Implication of diabetes mellitus in telomere length and alteration in some age related cell senescence markers of aged men in Kurdistan region-Iraq

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ABSTRACT

Indications of cell maturing including telomere length and mitochondrial work, just as oxidative pressure and incendiary markers affect one another and structure a complicated organization, which all influenced in age related sicknesses, for example, diabetes mellitus. For this cause, this current investigation was designed to study and compare of some oxidative and molecular markers in healthy aged and diabetic aged men older adults. The study design was the setting of a medium private laboratory with participants being common people, which was classified into three groups according to age and health complication, control young, healthy aged, and diabetic aged groups. Blood sera was obtained for oxidative status including catalase (CAT), superoxide dismutase (SOD), Total antioxidant capacity (T-AOC), 8-hydroxy-2'-deoxyguanosine (8-OHdG), and malondialdehyde (MDA) and molecular markers (including telomere length (T/S) and mitochondrial DNA copy number (mtDNA)). The findings highlighted that oxidative and molecular markers have significant changes with ageing and high changes significantly associated with diabetes mellitus of aged group. In conclusion, various oxidative stress, cell and mitochondrial biomarkers have been affected by normal ageing and the effects of ageing were complicated significantly in diabetic aged elderly.

Keywords: Oxidative stress markers, molecular markers, diabetic mellitus adults, aging adults, man.
1. INTRODUCTION

More recently, aging is described as decreasing and deteriorating functional characteristics at the stage of cells, tissues and organs [1]. Some consider aging to be continuous development, which includes embryonic development, maturation, adult period of activity, and aging; although this is controversial [2].

In the previous two decades, the importance of chronological and biological aging has acquired significant recognition. A complicated feature of wide science concern is also the biological foundation of aging, particularly due to its inherent link with prevalent human illnesses [3]. In comparison, chronological age is the quantity of years an individual has been alive, while biological age refers to the age of a person who appears. Biological age, also known as physiological age, requires consideration of many way of life parameters, including exercise, sleep practices and diet [4].

Since aging requires an uncertain relationship at the level of cells, tissues, organs, and processes, it is difficult to have an evidently specific biomarker for age-related illnesses. That is why it is frequently believed that there is no best quality standard instrument for measuring high aging. This is why a large group of biomarkers is used to examine the aging process. The plausibility of complementing biomarkers used to assess physiological ability with molecular-based markers has increased the range of predictions for safe aging [5-7].

Growing old is the important danger factor for cardiovascular diseases, reactive oxygen species, and oxidative stress have long been related to ageing, and elderly sicknesses inclusive of diabetes [8].

To explain the maturing system, various speculations have been executed. One theory is that the strange collection of natural waste product inside the life form is responsible for the senescence of organs or tissues [9]. The hypothesis of glycation shows that glucose fills in as a maturing middle person. Glycation is a programmed non-enzymatic reaction to the decrease of free sugar with free amino gatherings of proteins, DNA and lipids that make up the results of Amadori. Amadori items go through various irreversible drying out and adjustment responses, bringing about cutting edge glycation final results [9]. Such effects can be practically identical to high groupings of glucose and more limited life expectancies in individuals with Alzheimer's sickness and diabetes [10].

The course of glycation brings about a diminishing of protein work and harmed tissue adaptability, for example, veins, skin and ligaments [11, 12]. In the presence of hyperglycemia and tissue oxidative pressure, the glycation reaction is amazingly improved [13]. This involves it in the pathogenesis and aging of diabetic complications [14]. In view that there aren't any enzymes to separate glycated merchandise from the human frame, the approach of glycation is in keeping with the hypothesis that metabolic waste accumulation promotes aging [9].

The principle point this research a bunch of binding together signs of Glycation and free radical hypothesis of aging that has been characterized giving a framework to knowing the course of mitochondrial aging and oxidative in typical aging and diabetic aging at more established grown-ups work through check some atomic and oxidant status.

2. METHODS AND MATERIALS

Experimental design
The experiment design is that of a cohort study within the setting of a medium private laboratory with sixty participants being common male people were equally classified into three groups according to age and healthy status of participants: A) control young group (20-40 years old) (n=20); B) healthy aged group (60-80 years old) (n=20) and C) diabetic aged group (60-80 years old) (n=20). All samples were obtained from Sulaimani Nursing House and New Medical Center (Private Laboratory) from December-2016 to April-2017.

