International Journal of Biology Research
ISSN: 2455-6548; Impact Factor: RJIF 5.22
Received: 19-06-2019; Accepted: 22-07-2019
www.biologyjournal.in
Volume 4; Issue 4; September 2019; Page No. 84-91

Effect of Lutein (Lute-gen®) on proliferation rate and telomere length *in vitro* and possible mechanism of action

S Mehkri[1], Diego Perez[2], Pilar Najarro[3], Menelaos Tsapekos[4], KN Bopanna[5*]

[1] Bio-gen Extracts Pvt. Ltd., 57, Sompura Industrial Area, Dobaspet, Bangalore, Karnataka, India

[2, 3, 4] Life Length, C/Miguel Ángel, 11, Madrid, Spain

[5] Consultant Pharmacologist, 9b Sobha Emerald Jakkur, Bangalore, Karnataka, India

Abstract

Background: Many studies have reported that lutein could exert its biological activities, including anti-inflammation, anti-oxidative and anti-apoptosis, through its effect on reactive oxygen species (ROS). Thus, lutein may prevent the damaging effect of ROS in cells.

Telomere length is one of the most important biomarkers of aging. It is known that oxidative stress can accelerate telomere shortening, whereas antioxidants like lutein can delay their attrition through their antioxidant activity.

Aim of the study: This study was conducted to assess the effect of Lute-gen® (Lutein) on telomere length and cellular proliferation rate in cultures of human adult primary fibroblast cells grow under standard or oxidative stress conditions.

Method: The current study investigated the effect of Lute-gen® on telomere length. For this purpose, a primary cell line was treated with different concentrations of Lute-gen® (10; 5; 1 µg/ml) in the presence or absence of the pro-oxidant hydrogen peroxide (H_2O_2). Different concentrations of lutein were included along with positive (H_2O_2) and negative (no treatment) controls.

Determination of the telomere shortening rate along with the evaluation of the median telomere length, 20th percentile length and the percentage of telomeres below 3 kilo base pairs (Kbp) was performed using an optimized, analytically validated HT-Q-FISH methodology.

Results: Lute-gen was observed to exert a significant protective effect on telomere length erosion *in vitro* under oxidative stress conditions after eight passages.

Conclusions: Lute-gen's effect on telomere attrition indicates that the antioxidant activity of lutein is beneficial and counteracts the effects of oxidation on telomeres in human primary cells. The results grant further investigation in vivo, on a controlled human clinical trial context. The use of *in vitro* modelling is beneficial to investigators developing natural products with anti-oxidant claims such as lutein and their effect on ageing.

Keywords: oxidant, counteracts, modelling, telomeres

Introduction

In human populations, telomere length is a biomarker of aging for a whole organism and a biomarker of aging in specific tissues [1].

The contribution to oxidation and/or inflammation to telomere shortening and hence aging requires longitudinal studies in which individuals, monitored over decades have to be followed. This approach requires time course determination of telomere length, in well-characterized populations that measure social, behavioural, medical, and biological factors.

As cells age, they lose a certain number of base pairs, it is estimated that roughly 50 base pairs are lost during cell division because of the end-replication problem [15]. When telomeres have decreased to a critical length, cell division ceases, although cell senescence may continue for a time. This finite ability to replicate is known as the "Hay flick limit," and has been seen in normal cultured mammalian cells. Summarily, each time a cell divides, telomeres get shorter. When they get too short, the cells become "senescent" and eventually dies. Cell senescence is associated with aging and this could be due to ROS or oxidative stress [2, 5].

In vitro studies conducted in primary cells to determine the role of lutein (xanthophyll carotenoid) and its action on telomere length are required to investigate its mechanism of action. Here we focus on Lute-gen® and its effect on ROS. In order to establish the effect of oxidative stress induced in the presence of hydrogen peroxide was used as a pro-oxidant. Cellular proliferation rate and telomere length were measured in cultures of human adult primary fibroblast cells treated with or without lutein under both standard and oxidative stress conditions.

Materials and methods

Lute-gen® (Lutein) is manufactured from the *Tagetes erecta* species of non-GMO marigold flowers using a patent-pending manufacturing process. Lute-gen® is standardized for Lutein content by HPLC and is manufactured in a GMP, ISO and HACCP certified manufacturing facility. The formulation of Lute-gen is done with the use of natural ingredients only, including natural vitamin E from sunflower that serves as an antioxidant. The ingredient is Kosher and Halal certified, free of residual solvents, soy-free and dairy-free, thereby making it suitable for all population groups.

