

## Evaluation of telomere length in granulosa cells; effects of ketogenic and western diet

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### Abstract

**Objective:** In this study, it was aimed to research the effects of nutrition with different diets on the telomere length of granulosa cells.

**Material and Methods:** In the study, 21 BALB C female rats (in each group; n = 7) were divided into three groups as the standard diet group (SD), the ketogenic diet group (KD) and the western diet group. The animals in the SD group were fed with standard mouse feed, while the KD and WD groups were fed with specially prepared diets. At the end of the experiment, a controlled superovulation stimulation protocol was applied to both ovaries. Smear preparations were ready for telomere length (GLT) and apoptotic (caspase-3) evaluations from granulosa cells taken from oocytes collected from ovaries.

**Results:** There was a negative correlation between GLT and body weight ( $r = -0.424$ ;  $p = 0.056$ ). However, this correlate was not statistically significant. At the end of the experiment, body weight was significantly higher in the WD group compared to the KD group ( $p < 0.05$ ). GLT value of KD group was higher compared to WD and SD groups ( $p = 0.000$ ). There was a significant negative correlation between Caspase 3 activity and GLT ( $r = -0.594$ ;  $p = 0.004$ ). Caspase-3 H score was statistically significantly lower in the KD group compared to SD and WD groups ( $p < 0.05$ ).

**Conclusion:** As a result of the study, it was observed that diet types cause changes in GLT, and there was a negative relationship between "body weight and caspase-3 activity" and GLT. It has also been observed that low KD prevents cells from undergoing apoptosis by increasing GLT and decreasing caspase 3 activity.

**Keywords:** Granulosa telomere length, infertility, ketogenic diet, western die

### Introduction

Telomeres which are hexameric cascading repeats at the ends of chromosomes maintain chromosome stability and genome integrity. Telomere lengths are preserved during growth, and their role depends on their length and structure. In somatic cells, telomere shortening occurs with each successive round of replication inducing ageing in vitro and in vivo. Short telomeres result in meiotic arrest, dissociation and separation abnormalities leading to an increased incidence of aneuploid germ cells. Also, shortened telomeres in men cause apoptosis of germ cells, while in women, it causes meiotic arrest (1). Telomere length may be an indicator of replicative ageing in somatic cells as well as reproductive ageing. In cultured cells, reducing telomere length to a critical threshold halts cell division triggering replicative ageing and can also cells may undergo apoptosis. Cells that do not have a certain length of telomerase are not involved in mitosis; they enter apoptosis in S phase.

Therefore, telomere length can be viewed as a mitotic clock indicating the remaining replicative life of a cell (2). Telomere length differs from cell to cell. Telomeres are longer in spermatogonia and stem cells. (3).

One of the most common causes of infertility in women is problems with ovulation and oocyte quality. Pre-ovulation follicles contain granulosa cells and cumulus cells (4). Granulosa cells play a regulatory role during oocyte maturation by promoting or delaying oocyte maturation (5). The function of granulosa cells is closely related to oocyte maturation. Granulosa cells have important functions such as preserving the integrity of the oocyte at the beginning and after ovulation, feeding oocyte and producing steroid hormones, mainly progesterone under the influence of estrogen of theca lutein cells during developing follicle after ovulation (6). The presence of estrogen stimulates the production of telomerase and causes a decrease in reactive oxygen radicals.



By affecting the number of possible cell divisions, telomere length may in part determine the number of primordial follicles and thus the overall reproductive potential of women. It has been reported that decreasing estrogen levels and slowing down cell renewal in postmenopausal women are effective in accelerating the shortening of telomere length (7).

There are many different genetic and physiological factors that affect telomere length. Exercise, the Mediterranean diet, and a low-calorie diet, antioxidants, and a healthy social / spiritual life have positive effects on telomere length (8). Considering the relationship between telomere length and nutrition, it has been found that a healthy diet with a Mediterranean diet contributes to telomere length, and consumption of foods with antioxidant and anti-inflammatory properties is associated with longer telomere length. Telomere length and attrition of telomeric repeats are affected by nutrition in human and animal models (4, 9). Most of the studies investigating the relationship between telomere length and nutrition in the literature were conducted with leukocytes. Some studies provide information on human ovarian cell telomere length. However, no studies are investigating the relationship between granulosa cell telomere length and diet types. Therefore, in this study, we aimed to examine the relationship between granulosa cell telomere length, which has important effects on oocyte and embryo development using different diet models.