Data collection
Reagents and equipment were purchased from Roche (Germany). Fasting blood samples (10 ml) were obtained of all participants divided in to two parts, 5 ml of blood were gathered for
evaluation of molecular markers (mtDNA and T/S), and HbA1c in container tubes with EDTA as an anticoagulant at a concentration of 50 microns. 5 ml into vacuum tubes (non-heparinized) for the assay of serum. The serum was separated by centrifugation (15,000×g for 10 min), kept at −80 °C and assessed by Human ELISAs kit, as recommended by the manufacturer. The serum concentration of each CAT, SOD, TAOC, 8-OHDG and MDA were estimated using the ELISA Kit (Elabscience Biotechnology Inc.) they reagent with BioTek ELx800 UV (BioTek Instrument, Inc./USA) ELISA microplate reader.

Mitochondrial biomarkers (mtDNA) assay

Isolation of DNA

The DNA extraction procedure for the 50 blood samples was performed as follows. The conventional phenol-chloroform technique has been implemented with certain changes particular to the laboratory standard operating procedure.

1. 1 ml of each blood sample was transferred to a 15ml polypropylene tube.
2. 7ml distilled water was added to samples, then the mixture was shaken vigorously for 10 minutes.
3. This was followed by centrifugation of the mixture at 3000rpm for 10 minutes.
4. The supernatant was discarded and another 7ml distilled water was added to the pellet.
5. Steps 1 to 4 were repeated 3 times to achieve a clean and colorless supernatant.
6. The supernatant was discarded and 300ul lysis buffer plus 50ul proteinase K was added to the pellet, followed by incubation of the mixture at 37°C overnight.
7. 300ul of phenol was added to the mixture.
8. The new mixture was inverted several times then transferred to a 1.5ml polypropylene tube, followed by centrifugation of the mixture at 14000g at 4°C for 13 minutes.
9. The upper phase was transferred to a new tube.
10. 300ul chloroform was added.
11. The mixture was inverted several times then centrifuged at 12000g at 4°C for 11 minutes.
12. The upper phase was transferred to a new tube.
13. 300ul of isopropanol was added to the mixture.
14. 40ul sodium acetate was added to the mixture.
15. The mixture was inverted several times and then incubated at -20°C, followed by centrifugation of the mixture at 12000g at 4°C for 10 minutes.
16. The supernatant was discarded.
17. 500ul of ethanol 70% was added to the DNA pellet.
18. The mixture was inverted several times and then centrifuged at 14000g at 4°C for 6 minutes.
19. Step 17 was repeated for a second time to achieve cleaner or pure DNA.
20. The supernatant was discarded and let the pellet to dry at room temperature for 7 minutes.
21. 40ul double-distilled water was added to the pellet.
22. The mixture was incubated at room temperature to achieve full resolution of the pellet.
23. UV-visible spectrophotometry was applied for the quantification of the DNA samples.
24. Finally, DNA samples were stored at -20°C for further analysis by real-time PCR.

Designing oligo primers specific to mitochondrial 16srRNA and β2 microglobulin genes

In order to quantify mitochondrial counts in samples, mitochondrial 16srRNA genes have been chosen to be amplified by real-time PCR. Also, the nuclear β2 microglobulin gene was chosen as a single copy normalizer gene. Primers have been built using the Gene Runner software. The sequence for forward and reverse primers for the genes referred to above is shown in Table 1.
Table 1: Primers used for mitochondrial 16srRNA and β2 microglobulin genes qPCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer</th>
<th>Sequence (5’ to 3’)</th>
<th>Amplicon size (bp)</th>
<th>Annealing (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>mt16srRNA A</td>
<td>Forward</td>
<td>TATCATTTTCGGGGGAAGGG</td>
<td>104</td>
<td>60</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>CGTAGTAATCCAGGTCGGG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>gb2m</td>
<td>Forward</td>
<td>TGCTGTCTCCCCCTTTGATGTATCTTCT</td>
<td>86</td>
<td>60</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>TCTCTGCTCCCCACCTCTAAGT</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

qPCR setup
For each sample, the qPCR experiment was performed in duplicates using RealQ Plus 2X Master Mix Green on the AB StepOne Plus real-time PCR instrument. The general procedure was enacted by Venegas and Halberg [15].

qPCR data analysis
After the acquisition of Ct values for mt16srRNA and β2 microglobulin genes from real-time PCR, information was evaluated using the following formula [16, 17].

Relative mitochondrial DNA content = $2 \times 2^{-\Delta CT}$
Patient control mitochondrial count = mtDNA patients / mtDNA controls

Telomere length T/S by using real-time PCR
After extracted the DNA, Designing oligo primers specific to telomeres 16srRNA and β2 microglobulin genes. Telomeric hexamer repeats were targeted in order to quantify the length of telomeres in the samples. The longer the telomere length, the more binding sites available for such primers. As a result, no particular amplicon size is expected. Beta globin was chosen as a single copy normalizer gene. Primers have been accepted from Cawthon, 2009 [18]. The sequence of forwarding and reverse primers for the genes referred to above are presented in table 2.