Telomere analysis technology (TAT®)

All telomere length measurements were performed using Life Length's proprietary technology TAT. TAT is a robust and reproducible high-throughput quantitative fluorescent *in situ* hybridization (HT Q-FISH) technology that allows measuring telomere length in individual chromosomes in interphase cells. TAT has significant advantages over other telomere testing methodologies. TAT is able to determine telomere length in absolute units (base pairs – bp), report the distribution and 20th percentile of telomere length and inform of the % of short telomeres, allowing a more comprehensive analysis of each sample. Each test sample is plated in 5 replicates per plate to achieve statistical significance. Fine tuning of the methodology has accomplished high level of reproducibility; CV < 5% in inter-plate repetitions.

Cell culture

Primary dermal fibroblasts; Normal, human, adult (HDF_a) (ATCC® PCS-201-012™) were used for this study. Cells were seeded at $2x10^3$ cells/cm^2 in Gluta Max ^{TM} high glucose Dulbecco's modified Eagle's medium (DMEM, Gibco.) and supplemented with 10% fetal bovine serum (FBS, HyClone, Thermo Scientific), penicillin (100 U/ml) and streptomycin (1000 U/ml). Media was renewed every 2-3 days and cells passaged at sub-confluence (70-80%) every seven days. Cell growth was monitored for each condition by counting cell numbers at each passage using a Countess™ cell counter (Invitrogen). Population doubling (PD) was calculated with the formula PD = (log (Nn/Nn−1))/log $_2$ where n is the passage; and N the number of cells. One PD is equivalent to one round of cell replication. After passage four and eight, cells treated with Lute-gen® ® and controls were frozen in liquid nitrogen until their use.

Treatments

Human primary fibroblasts were treated with Lute-gen® during eight weeks at three different concentrations (10, 5 and 1 µg/ml) under standard and oxidative (10 µM H_2O_2) cell culture conditions. Fresh treatments were prepared and added to the cells at every passage and also when media was renewed. Stock solution of 10 mg/ml of the compound was prepared in DMSO and the different treatments were prepared from that stock. The final concentration of DMSO was 0.5% at which no detrimental effect on cell growth or toxicity was detected (data not included).

HT - Q Fish

Cell were thawed at 37º C and cell counts and cellular viability were determined. Cells were seeded in clear bottom black-walled 384-well plates at 12,000 cells per well in five replicates for each sample and eight replicates for each control cell line. Telomere length was measured by the High Throughput Quantitative Fluorescence *In Situ* Hybridization (HT-Q-FISH) technology (Canela *et al*., 2007) [6] on interphase nuclei. The assay was conducted according to quality standards of the Clinical Laboratory Improvement Amendments (CLIA) and ISO15189. Each telomere is hybridized with a fluorescent telomeric probe (PNA) that recognizes a fixed number of telomeric sequences (bp). The fluorescence intensity of the telomeric probes is directly proportional to telomere length.

Image acquisition and processing

The OPERA High Content Bioimager (Perkin Elmer) was used for automated image acquisition in combination with the acapella software, version 1.8 (Perkin Elmer). Images were captured, using a 40 x 0.95 NA water immersion objective. UV and 488 nm excitation wavelengths were used to detect the DAPI and A488 signals respectively. With constant exposure settings, 15 independent images were captured at different positions for each well. Cells were stained with DAPI to facilitate autofocus of the microscope and to define the region of interest for each cell, measuring telomere fluorescence intensity of the A488 image in each one.

Data analysis

An image algorithm was applied to allow cell nucleus segmentation based on a local threshold. The intensity results for each foci were exported to the Columbus 2.4 software (Perkin Elmer).

As mentioned above the fluorescence intensity from the telomeric PNA probe that hybridize to a given telomere is proportional to the length of that telomere. These intensities were translated to base pairs through a standard regression curve generated using control cell lines of known telomere length.

Quality control analysis in each run includes an intra-plate standard curve and 5 replicates per sample. The coefficient of variation (CV) of the technique is < 5%.

Results

Proliferative Analysis

In order to determine the effect of Lute-gen on cellular proliferation human primary fibroblasts were treated with Lute-gen® at three different concentration (10, 5; and 1 µg/ml) during a period of eight weeks. Cumulative population doubling (CPD) in standard conditions did not present differences between the control group and groups treated with Lute-gen®, except in the case of cells treated at 5 µg/ml, that showed a lower CPD compared to the rest of the groups after six weeks (Fig 1).

