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EFFECT OF MELATONIN ON TELOMERE IN EXPERIMENTAL TYPE 1 DIABETIC RATS

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ABSTRACT

Animal models have historically played a critical role in the exploration and characterization of disease pathophysiology and target identification and in the evaluation of novel therapeutic agents and treatments in vivo. Diabetes mellitus disease, commonly known as diabetes, is a group of metabolic disorders characterized by high blood glucose levels for a prolonged time. Due to its chronic symptoms, new treatment strategies need to be developed, because of the limited effectiveness of the current therapies. We reviewed the pathophysiological and genetic features of diabetes in relation to its complications in type 1 rat along with rat models. This study was conducted to measure the effects of melatonin loaded on rats in diabetes 2 model. Consequently, this study aimed to confirm whether the length of leukocyte telomere length was associated with the administration of melatonin to a type 1 diabetic rats and whether the number and presence of diabetic complications were related. This is a experimental study. Thirty two rats were used with type 1 diabetes. Eight diabetes rats(D), eight melatonin injected diabetic rats (DM), eight healthy melatonin injected rats (M), and eight controls (C). The control group was fed standard rat chow and received no additional treatment. The rats in the D and DM, groups were injected with STZ at a single dose of 60 mg/kg intraperitoneally (i.p). DM and M groups were given a dose of 10 mg / kg / day melatonin (i.p) for six weeks. Using Southern-blot analysis we determined mean terminal restriction fragment (TRF) length, a measure of average telomere size, in leucocyte DNA and chromosome telomere was measured by using a PCR method. Rats with diabetes complications had significantly shorter leukocyte telomere length than both rats without diabetes complications and healthy control subjects. Mean (± SE) TRF lengths of the Type 1 diabetic rats (58.00± 0.2 kb) were significantly shorter than those of the control subjects (112,90 ± 0.6 kb) (P =0.0001). Among the blood glucose level, melatonin injected diabetic rats showed a positive correlation with shortened telomeres in the diabetic subjects. However, telomere lengths in the control group were positively correlated in melatonin-injected rats and melatonin-injected diabetic rats. The results of the study support the hypothesis that telomere attrition may be a marker associated with the presence and the number of diabetic complications. It has also been observed that melatonin prevents shortening of telomere length in rats with diabetes.

KEYWORDS: Chromosome, Melatonin, Diabetes Mellitus, Telomere

It is common to use experimental animal models to understand the pathogenesis of various diseases and to examine the prevention and treatment opportunities (Animal Models for the Study of Human Disease, 2013). The main factors that make it rational to work with animal models are the ability to use controls to determine the effects of environmental factors, genetically select animal species suitable for the pathology under investigation, and study in a number of samples that allow meaningful statistical evaluation (Deneysel *et al.*, 2015) (Testa *et al.*, 2011). In addition, researches conducted by creating experimental diabetes record important findings in the treatment of diseases. Creation of experimental diabetes in experimental animals can be done with chemical agents, spontaneously or via a variety of viruses. (Chiu *et al.*, 2016)

Free radicals play an important role in the pathogenesis of many degenerative diseases such as atherosclerosis, cancer and diabetes mellitus. In diabetes mellitus, the functions of the antioxidant system occur negatively (Meza-Rios). In Type 1 diabetes, which is vital in metabolism and causes complicated symptoms, antioxidant use is widely investigated in order to make the damages of oxidative stress more moderate (Adaikalakoteswari *et al.*, 2005). The beneficial effects of various antioxidants in preventing the damage of oxidative stress have been shown (Booth *et al.*, 2018). It has been reported that the use of vitamin C and vitamin E as antioxidants in the experimental diabetes model created in rats has positive effects in preventing oxidative stress damage in erythrocytes (Furumoto *et al.*, 2008) (Wadley and McConell, 2010).