## Material and Methods

Approval was obtained from Sakarya University Ethics Committee for Animal Care and Use for the experimental protocols. (Approval date and decision no: 01/07/2020-33) All applications on animals were carried out in Sakarya University Animal Laboratory following international guidelines. At the end of the experiment, animals were sacrificed by cervical dislocation after the surgical procedures were completed under general anesthesia with 65 mg/kg (i.p.) ketamine and 7 mg/kg xylazine (i.p.) injection.

### Study Design and Creating Groups

In the study, 21 BALB C female rats weighing 15-17 grams of 10-12 weeks of age were used. The animals were kept in wire cages under standard laboratory conditions with a 12/12 hour light / dark-light cycle, a temperature of 22 ° C and a humidity of 50-60% during the experiment. All rats were fed tap water and specially prepared ad libitum diets. The rats were randomly divided into 3 groups, with 7 animals in each group

**Standard Diet (SD) Group;** The rats in this group were fed a regular standard diet for 4 weeks. (77.3% of calories consisting of carbohydrates, 2.7% fat and 20% protein).

**Western Type Diet (WD) Group;** The rats in this group were fed a western diet consisting of 39.70% of calories from carbohydrates, 39.51% from fat, 19.53% from proteins and 1.26% of other ingredients for 4 weeks.

**Ketogenic Diet (KD) Group;** In this group, the rats were fed a ketogenic diet consisting of 4.95% of calories from

carbohydrates, 74.24% from fat, 19.53% from protein and 1.28% from other components for 4 weeks.

### Controlled Ovulation Stimulation and Collection of Oocytes

Both ovaries of female rats were stimulated intraperitoneally (i.p). First injection, 15 international units (IU) Pregnant Mare Serum Gonadotropin (PMSG) was applied. The superovulation protocol was completed by performing the second injection with 15 IU Human Chorionic Gonadotropin (HCG) hormone 48 hours after the first injection. 15 hours after the second injection, the rats were sacrificed and oocytes collected. For the incubation of the oocytes, Human Tubal Fluid (HTF) medium with 4 mg / ml Human Serum Albumin (HSA) was cultured in an incubator at 37°C, 5% CO<sub>2</sub> concentration from one day before. Culture drops were prepared in the form of group cultures on the culture tooth under mineral oil

### Preparation of Granulosa Cell Preparations

After the collected oocytes were incubated for at least 2 hours, they were treated with Irvine Scientific TM Hyaluronidase solution to separate them from cumulus cells. Smear preparations were prepared by taking 2 separate polylyzed slides of cumulus cells purified from the enzyme. The smear preparations were fixed in the fixation solution at -20 ° C for 30 minutes and kept at +4 ° C for a maximum of 1 week until immunohistochemical staining.

### Immunohistochemical Staining Protocol

The preparations were fixed in the fixation solution at -20 ° C for 30 minutes. The fixed preparations were washed with Phosphate Buffered Saline (PBS) 3 times for 5 minutes. Triton X-100 was dropped and kept for 10 minutes for permeabilization. They were treated with 3% H<sub>2</sub>O<sub>2</sub> (Hydrogen Peroxide) solution for 20 minutes; and were held at 37 ° C for 1 hour, covered with Primary Antibody (Thermo Fisher Scientific; cat no: PA5-16335). They were washed 3 times for 5 minutes with PBS. They were kept covered for 10 minutes with secondary antibody. Then they were washed 3 times for 5 minutes with PBS. Streptavidin Peroxidase was dropped and kept for 10 minutes covered. They were washed 3 times for 5 minutes with PBS. They were treated with DAB for 2 minutes and washed in running water. Counter-staining was applied with hematoxylin for 1 minute. Then they were washed in distilled water for 5 minutes. After drying, Mounting Medium was dropped on them and covered with a coverslip. For negative control, Antibody Diluent was dropped instead of Primer Antibody. The stained preparations were examined with an Olympus BX53 model light microscope, and counting operation was performed.