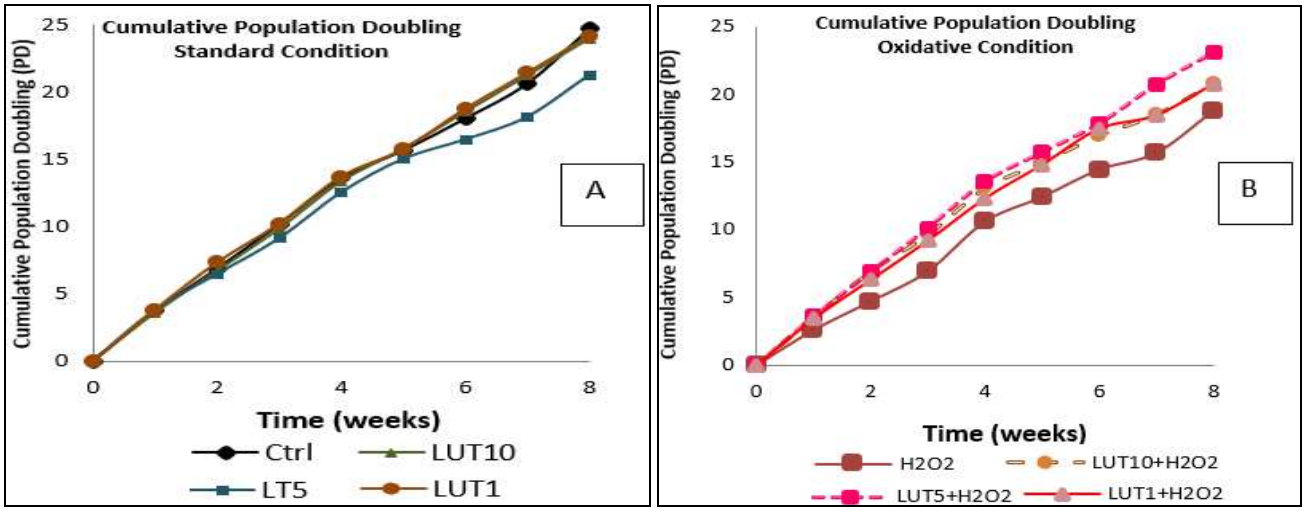


Fig 1: Growth curves of human adult primary fibroblasts untreated (ctrl-DMSO), treated with Lutein (10.0, 5.0 and 1.0 µg/m) under standard and oxidative (10µM H_2O_2) condition. Each point on the population curve represents one passage.

Telomere Length measurements - Standard Conditions

A time course assay was conducted such that fibroblast cells expanded under standard conditions as described in the method section were detached from the cell culture flasks at zero and then after four weeks and eight weeks. Samples were frozen and analyzed at the end of the incubation period. Data obtained following HT-Q-FISH analysis for each of the variables determined are presented in the graphs in Figure 2.

Compared to control attrition rates, after 4 weeks of treatment with lutein at different concentrations we observed a, statistically significant shortening in the median telomere length variable for group treated with Lute-gen® at 1 µg/ml. Additionally, significant telomere shortening was identified in the 20th percentile length variable, between the control group and the group treated with Lute-gen® at 5 µg/ml.

Following 8 weeks of treatment with lutein at 10 and 1 µg/ml, statistically significant differences were detected in the 20th percentile length as well as in the % of telomeres < 3 kbp variables when compared to the control group. The statistical analysis and their p-values are summarized in Tables 1-3.