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Melatonin is a hormone secreted in the diurnal rhythm of pinealocyte cells in the pineal gland, the synthesis of which is regulated by dark-light signals received by light stimulation (Gurel-Gokmen *et al.*, 2018). Although many interactions have not been elucidated until today, it is known that melatonin is also associated with the immune system. Positive immunological effects include anti-inflammatory effect, cytokine release enhancing effect, and positive effects detected in the infection detected in some studies (Kahya *et al.*, 2015). It has been stated that melatonin taken orally has a protective effect against diabetes development in diabetic rat species fed with high-fat diet and contributes to the normal maintenance of triglyceride and cholesterol values and blood glucose level homeostasis (Duarte *et al.*, 2005). It has been stated that due to the single nucleotide polymorphisms occurring in melatonin receptors in peripheral tissues, blood sugar and Hb A1c levels are increased and susceptibility to gestational diabetes and type 1 diabetes have increased significantly (Emden, 2014). However, the role of melatonin in glucose metabolism has not been fully elucidated yet (ADA, 2012).

It consists of sequential repeats of guanine-rich short sequences such as telomere, TTAGGG, and some related protein complexes at the ends of the chromosomes. Different organisms and different cells and tissues of the same organisms have different telomere lengths. Telomere contributes to the maintenance of chromosomal stability by shortening the ~ 50-120 base during DNA replication. Telomere length was measured by several different methods as well as Southern blot, which is considered the gold standard in the studies to understand the importance of telomere in aging, type 1 diabetes and normal cell biology. These methods has specific superior and limitations to himself. In this study, The aim of this study is to show whether there is telomere shortening for type 1 diabetes and melatonin given type 1 diabetes, we can use to measurement of telomere length of rats and can also be applied in subsequent studies, and to obtain information about the telomere lengths (Ali *et al.*, 2021).

In our study, the aim of this study is to show whether there is telomere shortening for type 1 diabetes and melatonin given type 1 diabetes. İt will be the basis for later studies in the measurement of telomere length of rats, which are frequently used as experimental animals, and to obtain information about the telomere lengths of diabetic rats using this protocol.

MATERIALS AND METHODS

Experimental Design

In the study, Thirty-two Sprague-Dawley male rats weighing 250-300 grams of 10-12 weeks of age were used. Rats were placed in cages under standard laboratory conditions at 22 ° C and 50-60% humidity for six weeks in light and dark cycles for 12/12 hours. All rats were given standard tap water and fed with pellet feed. The rats were randomly divided into groups and four groups were made, with eight rats per group.

Control (C)

The animals in this group received a normal diet and no other treatments were applied to the rats.

Diabetes Mellitus Group (D)

The group is in which diabetes mellitus was created with a dose of 60 mg / kg by STZ intraperitoneal (i.p.) injection.

Melatonin Group (M)

Melatonin at a dose of 10 mg / kg was injected subcutaneously (s.c.) for 6 weeks in this group.

Diabetes Mellitus and Melatonin Group (DM)

Rats became diabetes mellitus with i.p injection of 60 mg / kg STZ. Melatonin s.c injection at a dose of 10 mg / kg per day for 6 weeks was administered to the rats in this group.

Diet and tap water were given to the control group and treatment groups regularly during the experimental period of 6 weeks. The animals were given 50 mg / kg of STZ (Cayman Chemical product number, 13104, USA) one day before (12 hours) to induce diabetes. Rats were injected with a single dose i.p. at pH 7.2 PBS. Two days after STZ administration, fasting blood glucose levels were measured from tail vein blood samples. Rats with blood glucose above 250 mg / dL were considered diabetic. While STZ injection was continuing, treatment was started on the third day and this day was determined as the first day of treatment. For six weeks after the diabetes was induced, Melatonin (Cayman Chemical East Ellsworth rd. Item No. 14427, USA) was prepared in isotonic NaCl solution containing 10% ethanol daily. and s.c. Injection at a dose of 10 mg / kg.

When the experiment was over, the rats were killed by injecting 65 mg / kg (i.p.) ketamine and 7 mg / kg xylazine (i.p.) under general anesthesia. Blood samples of rats were collected by cardiac puncture and

tissue samples were taken and stored in a suitable environment.

And than Telomer analysis was performed using a quantitative polymerase chain reaction (qPCR)-based method with slight modifications (Dlouha *et al.*, 2014). The relative telomere length was calculated as the ratio of telomere repeats to a single-copy gene (SCG) (T/S ratio). The acidic ribosomal phosphoprotein PO (36B4) gene was selected as the SCG. For each sample, the quantity of telomere repeats and the quantity of SCG copies were determined by comparison to a reference sample (Cawthon, 2002).