Table 2: Primers used for telomeres 16srRNA and β2 microglobulin genes qPCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer</th>
<th>Sequence (5’ to 3’)</th>
<th>Amplicon size (bp)</th>
<th>Annealing (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Telomere</td>
<td>TelG</td>
<td>ACACTAAACGTTTGGGTTGTTGGGTTGGGGT</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>TTGGGTTAGTGT</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>TelC</td>
<td>TGTTAGGTATCCCCCTATCCCTATCCCTATCCCTATC</td>
<td>-</td>
<td>62</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CCTATCCCCATAACA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hbgu</td>
<td></td>
<td>CGCCGCCGCGCCGCGCGGCGGCTGGGGCGG</td>
<td>106</td>
<td>84</td>
</tr>
<tr>
<td></td>
<td>Hbgd</td>
<td>AGGAGAAGTGCTGAGTTAAGT</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

qPCR setup
qPCR experiment was performed for each sample in duplicates using RealQ Plus 2X Master Mix Green on an AB StepOne Plus real-time PCR instrument. The general procedure was adopted from Cawthon, 2009 [18].
qPCR data analysis
After the acquisition of Ct values for telomere length and single-copy gene from real-time PCR, data were analyzed using the following formula [18].
\[
T/S \text{ ratio} = 2^{-\Delta CT}
\]
\[
T/S \text{ ratio among samples} = (T/S)_{\text{cases}} / (T/S)_{\text{controls}}
\]

Statistical Analysis
SPSS 22 software was used to analysis the results. ANOVA test was used to differentiation between groups. The outcomes had been expressed because the mean ± standard deviation (SD). Differences with values of p<0.05 had been considered statistically significant.

3. RESULTS

Glucose and HbA1c levels in male aged groups
The Glucose level increased significantly in the diabetic aged group (127.8±6.84 mg/dl) in comparison to its level in control group (99.08±9.31 mg/dl) and healthy aged group (102.6 ± 8.36 mg/dl).
The high HbA1c level (6.419% ± 0.12) was found in the diabetic patient group, while HbA1c level in healthy aged groups were close to control group (table 3).

Table 3: Glucose and HbA1c levels in men aged groups.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Parameters</th>
<th>Control young A</th>
<th>Healthy Aged B</th>
<th>Diabetes C</th>
<th>P-value</th>
<th>A vs B</th>
<th>A vs C</th>
<th>B vs C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose (mg/dl)</td>
<td></td>
<td>99.08 ± 9.31</td>
<td>102.6 ± 8.36</td>
<td>127.8 ± 6.84</td>
<td>0.170</td>
<td>0.0001</td>
<td>0.0001</td>
<td></td>
</tr>
<tr>
<td>HbA1c (%)</td>
<td></td>
<td>5.304 ± 0.19</td>
<td>5.515 ± 0.19</td>
<td>6.419 ± 0.12</td>
<td>0.410</td>
<td>0.0001</td>
<td>0.0001</td>
<td></td>
</tr>
</tbody>
</table>

Oxidative stress markers in male aged group.
Table 4 showed that the level CAT in diabetic aged group is strongly significant decrease (P=0.0001) and decreased significantly with aging. Regarding the SOD, the result showed that the control aged group had the highest value (4.022±1.12 U/mL) in contrast the lowest level was found in diabetic aged group when compared to healthy aged and control groups.

TAOC results in this study revealed a maximum level (1.421±0.76 mmol/L) in the control young group (20-40) and non-significantly decreased with aging. Its level increased significantly in the diabetic aged group (1.090 ± 0.09 mmol/L) in comparison to its level in healthy aged group (1.357 ± 0.13 mmol/L).
The result in the table 4 showed that the highest level of 8-OHDG and MDA were found in the diabetic groups

Table 4: Oxidative stress markers in men aged group.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Parameters</th>
<th>Control young A</th>
<th>Healthy Aged B</th>
<th>Diabetic aged C</th>
<th>P-value</th>
<th>A vs B</th>
<th>A vs C</th>
<th>B vs C</th>
</tr>
</thead>
<tbody>
<tr>
<td>CAT (pg/mL)</td>
<td></td>
<td>135.3 ± 20.4</td>
<td>120.1 ± 14.63</td>
<td>106.1 ± 13.6</td>
<td>0.0001</td>
<td>0.0001</td>
<td>0.000</td>
<td></td>
</tr>
<tr>
<td>SOD (U/mL)</td>
<td></td>
<td>4.022 ± 1.12</td>
<td>3.595 ± 0.14</td>
<td>3.184 ± 0.16</td>
<td>0.0001</td>
<td>0.0001</td>
<td>0.727</td>
<td></td>
</tr>
<tr>
<td>TAOC (mmol/L)</td>
<td></td>
<td>1.421 ± 0.76</td>
<td>1.357 ± 0.13</td>
<td>1.090 ± 0.09</td>
<td>0.0001</td>
<td>0.0001</td>
<td>0.761</td>
<td></td>
</tr>
<tr>
<td>8-OHDG (ng/mL)</td>
<td></td>
<td>0.418 ± 0.12</td>
<td>1.119 ± 0.18</td>
<td>1.691 ± 0.12</td>
<td>0.0001</td>
<td>0.0001</td>
<td>0.000</td>
<td></td>
</tr>
<tr>
<td>MDA (µmol/L)</td>
<td></td>
<td>0.582 ± 0.10</td>
<td>0.611 ± 0.01</td>
<td>0.710 ± 0.09</td>
<td>0.0001</td>
<td>0.0001</td>
<td>0.000</td>
<td></td>
</tr>
</tbody>
</table>
Mitochondrial genetic markers in male aged group.