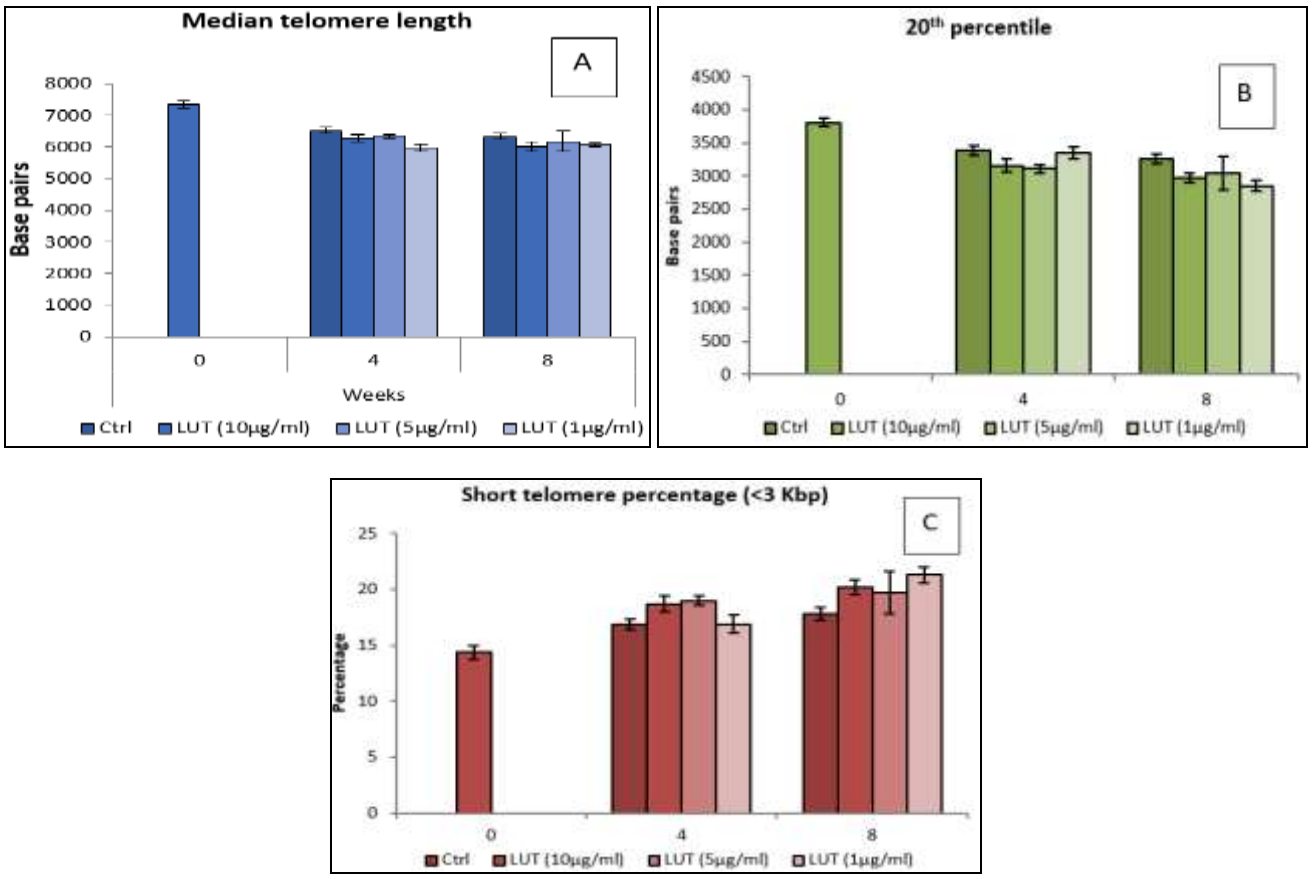


Fig 2: Figure shows bar graphs for all variables measured; median telomere length (A), 20th percentile length (B) and percentage of short telomeres (<3 kbp) (C) in standard condition

Table 1: Statistical analysis and p-value for median telomere length at different time points for each treatment compared to untreated, paired samples. Significant differences: *p<0.05; **p<0.01, ***p<0.001, ****p<0.0001.

Two-Way ANOVA	Median Telomere Length			
Passage 4	Mean 1	Mean 2	Mean Difference	Significance
Ctrl vs. LUT10	6515	6272	243	No
Ctrl vs. LUT5	6515	6340	175	No
Ctrl vs. LUT1	6515	5984	531	Yes (****)
Passage 8	Mean 1	Mean 2	Mean Difference	Significance
Ctrl vs. LUT10	6333	6008	326	No
Ctrl vs. LUT5	6333	6177	157	No
Ctrl vs. LUT1	6333	6071	263	No

Table 2: Statistical analysis and p-value for 20th percentile length at different time points for each treatment compared to untreated, paired samples. Significant differences: *p<0.05; **p<0.01, ***p<0.001, ****p<0.0001.

Two-Way ANOVA	20th percentile telomere length			
Passage 4	Mean 1	Mean 2	Mean Difference	Significance
Ctrl vs. LUT10	3393	3160	232	No
Ctrl vs. LUT5	3393	3110	282	Yes (*)
Ctrl vs. LUT1	3393	3356	36	No
Passage 8	Mean 1	Mean 2	Mean Difference	Significance
Ctrl vs. LUT10	3259	2970	289	Yes (*)
Ctrl vs. LUT5	3259	3044	216	No
Ctrl vs. LUT1	3259	2847	412	Yes (***)

Table 3: Statistical analysis and p-value for percentage of telomeres < 3kbp at different time points for each treatment compared to untreated paired samples. Significant differences: *p<0.05; **p<0.01, ***p<0.001, ****p<0.0001.

Two-Way ANOVA	% short telomeres (<3 kbp)			
Passage 4	Mean 1	Mean 2	Mean Difference	Significance
Ctrl vs. LUT10	16,9	18,7	-1,8	No
Ctrl vs. LUT5	16,9	19,0	-2,1	No
Ctrl vs. LUT1	16,9	16,9	0,0	No
Passage 8	Mean 1	Mean 2	Mean Difference	Significance
Ctrl vs. LUT10	17,9	20,2	-2,4	Yes (*)
Ctrl vs. LUT5	17,9	19,7	-1,8	No
Ctrl vs. LUT1	17,9	21,3	-3,5	Yes (***)