### Immunohistochemical Evaluations

For immunohistochemical evaluation, each prepared preparation was examined with Olympus BX53 model light microscopy at 100, 200 and 400x magnification in different areas, at least 200 cells per preparation were counted. Morphologically, good and bad cells were detected in the groups. In the cell count, the areas of the cells showing uniform distribution in the preparation were evaluated, as

the areas where the cells cluster were not. Results were calculated semi-quantitatively by determining the H score (10).

### Chromosomal Preparation Procedure

In this study, the method was used as a basis for chromosomal preparation. Accordingly, all steps are optimized step by step. In all stages, the metaphase spread was photographed under 1000X (oil immersion) using an Olympus BX53 microscope. Slides were incubated in freshly prepared 4% (for 6 min.) and 10% Giemsa (for 15 min) solution. The optimum concentration and duration of Giemsa stain were chosen based on the visibility of chromosomes, background clarity, and distinction between chromatids within a single chromosome. Chromosome spread counts were arcsin-square-root transformed to improve normality and homogeneity of variance. Prior to running ChAS 4.1 analysis, normality and homogeneity of data had been tested. ChAS enables you to view and summarize chromosomal aberrations across the genome. Chromosomal abnormalities may include copy number gain or loss, mosaicism, or loss/absence of heterozygosity. All values comparisons have been made through the program. In the program, using the normalization workflow for chromosome sequences, the samples were manually re-centred when necessary by automatically correcting the non-diploid status. By comparing the chromosomes with the program, measuring their lengths and comparing the standard lengths and samples, the shortenings were calculated.

### Statistical Analysis

Statistical analysis was performed using SPSS 22.0 package program (SPSS Inc. and Lead Tech. Inc. Chicago. USA). Numerical data were given as mean  $\pm$  standard deviation (SD). Normal distribution of data was performed using the Shapiro Wilk test. Pearson's correlation test was used to compare body weights and telomere length, and Spearman correlation test was used for caspase-3 H score comparison. One-way ANOVA and Kruskal Wallis test (caspase-3) were used to compare more than two variables (telomere length, body weights).

TUKEY HSD test was used for variables with homogenous variances of significance within the group. Significance limit was accepted as  $P < 0.05$ .

### Results

Average body weights of the groups at the beginning and end of the experiment are given in Figure 1. Before starting the study, there was no significant difference between all groups in terms of body weight ( $p > 0.05$ ).

At the end of the experiment, it was observed that the highest body weight was in the WD group ( $17.44 \pm 0.47$ ) and the lowest in the KD group ( $16.81 \pm 0.52$ ). When compared with the KD group, a statistically significant increase was found in the WD group ( $p = 0.036$ ).

There was a moderately negative correlation between telomere length and body weight (Figure 2A). However, this correlation was not statistically significant ( $r = -0,424$ ,  $p = 0.056$ ). Mean GTL was calculated as  $12.75 \pm 0.32$  kb in the SD group,  $12.70 \pm 0.31$  kb in the WD group and  $13.60 \pm 0.34$  kb in the KD group (Table 1).

In the comparisons between the groups, the telomere length of the KD group was found to be statistically significantly higher compared to the WD and SD groups ( $p = 0.000$ ).

Mean Caspas 3 H score was  $21.54 \pm 1.72$  in the SD group and  $25.18 \pm 4.32$  in the WD group and  $16.38 \pm 1.28$  in the KD group. In the comparisons between groups, a statistically significant decrease was found in the KD group compared to the SD and WD groups ( $p < 0.05$ ) (Table 1).

