves includes the TSR dilutions (amoles), corresponding copies of TSR8, log values, and the experimental average count, as described in the manual. Telomerase activity is then calculated according to the generated standard curve (average Ct vs log copy number, Figure 1).²¹ Based on the standard curve the results were transformed and expressed as "copies".^{21,22}

Peripheral blood mononuclear cells were separated from blood, and the cellular protein extracts were prepared in CHAPS lysis buffer provided in the kit and stored at -80°C until used in the TRAP assay. Nano drop was used to measure the protein concentrations. For the TRAP assay, we used the Realplex Sequence Detection System (Eppendorf, Hauppauge, NY, USA). The reaction mixture consisted of 5 μl 5X TRAPeze RT Reaction Mix, 50 X TITANIUM Taq DNA polymerase (Clontech, Merck, Darmstadt, Germany) and 17.6 μl PCR-grade water and 2 μl sample for a total reaction volume of 25 μl . The PCR protocol started with the following cycling parameters with an extension of telomerase substrate for

30 min at 30°C, 2 min at 95°C, and extension of PCR amplification for 45 cycles for 15 seconds at 94°C, 1 min at 59°C and 20 seconds at 45°C. A TSR8 control template serially diluted was used as a standard curve.

Telomerase activity (copies) is shown by the amount of TRAP products generated. In the first step of the reaction, the telomerase enzyme present in the sample adds a number of telomeric repeats (TTAGGG) onto the 3'-end of a substrate oligonucleotide (TS). In the second step, the extended products are amplified by the Taq polymerase, using RT-PCR. The activity of each sample was detected by using fluorescence energy transfer (ET) primers generating fluorescently labeled TRAP products. This method allowed for detection and quantification of telomerase activity since the fluorescence emission produced is directly proportional to the amount of TRAP products generated.

Statistical Analyses

Baseline characteristics of participants were reported using relative frequencies for categorical measures and means and standard deviations for continuous measures. The distribution of telomerase activity, defined as copies, was examined and was approximately normally distributed. Telomerase copy number was analyzed using the linear mixed-effects model (LMM) and the generalized estimating equations (GEE) to examine the treatment effects at each blood draw, controlling for blood draw, age, systolic and diastolic blood pressure (BP), statin use, and baseline telomerase activity (copies).²³ In the LMM, the unstructured correlation option was used to impose less constraint on within-subject variations of telomerase copies. GEE was used to ensure valid inference since it requires no mathematical distribution such as normality, allowing for a broader class of data distributions. When discrepancies arose between the two models, GEE results were reported. Values for blood draws 2 through 6 were compared individually to baseline draw 1 using p-values, with $p \leq 0.05$ considered statistically significant. Data were also evaluated for trends where apparent using crude (without controlling for covariates) and adjusted (controlling for covariates) methods. All statistical procedures were done using the Statistical Analysis Software (SAS) version 9.3 (SAS Institute, Inc., Cary, NC).

Results

Details of participant demographics were previously reported.²⁰ In summary, participants had an average age of 56.6 years, half (50.0%) were male, mostly white (56.7%), and with at least a high school education (80.0%). Most participants were never smokers (63.3%), did not consume alcohol (76.7%), ate fish less than once per week (69.9%), and used metformin to control their diabetes (83.3%). Baseline mean systolic and diastolic blood pressures were 132.9 and 76.0, respectively, and mean BMI was 34.6. Figure 2 illustrates the effects of ingesting aspirin alone, fish oil alone, and aspirin+fish oil on telomerase activity (expressed as copies), relative to baseline. Overall, results from LMM indicate that no significant effects were observed when comparing phlebotomies 2 ($p=0.85$), 3 ($p=0.32$), 4 ($p=0.26$), 5 ($p=0.82$), and 6 ($p=0.34$) individually to baseline. After 28 days of fish oil ingestion alone, telomerase copies increased by 1760.9 units relative to baseline, then exhibited a time-dependent decrease to 2106 units below baseline with the addition of aspirin consumption.

Crude (F-stat=2.09, $p=0.13$) and adjusted (F-stat=2.20, $p=0.14$) analyses of this decrease showed signs of a trend. As GEE yielded quite similar and consistent results, they were not reported.