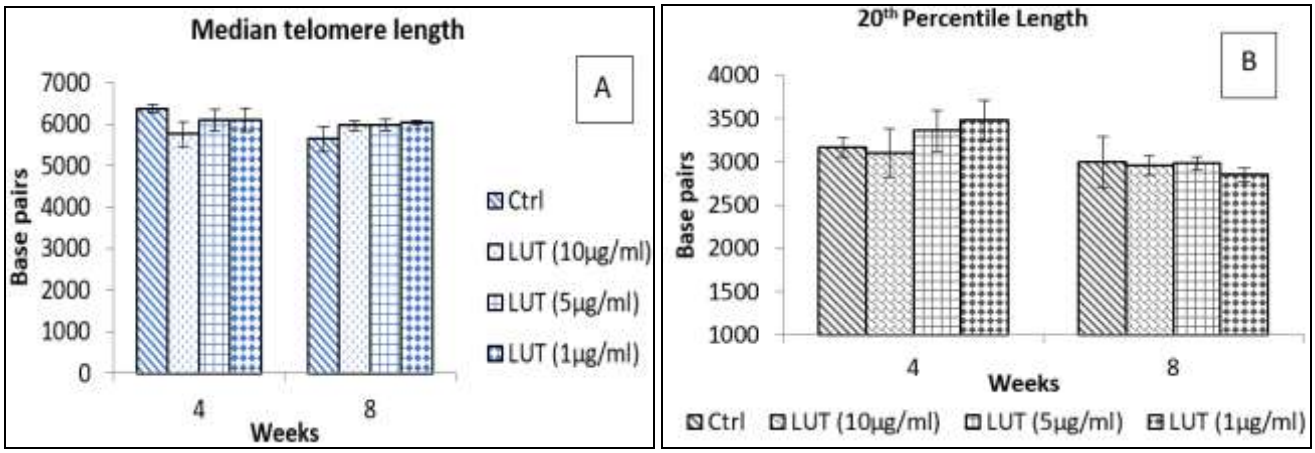
Telomere Length measurements–Oxidative Stress Conditions

We wanted to determine the effect of oxidative stress in telomere length attrition in primary fibroblasts cultures and evaluate the rate of telomere shortening of the same cultures in the presence of lutein. This is more/less/equal than the total shortening observed in the absence of H_2O_2 (Fig 2 A).

Following treatment under oxidative conditions in the presence or in the absence of lutein telomere length variables were determined at 4 and 8 weeks. Data for all variables determined are presented in the graphs in Figure 3.

Telomere length measurement obtained in oxidative conditions alone, compared to lutein treated at 4 weeks were found to be lower in the later (Table 4). Concomitantly an increase can be observed in the 20th percentile as well as a decrease in the percentage short telomeres (<3 kbp) (Tables 5 and 6).

After 8 weeks of treatment with Lute-gen® statistical differences were observed in the median telomere length values. All Lute-gen® treated groups (10, 5 and 1 µg/ml) presented a higher telomere length compared to control (Table 4).



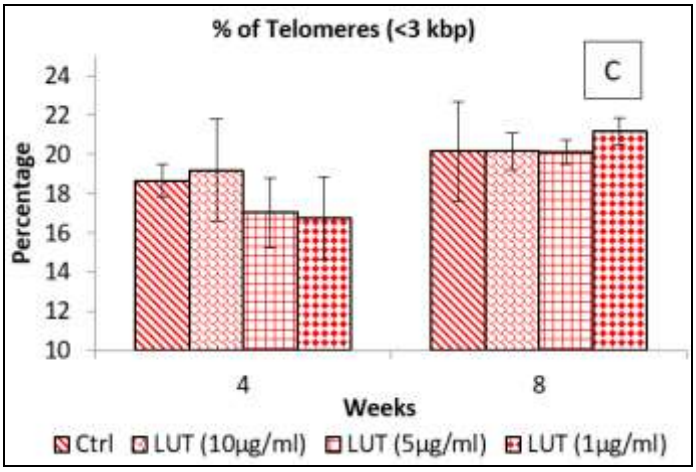


Fig 3: Figure shows bar graphs for all variables measured; median telomere length (A), 20th percentile length (B) and percentage of short telomeres (<3 Kbp) (C) in oxidative condition

Table 4. Statistical analysis and p-values for median telomere length at different time points for each treatment compared to the oxidative paired sample. Significant differences: *p<0.05; **p<0.01, ***p<0.001, ****p<0.0001.

Two-Way ANOVA	**Median Telomere Length**			
Passage 4	Mean 1	Mean 2	Mean Difference	Significance
H2O2 vs. LUT10+H2O2	6360	5758	602	Yes (****)
H2O2 vs. LUT5+H2O2	6360	6085	275	No
H2O2 vs. LUT1+H2O2	6360	5980	380	Yes (*)
Passage 8	Mean 1	Mean 2	Mean Difference	Significance
H2O2 vs. LUT10+H2O2	5636	5972	-336	Yes (*)
H2O2 vs. LUT5+H2O2	5636	5980	-344	Yes (*)
H2O2 vs. LUT1+H2O2	5636	6045	-409	Yes (**)

Table 5. Statistical analysis and p-values for 20th percentile length at different time points for each treatment compared to the reference paired sample. Significant differences: *p<0.05; **p<0.01, ***p<0.001, ****p<0.0001.