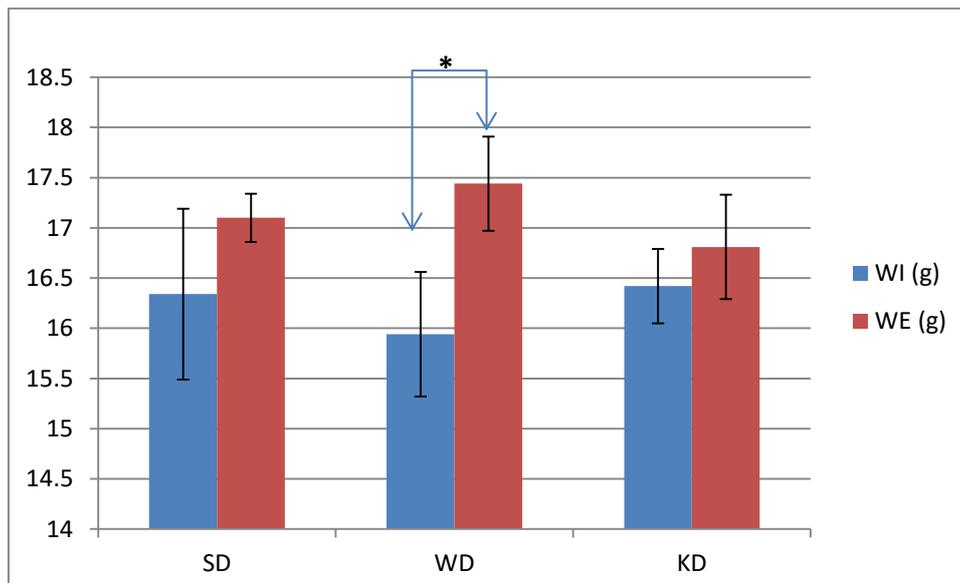
It was found that there was a moderate negative correlation between telomere length and caspase-3 H score, and this relationship was statistically significant ( $r = 0, -594$ ;  $p = 0.004$ ) (Figure 2B).

Caspase-3 positivity of the groups is presented in Figure 3. As a result of the immunohistochemical evaluation, it was seen that the highest Caspase-3 activity was in the WD group cells (Figure 3C) and the lowest in the KD (Figure 3A) group cells.

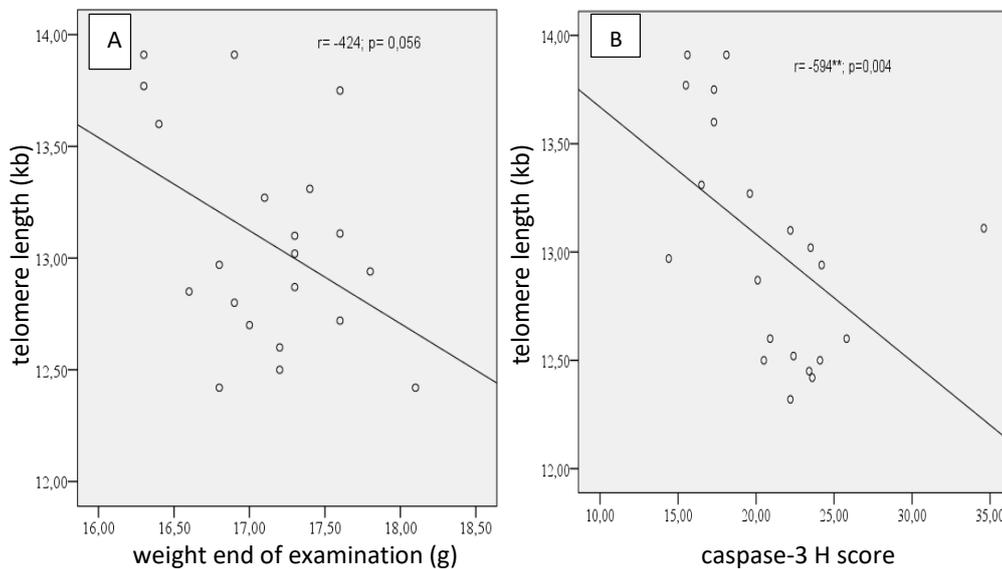
**Table 1.** Comparison of the Caspase-3 H score and the telomere length of Granulosa cells in experimental groups.