Approval was obtained from the ethics committee (date; 01/07/2020 and decision number; 32) of Sakarya University for animal study Sakarya University Experimental Medicine App. and Research.

Chromosome Painting Technique (FISH)

Fluorescence in situ Hybridization, ie FISH, is a general method involving the staining of DNA or various RNA types (mRNA, non-coding RNA) with fluorescent dyes. It is used to investigate the presence of specific DNA sequences in chromosomes and to determine and locate telomere length.

Cell culture is based on the stimulation of small leucocyte in the blood, placed in their appropriate media, with fitohemaglutinin, a mitogenic substance. In a 37 ° C oven, at the end of 72 hours, colcemide added to the dividing cells produces metaphor plates. Then, the chromosomes are separated from each other with the hypotonic solutions added to the medium.

Identification of Primers

In order to normalize the operation of genomes and PCR operation in the samples, it was necessary to identify a reference gene that was expressed in the leukocyte. For this purpose, the "acidic ribosomal phospoprotein P0 (36B4)" gene, which is expressed as the only copy on the 12th chromosome in the rat genome and is not pseudogene, that was selected. Using the LightCycler Probe Design Software 2.0 program, 36B4 Standard primers(Genbank accession no: NW 047376.1) that form 453 base pairs (bp) length amplicon and 36B4 primers(Genbank accession no: NW 047376.1) that will form 80 bp amplicon out of the 36B4 standard sequence of 453 bp were determined. All primers used in the study are shown in Table 1.

Table 1: PCR primer list

Name	Primer Sequence	Primer length (Kb)	PCR Product length (kb)
Telomere	Forward:5'-GGTTTTTGAGGGTGAGGGTGGGGTGAGGGT-3'	37	76
	Revers:5'-TCCCGACTATCCCTATCCCTATCCTATCCCTA-3'	39	
36B4	Forward:5'-CAGCAAGTGGGAAGGTGTAATCC-3'	23	63
	Revers:5'-CCCATTCTATCATCAACGGGTACAA-3'	25	

Telomere Length Measurement

To measure TL and Terminal restriction fragment (TRF) analysis and polymerase in epidemiological studies chain reaction (PCR) are the two most common methods.

Q- PCR Application

The number of 36B4 copies in the rat leukocyte was determined to use as a nuclear genome standard. The absorbance of DNA at 260 and 280 nm was recorded using an ultraviolet spectrophotometer, and the DNA concentration was measured using a Nano-Drop 2000 spectrophotometer. The relative length of telomere was measured using quantitative polymerase chain reaction (qPCR), with the primers synthesized by Sango Biotech Co., Ltd. (Shanghai, China). DNA from the leukocytes of rats was isolated using DNA extraction kit from plate (Tiangen, Beijing, China). Relative telomere lengths were measured by qPCR method with modifications In brief, DNA samples were used for telomeres and single-copy

gene (36B4) qPCR, which was amplified by Power SYBR Green in 20 µL total volume (Dumont, 2000).

The analysis was conducted using the integrated software to determine the threshold cycle (Ct) value by negative control and baseline, and to determine the validity of Ct value according to the dissolution curve. The triplicate reactions were performed in parallel for each sample, and the average Ct value was calculated. The negative control reaction was also performed for each round of PCR. The ratio of telomere/copy (T/S) was calculated using the following formula:

$\Delta Ct1 = Ct_{telomere} - Ct_{36B4}$, relative T/S ratio = $2^{-(\Delta \text{ Ct telomere} - \Delta \text{ Ct 36B4})} = 2^{-\Delta\Delta Ct}$.

ΔCt1 stands for T/S ratio, and ΔCt2 stands for the T/S ratio of the reference DNA.

TRF Analysis

TRF analysis is the first and the traditional method to measure TL (Cawthon, 2002). The methods developed afterwards, such as PCR and in situ hybridisation based methods have used TRF analysis, which is still considered as the gold standard, as a reference or for calibration.