Two-Way ANOVA	**20th Percentile Telomere Length**			
Passage 4	Mean 1	Mean 2	Mean Difference	Significance
H2O2 vs. LUT10+H2O2	3166	3106	60	No
H2O2 vs. LUT5+H2O2	3166	3359	-193	No
H2O2 vs. LUT1+H2O2	3166	3371	-205	No
Passage 8	Mean 1	Mean 2	Mean Difference	Significance
H2O2 vs. LUT10+H2O2	2998	2964	34	No
H2O2 vs. LUT5+H2O2	2998	2983	14	No
H2O2 vs. LUT1+H2O2	2998	2854	144	No

Table 6. Statistical analysis and p-values for percentage of telomeres < 3kbp at different time points for each treatment compared to the reference paired sample. Significant differences: *p<0.05; **p<0.01, ***p<0.001, ****p<0.0001.

Two-Way ANOVA	**% short telomeres (<3 kbp)**			
Passage 4	Mean 1	Mean 2	Mean Difference	Significance
H2O2 vs. LUT10+H2O2	18,65	19,19	-0,5401	No
H2O2 vs. LUT5+H2O2	18,65	17,01	1,639	No
H2O2 vs. LUT1+H2O2	18,65	16,77	1,882	No
Passage 8	Mean 1	Mean 2	Mean Difference	Significance
H2O2 vs. LUT10+H2O2	20,15	20,14	0,00514	No
H2O2 vs. LUT5+H2O2	20,15	20,1	0,05202	No
H2O2 vs. LUT1+H2O2	20,15	21,15	-1,005	No

Telomere shortening rate

Due to the fact that cell replication is one of the principal causes of telomere shortening, the telomere length measurements performed were normalized by the population doubling levels (cell replication) for each condition and time point. The telomere shortening rate was calculated using the following formula; *Median telomere length (initial-final) / Population Doubling.*

After 8 weeks under oxidative cell culture conditions (10 µM-H_2O_2), a decrease in telomere shortening was observed (Figure 4 B). This decrease was observed in all concentrations tested and was detected as significant by the statistical analysis (Table 7).

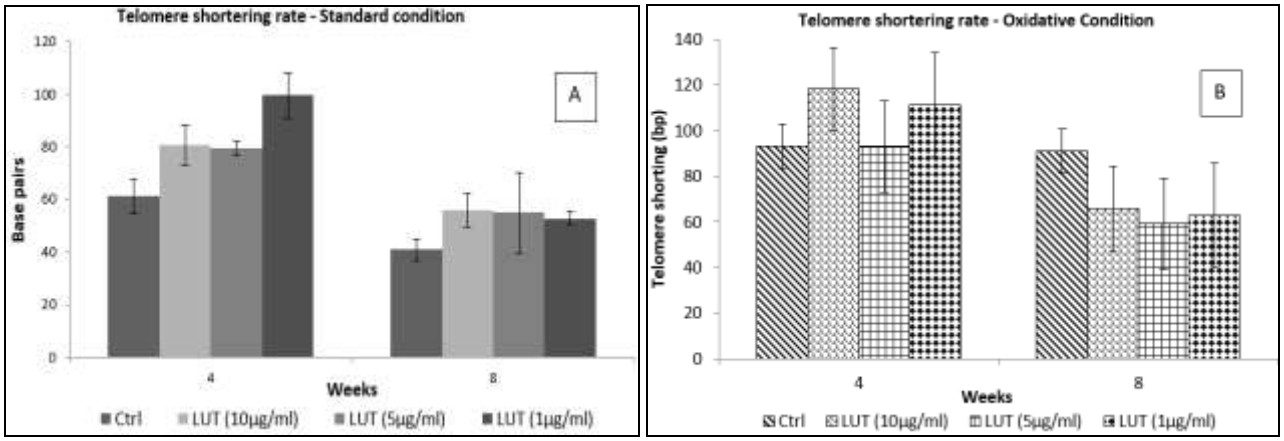


Fig 4: The bar chart below displays the average telomere shortening rates of the median telomere length of each condition (control and treated groups), in standard (A) and oxidative (B) condition

Table 7. Statistical analysis p-value for telomere shortening rate at different time points for each treatment compared to the reference paired sample. Significant differences: *p<0.05; **p<0.01, ***p<0.001, ****p<0.0001.