Discussion

The current study evaluated telomerase activity associated with aspirin and fish oil supplementation in 30 patients with type 2 diabetes mellitus. A non-statistically significant increase in telomerase activity (expressed as copies) after 28 days of fish oil ingestion is consistent with reduced oxidative stress effects. The ingestion of low-dose aspirin along with fish oil led to a reduction in telomerase, which trended toward being significant, whereas no similar trend was observed when aspirin was ingested before fish oil. The current study provides support for a reduction in disease progression or consequences through fish oil supplementation or related fatty acids but does not support the use of aspirin to obtain such effects, especially in light of possible bleeding risks. These results may be useful for future studies informing interventions for diabetic, pre-diabetic patients, or patients with other metabolic conditions, particularly with regards to nutrition.

A study conducted by Kiecolt-Glaser, et al. randomized 106 healthy sedentary overweight middle-aged and older adults to 2.5g/day n-3 PUFAs, 1.25g/day n-3 PUFAs, or placebo capsules that mirrored the proportions of fatty acids in the typical American diet. Results did not reveal any significant differences in telomerase or telomere length changes between groups but did suggest an inverse relationship between telomere length and n-6 (omega-6):n-3 fatty acid ratio.¹⁸ Farzaneh-Far, et al. have shown, in a cohort of patients with coronary artery disease (~25% had type 2 diabetes), an inverse relationship between baseline blood levels of EPA and DHA and the rate of telomere shortening over 5 years.²³ We have not found any other studies that have investigated the effect of n3 fatty acid and/or aspirin ingestion on telomerase in humans with diabetes mellitus. Thus, these results provide additional evidence to a thin body of literature, in a population where such work may be particularly relevant.

A major limitation is the small sample-size, lowering statistical power to find differences. In addition, it should be noted that this study was conducted using stored blood from a previous study, which was short in duration. As such, we were unable to look at changes in telomerase activity (expressed as copies) over a longer period of time, though our results comparing acute (post 4hrs ingestion) and chronic (7 day ingestion) do not seem to indicate activity. Still, it is possible that over time such supplementation could yield changes we were not able to assess, particularly when considering ageing. Strengths include the statistical approach taken to look for a trend across interventions. The dose of EPA and DHA consumed altered the plasma concentrations of these n3 fatty acids significantly.²⁰

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Highlights

- Diabetes mellitus is associated with aging and shortened telomere length
- Aspirin and the n3 fatty acids are commonly used therapies yet their relationships to telomerase activity are understudied
- An observed increase in telomerase activity following omega-3 fatty acid treatment and then a reduction after aspirin ingestion is consistent with altering the oxidative stress paradigm
- The current study provides support for reduction in disease consequences through fatty acid supplementation

Figure 1.
legend – Standard curve for log copy number and average threshold cycle

Figure 2.

The effects of aspirin alone (BD2–3), fish oil alone (BD4), and aspirin + fish oil (BD5–6) relative to baseline (BD1, as zero) on telomerase copies. Data are expressed as mean change from baseline with standard error bars.

Table 1.

Study timeline of activities

	Day	Study Visit	Activity
	1		<ul style="list-style-type: none"> • Recruitment and screening • Collection of baseline demographic and clinical data
	1–10		<ul style="list-style-type: none"> • Aspirin-free period of 10 days prior to Study Visit 1
Aspirin only	11	1	<ul style="list-style-type: none"> • Blood draw 1 (baseline, before aspirin) • Ingestion single 81 mg dose aspirin • Blood draw 2 (4 hours post aspirin ingestion)
	12–17		<ul style="list-style-type: none"> • Single 81 mg aspirin/day
Fish oil only	18	2	<ul style="list-style-type: none"> • Blood draw 3 (7 days aspirin ingestion) • Begin fish oil 4g/day and discontinue aspirin unless subject takes it as prescribed by their doctor • 28 days of fish oil 4g/day
	19–45		<ul style="list-style-type: none"> • Aspirin-free period of 10 days prior to Study Visit 3
	36–45		
Aspirin + fish oil	46	3	<ul style="list-style-type: none"> • Blood draw 4 (4 weeks fish oil ingestion, before aspirin) • Continue fish oil and a single dose 81 mg aspirin • Blood draw 5 (4 hours post aspirin + fish oil ingestion)
	47–52		<ul style="list-style-type: none"> • Continue aspirin and fish oil 4g/day
	53	4	<ul style="list-style-type: none"> • Blood draw 6 (7 days aspirin + fish oil ingestion) • Discontinue fish oil and aspirin