The main drawback of this method, especially for large scale studies, is that it requires large amounts of DNA (0.5–5 g/sample) which is equivalentto genomic DNA from around 105 cells.There are different alternatives for restriction enzymes. TL of individuals may vary as much as 5% due to presence of polymorphisms in the subtelomeric restriction sites and/or polymorphisms in the subtelomeric regions. Previous studies have shown that the amount of subtelomeric region present in TRF can vary 2.5–6 kilo-base-pairs (KB) in unrelated individuals (Zhang *et al.*, 2008).

This confounds determination of true interindividual variability of TL. Independent of the enzyme selection, presence of subtelomeric DNA should be taken into account when calculating the absolute TRF length.

Statistical Analysis

The covariance analysis of repeated measurements by the mixed model approach was used for the telomere length analysis. The comparison of averaged data among groups was performed using least significant difference (LSD) and analysis of variance (ANOVA).

RESULTS

Telomere length measurement using real-time quantitative PCR was evaluated for reproducibility.

The body weight of rats increased significantly ($p < 0.001$) at the day 45th day. After injection of melatonin the body weight started to decrease gradually in all the groups differently D, DM, M and C until the end of the experiment. The final body weight of rats among all groups was significantly differed than C but the weight of D rats was the lowest. The weight percentage of M were found height in D group which significantly ($p < 0.001$) differed than C group. The melatonin performance were significantly ($P < 0.001$) increase (61%) in DM group than D group which indicating the fatigue syndrome induced by diabetes mellitus. Between the DM and M weight differences were mostly corrected by combined action of melatonin treatment. Especially In the study, it was seen that 6 th and 8 th rats from the DM group rats came out of diabetes mellitus.

İn the study melatonin administration with or without insulin also significantly increased the body weights compared to D group. Rats treated with insulin and with both insulin and melatonin revealed decreased diabetic effects when compared to rats in DM group (Graakjaer *et al.*, 2003).

Telomere Length

To date, various key factors related to cellular aging have been identified (Naoki *et al.*, 2019). One of these is the telomere, a region of repetitive DNA at the ends of eukaryote chromosomes that protects the chromosomes from attrition and damage (Naoki *et al.*, 2019) (Butt *et al.*, 2010). Telomeres shorten with each cell division because of the end replication problem. Telomerase, an enzyme that prevents telomere shortening, consists of telomere reverse transcriptase (TERT) and a telomerase RNA component (TR) (Butt *et al.*, 2010). Telomere length measurement using quantitative PCR was evaluated for reproducibility. Between the groups there was significant difference ($P < 0.05$) between telomere length measurements. We found relationship between TL and D, DM, M with the longest TL detected in the Melatonin injected rats.

There was high variability in the relative telomere length between rats. We found an inverse relationship between TL and D with the longest TL detected in the M group However, the DM group is also very close to the M Telomere size. (Fig. 1)

Interestingly, the variability of TL was very high among the same rat in the group DM. In the DM group there were differences of up to 30%. The relative telomere length was significantly longer in M compared D ($p < 0.001$) but was not too different compared to and DM ($p < 0.001$). Fig. 1 The biggest TL variability was observed in the DM group; the smallest TL variability

was observed in the D group. TL length as seen in Fig. 2 Although there is a small affinity in all other groups, there is no corellation with the M group. We found a low TL correlation between group D and group M. (R=0.92)

Telomere Length in Diabetes

Our study confirmed that TL length decreased in Diabetes mellitus. in addition we observed a slightly faster decrease in TL was observed in D compared to DM and M. TL length, It was in the range of 10,42±0,07 for D and 17,24±0,45 for M. We compare, TL length also in degraded and intact DNA samples.

Melatonin treatment (P<0.001) significantly influenced telomere length (Jeanclos *et al.*, 1998). (T/S ratio) (P<0.001). Fig 2. Telomere lengths were negatively correlated with insulin resistance (D) and positively correlated with DM and M levels in the control subjects. Rats with diabetes had significantly shorter telomeres than Control rats. Fig 2. Rats with DM had longer telomeres than Control rats, especially rats 6 and 8 in the DM group exited diabetes as a result of administration. The telomere lengths of these rats were seen in proportion with the rats in the control group.