Groups (Each group n=7)	GTL kb	Caspas 3 H score
SD (G1)	$12.75 \pm 0.32$	$21.54 \pm 1.72$
WD (G2)	$12.70 \pm 0.31$	$25.18 \pm 4.32$
KD (G3)	$13.60 \pm 0.34$	$16.38 \pm 1.28$
P value	0.000 (G1-G2) 0.000 (G1-G3)	0.021 (G1-G2) 0.000 (G1-G3) 0.000 (G2-G3)

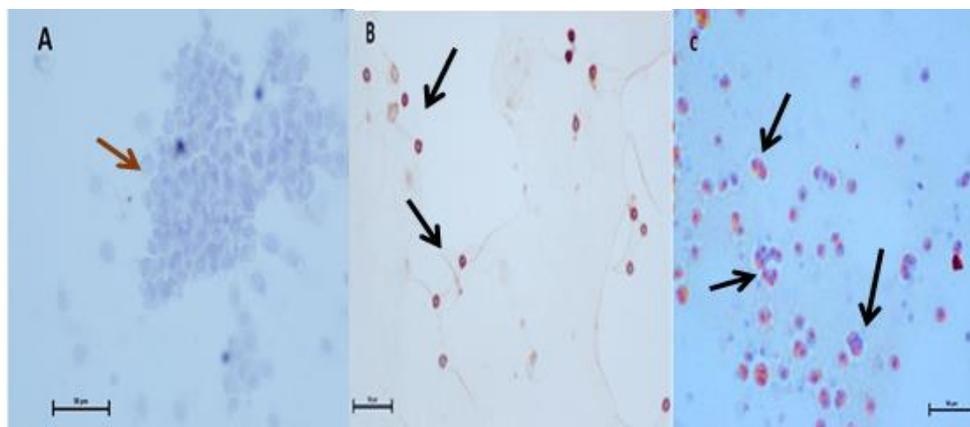
GTL: Granulosa cell telomere length; kb: kilobase pair. The mean difference is significant at the  $p < 0.05$  level.



**Figure 1:** Body weights of all groups at the beginning and the end of the experiment (gram). WI: Initial of experiment body weight; WE: Bodyweight at the end of the experiment; SD: standard diet; WD: Western diet; KD: Ketogenic diet; values are Mean±SD. The mean difference is significant at the  $p < 0.05$  level. \* Compared to the KD group;  $p = 0.036$



**Figure 2. A:** Correlation between telomere length and body weight after a 4-week diet; **B:** Correlation between telomere length and caspase 3 H score. \*\* Significant negative correlation between Caspase 3 activity and telomere length.



**Figure 3.** Immunohistochemical Caspase-3 staining of granulosa cells, at X400 magnification, 50 scale bar. Caspase-3 positivity indicates apoptosis of granulosa cells. **Figure 3A-** Low Caspas-3 activity granulosa cells of the KD group (brown arrow). **Figure 3B-** Brown stained Caspas-3 positive cells are seen around the blue granulosa cells of the SD group (black arrow). **Figure 3C-** Granulosa cells of the WD group with intense Caspas-3 positivity are seen (black arrows).

## Discussion

Of interest to nutritionists, it has been shown that telomere length is correlated with nutritional status in human and animal studies. Healthy lifestyles and diets are positively associated with telomere length. Changes in diet and lifestyle may regulate telomerase activity in peripheral blood mononuclear cells, but whether this reflects changes in telomere length remains unclear (11). Studies investigating the relationship between nutrition and infertility have greatly increased in recent years. Nutrition with healthy diets provides increased fertility in women and higher quality sperm formation in men (12). A ketogenic diet is a diet consisting of high fat, sufficient protein and low carbohydrate that mimics the metabolic changes of hunger in the body. This diet has been reported to have various health effects (13). There are multiple studies in the literature investigating to what extent KDs can affect fertility outcomes. All of these studies reported a significant improvement in the menstrual cycle and / or ovulation rates with a low-carbohydrate diet (14).