	Telomere shortening rate – Normal Condition			
Passage 4	Mean 1	Mean 2	Mean Difference	Significance
Ctrl vs. LUT10	61	81	-20	No
Ctrl vs. LUT5	61	80	-19	No
Ctrl vs. LUT1	61	100	-38	Yes (****)
Passage 8	Mean 1	Mean 2	Mean Difference	Significance
Ctrl vs. LUT10	41	56	-15	No
Ctrl vs. LUT5	41	55	-14	No
Ctrl vs. LUT1	41	53	-12	No

	Telomere shortening rate – Oxidative Condition			
Passage 4	Mean 1	Mean 2	Mean Difference	Significance
H2O2 vs. LUT10+H2O2	93	118	-25	Yes (*)
H2O2 vs. LUT5+H2O2	93	93	0	No
H2O2 vs. LUT1+H2O2	93	109	-15	No
Passage 8	Mean 1	Mean 2	Mean Difference	Significance
H2O2 vs. LUT10+H2O2	91	66	25	Yes (*)
H2O2 vs. LUT5+H2O2	91	59	32	Yes (***)
H2O2 vs. LUT1+H2O2	91	63	28	Yes (**)

Discussion & Conclusion

This study aimed to investigate the effect of lutein in telomere length during cell culture of primary cells using two general classifications: with and without the pro-oxidant hydrogen peroxide (H_2O_2). Different concentrations of Lutein were included along with positive (H_2O_2) and negative (no treatment) controls.

The findings of the study reveal that oxidative stress decreased proliferation capacity and increased telomere shortening rate in cultures of human primary fibroblast. For all treated groups in standard conditions a significant increase in telomere shortening rate was observed after LUT1 treatment. For all treated groups in oxidative culture conditions the observations were that a significant difference in the telomere shortening rate was identified between LUT10+H_2O_2, LUT5+H_2O_2 and LUT1+H_2O_2 compared to control (H_2O_2) after 8 passages.

Numerous studies have shown that oxidative stress is associated with accelerated telomere shortening and dysfunction [16, 17]. Due to the antioxidant properties of Lute-gen both normal and oxidative stress conditions were included in the study design in order to investigate if the compound exerts a stronger telomere protective effect under oxidative stress conditions.

According to the data from the study Lutein has a significant protective effect on telomere length erosion in vitro under oxidative stress conditions in 8 weeks.

The evaluation of changes in telomere length including analysis of the increase of telomeres of critical short length in cell cultures serves as the demonstration of the protective effects of lutein (Lute-gen®). Measurement of the rate of shortening of telomeres along with the accurate determination of median, 20th percentile and percentage of telomeres below 3 Kpb, was gathered to examine lutein impact on telomere dynamics.

A consistent reduction on the attrition rates observed in the presence of lutein indicates that it has a significant protective effect on telomere length erosion *in vitro* under oxidative stress conditions after eight weeks in culture during which time X cell divisions took place.

Telomere erosion occurs at a slow pace and can only be accurately monitored in non-immortalized cells that do not have constitutive telomerase activity. Although primary adult cell cultures are suitable to study the effect of culture conditions and compounds in telomere attrition their maintenance over long period of time to promote cell division is also limited. The data obtained following 8 weeks in culture is probably the most representative one given that the cells had time to undergo more divisions. This is observed at all three concentrations tested with similar

protective effect for all of them in this long-term experiment indicating that at concentrations as low as 1 ug/ml lutein can slow telomere erosion in vitro.

Under normal, non-oxidative conditions the presence of lutein seems to increase telomere shortening rates after 4 weeks in culture. This effect appears marginally significant for LUT1. It is unclear the reason why the treatment with lutein under normal conditions could have accelerated telomere shortening, one possibility is the side effects of the excipients present in the treatment during the first weeks of treatments. Telomere length could be affected by a number of different factors in addition to proliferation and oxidative stress, such as activation of nucleases, increased protein instability of the sheltering-complex (telomere protector), effect on the expression (transcription / translation) of different proteins involved in the regulation of telomere length etc. that were not determined in this study.

The results of the *in vitro* study performed indicate that there appears to be evidence that the antioxidant activity of lutein is beneficial to counter act the effects of oxidative stress on telomeres in humans. The use of human cell cultures to obtain proof of concept for the effect on cell health of compounds such lutein has been demonstrated with the data presented as they support and provide evidence that lutein has a direct effect on the proliferation capacity of fibroblast cells. Further investigation of this phenomena may lead to understanding of the mechanism of action of lutein as well as generating information that may lead to in vivo, clinical studies. The use of such *in vitro* modeling is beneficial to investigators attempting to make initial determinations as to potential effects of a substance under investigation [8, 9].

The rate of shortening is indeed affected by the treatments which is something expected. In the case of lutein treated groups under oxidative stress, we obtained data showing a slowdown of the shortening rates after eight weeks indicating a direct protective effect of the compound on telomeres under these conditions. Telomere attrition during cell proliferation is mostly caused by the end replication problem. [10, 11]. The process of telomere shortening is reduced by the enzyme telomerase, whose purpose it is to add nucleotides at the end of the DNA molecule. Telomerase activity is found in some cells (e.g., germ cells and stem cells), which divide continually and must maintain telomeres above a critical length in order to perform their functions [12].