Telomere of DM and M rats; D was significantly shorter than rats' telomeres (P <0.001). DM group rats telomere length was significantly correlated with bodyweight (Jeanclos *et al.*, 1998).

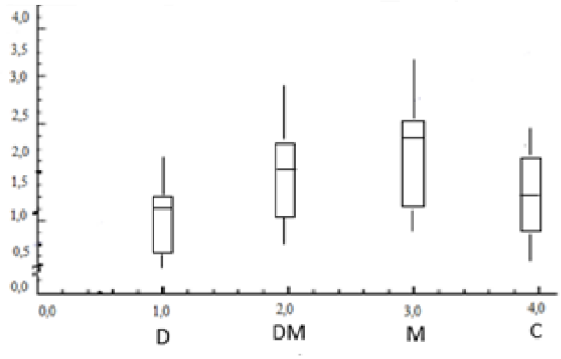
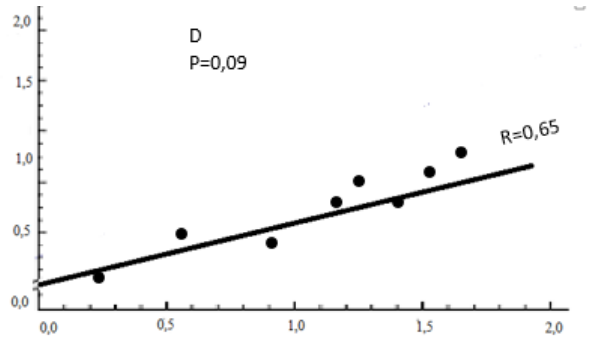
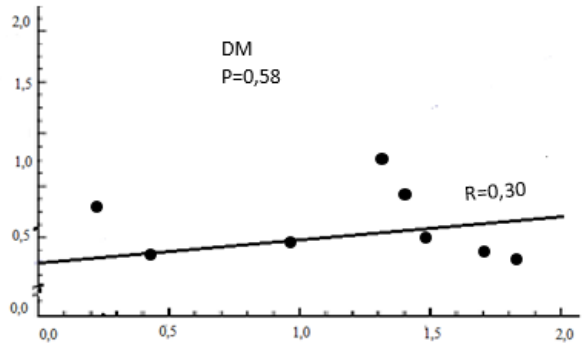


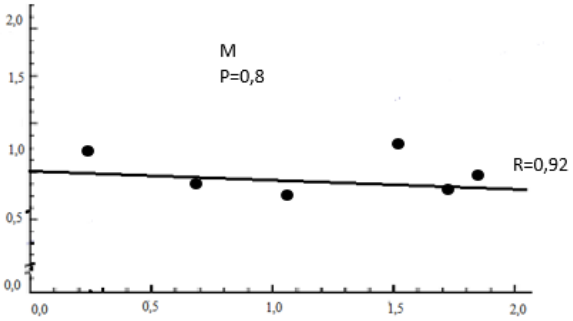
Figure 1: The range of relative T/S ratio measured in Telomere lenght of rats



(A)



(B)



(C)

Figure 2: TL length as seen in Fig A. Although there is a small affinity in all other groups, there is no corellation with the M(C) group. We found a low TL correlation between group D(A) and group M(C). In the DM (B) group there were differences of up to 30%

Correlation between mean TRF Length and Telomere Length

Diabetes based on telomere lengths Group D was significantly shorter than group DM (P = 0.02) in the control group. D, DM, M group's difference in TRF length was observed among the diabetic

Especially TRF sizes of rats 6 and 8 in the DM group, are proportional to the telomere lengths. When diabetic rats are associated with DM and M of subjects receiving melatonin only TRF lengths were significantly longer (P = 0.007). When diabetic rats are associated with D and DM of subjects receiving diabetes TRF lengths were significantly shorter. (Kreier *et al.*, 2007) (Table 2).

Result was performed to assess the correlation between TRF expression and relative telomere length in the diabetic rats. A significant relationship was found between TRF protein and telomere length. (P < 0.01). Testing was also done to evaluate the correlation between

TRF expression and relative telomere length in control rat($P > 0.05$).