In our study, we found that the telomere length of granulosa cells is affected by feeding with different diet types. At the study results, KD-fed rats had longer telomere lengths than SD and WD-fed rats. Various studies are investigating deeply the relationship between female infertility and telomere length. In addition to natural, chronological ageing; telomere shortening can be affected by genetic factors, physical activity, body mass index, smoking, chronic inflammation, hormone replacement therapy, oxidative stress, antioxidant foods and vitamins (15). Since obesity and smoking are important risk factors for age-related diseases, Valdes et al. investigated the leukocyte telomere lengths of 1122 white women. As a result of the study, they reported that obese and smoker women had significantly shorter telomeres than those of the lean and non-smokers group. This study shows that telomere length and possibly longevity can be affected by environmental factors (16). In previous studies, it was found that women with a healthy lifestyle had longer telomeres (17). In our study, where we performed a four-week diet program, we found a moderate positive correlation between weight and telomere length. There are pieces of evidence from large-scale observational studies that weight loss leads to ovulatory infertility improvement in obese patients. A prospective study of infertile obese women showed that weight loss resulted in the resumption of ovulation in 90% of women, whereas the rate of spontaneous pregnancy was 25%. In fact, it has been shown that a weight loss of only 5% of the body improves fertility in obese women (18). Researchers have reported a relationship between the amount of carbohydrate consumed and the risk of ovulatory infertility. Specifically, they reported a 78% higher risk of ovulatory infertility in women consuming high levels of carbohydrates and 20% higher in those consuming animal protein; with 43% lower risk in those consuming vegetable protein (19). Another study has shown that women who consume vegetable protein as a protein source have a lower rate of infertility compared to those consuming animal protein.

The type of protein in the diet has been clearly shown in the study results to influence the risk of ovulatory infertility (20).

In the present study, we evaluated the effect of nutrition type on apoptotic activity in cells with caspase 3 positivity. Caspase 3 activity was highest in WD group granulosa cells. There was a negative correlation between telomere length and Caspase-3 activity. This situation was compatible with the telomere length. This result suggests that shortening telomere length and reaching the critical threshold may slow down cell division or even halt it, leading cells to apoptosis. Caspase is cysteine proteases involved in apoptotic cell death. While caspase-3 was observed in atretic granulosa cells in the ovary, no caspase-3 activity was observed in the granulosa cells of healthy follicles. Apoptosis is genetically programmed cell death. Granulosa cell apoptosis is an active cellular event dependent on transcription and protein synthesis (21). Although a study reported that caspase activation causes telomere erosion and caspase inhibitors to reduce telomere loss, it has not been explained how this happens (22).

In the last few years, telomere biology has become an important issue in the reproduction field. Despite many hypotheses, there is little direct evidence about telomere dynamics in human gametes and embryos. Increasing evidence of the role of telomeres and telomere length in human reproduction has indeed broadened the historical view of seeing them simply as an indicator of ageing. Telomere length has been studied more frequently, especially in women with recurrent miscarriages or in-vitro fertilization (IVF). The mean oocyte telomere length was found to be longer in women who became pregnant (23). It has also been reported that the granulosa telomere length is shorter in women with ovarian insufficiency of unknown origin compared to those with tubal factor infertility (24). In a study by Czamanski-Cohen et al., It was found that lymphocytes of women who underwent in vitro fertilization (IVF) due to infertility had statistically significantly shorter telomeres at various stages of the menstrual cycle compared to healthy controls (25). Barha et al. found that estradiol, a potent antioxidant with increased levels during pregnancy, preserves telomere length (26). The presence of estrogen stimulates the production of telomerase and causes a decrease in reactive oxygen radicals. It has been reported that decreasing estrogen levels and slowing down cell regeneration are effective in accelerating telomere length shortening in postmenopausal women (27). Cumulus cell telomere length has been reported to be longer in mature oocyte cells than immature oocytes. When the obtained embryo quality and telomere length were compared, it was observed that there was a positive correlation with the embryo quality (28). In another study, it is found that granulosa cell telomere length increased during the follicular development process (29). In particular, a positive correlation has been observed between short telomere length in deficient quality oocytes and oocyte maturation and interrupted gap-junctions in stromal cells (30). These remarkable findings suggest that obesity alone may not be responsible for sub-fertility and that specific dietary components may increase or impair reproductive potential.

## Conclusion

In our study results, we have shown that diet types cause changes in granulosa cell telomere lengths, albeit for a short time; and there is a negative correlation between telomere length and weight gain. With recent advances in reproductive technology, it is clear that powerful predictive biomarkers are needed to improve the clinical strategy in infertile individuals. We think that telomere length can be a marker for defining reproductive capacity; nutrition and lifestyle changes with healthy diets can be effective in increasing reproductive functions, and thus infertility can be prevented. However, in order for such methods to be used efficiently in a clinical setting, it is critical to answer many questions first and to conduct further studies to understand the relationships of telomere and related structures with biological ageing and reproduction.

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