By promoting telomerase activity, it is possible to increase telomere length and consequently extend the number of cellular divisions that can take place without incurring in detrimental chromosomal aberration or telomere fusion. While this will not make cells immortal, it may extend their lifespan. We have been able to evaluate a protective effect on telomeres by comparing cell groups undergoing similar number of divisions. This theoretically means that the lutein treatment has a protective effect on telomeres, but this statement needs to be confirmed by taking into account the division of the cells during the expansion period. In the case that Lute-gen would halt the division of the cells, the cells in the control-untreated group would expectedly undergo several more divisions in comparison to the treated groups. In that case telomere length in the treated groups would be higher not because of the compound's protective effect on telomeres, but simply because it prevented the cells from dividing sufficient times. For this reason, the data was normalized taking into account the cell divisions in each group and the telomere shortening rate was thus calculated. It is very interesting that the protective effect under oxidative stress conditions after 8 weeks of treatment, is indeed observed after the correction factor is included in analysis of the study. We can therefore conclude that *in vitro* we are observing a protective effect under oxidative stress conditions and not simply a decline in the proliferation rate [13].

However, whether the administration of lutein reduces the level of ROS in other cell types and has a positive effect on healthy aging needs to be elucidated which may be beyond the scope of this work.

References

1. Estrada JC, Albo C, Bengur.a A, Dopazo A, L.pez-Romero P, Carrera-Quintanar L. *et al.* Culture of human mesenchymal stem cells at low oxygen tension improves growth and genetic stability by activating glycolysis. Cell Death and Differentiation. 2012; 02(19):743-755.
2. Spence AP. Biology of Human Aging, 2nd ed. Prentice Hall, Upper Saddle River, NJ, 1999.
3. Bryan TM, Reddel RR. Telomerase, Immortality and Cancer. Today's Life Science. 1996; 8:26-28.
4. Hayflick L. Theories of biological aging: Programmed Aging. Experimental Gerontology. 1985; 20:145-159.
5. Zhu H, Guo D, Li K, Pedersen-White J, Stallmann-Jorgensen IS. Increased telomerase activity and vitamin D supplementation in overweight African Americans. Int J Obes (Lond). 2012; 36:805-9.
6. Canela A, Vera E, Klatt P, Blasco MA. High-throughput telomere length quantification by FISH and its application to human population studies. Proc Natl Acad Sci USA. 2007; 104(13):5300-5.
7. Kimura M1, Stone RC, Hunt SC, Skurnick J, Lu X, Cao X. *et al.* Measurement of telomere length by the Southern blot analysis of terminal restriction fragment lengths. Nature Protocols. 2010; 5(9):1596-607.
8. Richards JB, Valdes AM, Gardner JP, Paximadas D, Kimura M. Higher serum vitamin D concentrations are associated with longer leukocyte telomere length in women. Am J Clin Nutr. 2007; 86:1420-5.
9. Chan R, Woo J, Suen E, Leung J, Tang N. Chinese tea consumption is associated with longer telomere length in elderly Chinese men. Br J Nutr. 2010; 103:107-13.
10. Wright WE, Tesmer VM, Huffman KE, Levene SD, Shay JW. Normal human chromosomes have long G-rich telomeric overhangs at one end. Genes Dev. 1997; 11:2801-2809.
11. Wright WE, Tesmer VM, Liao ML, Shay JW. Normal human telomeres are not late replicating. Exp Cell Res. 1999; 251:492-499.
12. Sen A, Marsche G, Freudenberger P, Schallert M, Toeglhofer AM. Association between higher plasma lutein, zeaxanthin, and vitamin C concentrations and longer telomere length: results of the Austrian Stroke Prevention Study. J Am Geriatr Soc. 2014; 62:222-9.
13. Xu Q, Parks CG, DeRoo LA, Cawthon RM, Sandler DP. Multivitamin use and telomere length in women. Am J Clin Nutr. 2009; 89:1857-63.
14. O'Callaghan NJ, Fenech M. A quantitative PCR method for measuring absolute telomere length. Biol Proced Online. 2011; 13:3.

15. Proctor CJ, Kirkwood TB. Modelling telomere shortening and the role of oxidative stress. Mech Ageing Dev. 2002; 123(4):351-363.
16. Kawanishi S, Oikawa S. Mechanism of telomere shortening by oxidative stress. Ann N Y Acad Sci. 2004; 1019:278-84.
17. Tchirkov A, Lansdorp PM. Role of oxidative stress in telomere shortening in cultured fibroblasts from normal individuals and patients with ataxia-telangiectasia. Hum Mol Genet. 2003; 12(3):227-32.