It was found that there was significant association between TRF length and telomere length. The test was performed to assess the correlation between TRF expression and relative telomere length of rats. The test was also performed to assess the correlation between TRF expression and relative telomere length in the control($P > 0.05$), D, DM and M rats.

Table 2: Correlation between TRF and telomere length

		TL D	TL DM	TL M	TL C
	Correlation coefficient	0.430**	0.610**	0.800**	0.800
TRF					
	Significance	0.000	0.110	0.200	0.200

**Correlation is significant at the 0.01 level

DISCUSSION

We focused on correlation in our study, this is the first study focused on Telomere length comparison analysis by applying melatonin to diabetic rats. There are a few studies that have focused on a similar topic, but they focused on a only telomere length in diabetes mellitus. Some studies observed for the Telomere length in different parts and different illness. In this study, Diabetes model was created in rats using STZ method and telomere length was determined and real-time fluorescence was measured using quantitative PCR. The correlation between relative T/S ratios measured by quantitative PCR and relative TRF length measured by the traditional southern blot approach.

During the STZ application to rats, it was observed that the 6th and 8th rats in tthe DM group given melatonin exited diabetes by controlling their glucose measurements. The subsequent qPCR study also supported this idea. As a result of the study, there was no shortening of Telomere and TRF length in the 6th and 8th rats in the DM group. It was observed that the group D had shortened telomere length faster than other groups. There are very slightly differences between the telomere length in group D itself. Although there was no significant difference between the M and C group telomere length, it was seen that the M group telomere length was slightly more than the C group. Melatonin therapy can be used to improve cell damage and reduce the incidence in diabetic patients (Ramesh and Pugalendi, 2006). Group M telomere length more than group DM but group DM shows different telomere lengths in itself. A positive improvement was observed in the telomere lengths of the DM group rats compared to the D group.

In this study, the possible effects of melatonin on the induced diabetic rats were investigated. The relationship between Telomere length (TL) in diabetic rats and Telomere length in melatonin injected rats was investigated (Butt *et al.*, 2010). At this work, body weight loss and increased plasma glucose levels were observed in untreated diabetic rats. Melatonin DM group animals on blood glucose level and weight loss compared to group D, we found its significant effect. During the study M and C the blood glucose level of the group was within normal limits and was not different from each other. In 30 days Weight gain in group C animals, statistically in group M animals we detected a slight weight loss. The body weight of diabetes group rats was decreased its might be due to the loss and degradation of structural protein which is responsible for the body weight and for complex metabolism (Tiwari *et al.*, 2013).

MELATONIN

In addition to the telomere shortening that occurs during each DNA replication period, some irreparable DNA damage and epigenetic factors rapid telomere shortening and aging can contribute to its formation

Some researchers in rats with type 1 diabetes melatonin lowers blood sugar levels, so blood sugar level they argued that he might have a role in his control. However, in the study mentioned, melatonin, administered as a preservative before the creation of type 1 diabetes with STZ in rats (Zephy and Ahmad, 2015). İn this study showed that; DM group telomere length, D and C group compared to, telomere administration of melatonin in diabetic animals it can be said that it has a positive effect on its length. Melatonin plays a crucial role in glycemic control by reducing FPG. Melatonin also ameliorates insulin resistance and enhances insulin sensitivity in the cells. In a nutshell melatonin treatment can be used to improve diabetic conditions in patients and reduce the incidence (Pirozzi *et al.*, 2015) .

A good treatment regimen is required to cure these diabetic conditions associated with oxidative stress. Melatonin confers protection against diabetes via many different mechanisms.

The results of our study should be analyzed with regard to the biology of the blood used. Our study examined telomere length in this major blood cell population. The fact that telomere length was significantly higher in blood compared to most of the Telomere length studied could reflect the expected life span of the different cell types. Diabetes increased oxidative stress and inflammatory cytokines were effectively ameliorated by the combined action of melatonin supplementation regimentation in DM group in this study. The relatively Telomeres shortened in group D diabetic rats, TL is gradually getting shorter due to complications related to diabetes as well as its progress. (Ekmekcioglu, 2006). In comparisons, telomere shortening rate of M group is close with DM but the difference with group D is quite high.

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