Regarding the mtDNA in the current study, results showed a marked reduction in diabetic patients aged groups, and healthy aged groups that moderately decreased in comparison to the control group (145.50±20.97), as seen in table 5.

In the table 5, the highest rate of T/S was found in the control group (701.0±23.72) in contrast to the healthy aged which showed a reduction in T/S (604.9±12.24), followed by renal disease group (570.5±22.87) and Parkinson's disease groups (522.9±12.40), they are its level moderately decreased, while there was the highest reduction in the level of the T/S (517.6±17.04 and 490.6±19.62) more found in diabetic patient and CVD groups consecutively. Statistically, all results showed a highly significant difference between each group (P=0.0001).

<table>
<thead>
<tr>
<th>Groups</th>
<th>Control young A</th>
<th>Healthy Aged B</th>
<th>Diabetic aged C</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>mtDNA</td>
<td>145.50 ± 20.97</td>
<td>127.5 ± 10.44</td>
<td>108.5 ± 9.96</td>
<td>0.0001</td>
</tr>
<tr>
<td>T/S</td>
<td>701.0 ± 23.72</td>
<td>604.9 ± 12.24</td>
<td>517.6 ± 17.04</td>
<td>0.0001</td>
</tr>
</tbody>
</table>

4. DISCUSSION

Glucose and HbA1c levels

The result of the current study revealed an increase in the level of the glucose by age advancing. A previous study proved that blood glucose level tends to increase with age [19]. Also in line with the research carried out in the USA in a community aged 65 years or older after testing, it accounted for more than 37% of the rise in glucose levels and was at danger of rising diabetes [20].

The present results revealed that glucose level increased in the aged diabetic patient. A previous study reported that elevated blood glucose can also be an indicator of metabolic syndrome [21]. Fasting of blood glucose is recommended as a proxy for the accumulation of risk factors, including obesity, hypertension and blood pressure associated with cardiovascular disease [22]. HbA1c concentration rises steadily until the age of 79 years [23]. Previous studies reported that sensitivity and specificity of HbA1c have been reported to be elevated in the older population (mean age of more than 69 years) and this study insists that elevated levels of HbA1c have been found in both sexes in (65-85) age groups [24, 25]. A previous study found that HbA1c sensitivity and specificity were improved in subjects with a median age of 49.9 years [26].

The current study showed that the high rate of HbA1c was found in the diabetic patient aged group, and a previous study reported that higher HbA1c levels could be a predictor of cognitive decline in people with diabetes [27]. This might not be irrelevant to the fact that HbA1c plays a substantial role in the regulation of glucose homeostasis and a stronger predictor for diabetes in general.

Oxidative stress markers

In this study, significant decrease was seen in the CAT levels in advanced aged groups and its level declined with aging. Previous reports found that the level of activity for CAT decreased with aging [28, 29]. Decreasing CAT level has been displayed to boost oxidative DNA and protein modification and hoist the improvement of a few diseases [30]. Also the lowest level of CAT was found in the diabetic aged group in comparison to the healthy aged group and a previous study measured CAT levels in the blood of the control group as for matters with cardiovascular and osteoarticular pathologies such as myoma, also risen CAT levels in hyperglycemia patients because hyperglycemia doubled the development of hydrogen peroxide and controlled CAT gene expression [31].
It is previously reported that the decrease in the level of SOD with aging [32, 33], decrease in SOD activities with age may further accelerate the aging process. The role of SODs in signalling pathways that modulate aging is increasingly understood. SOD genes are necessary for normal oxidative stress resistance and life span and have been targets for the development of aging mechanisms [34]. Herbal dietary merchandise with antioxidant interest have acquired special interest in latest a long time because of their function inside the regulation of oxidative stress correlated with brain getting older and persistent situations [35].

The T-AOC level in this study showed a significant decrease with aging. The previous study showed that the activity of T-AOC is significantly lower in the elderly age group than the middle-aged group [36], which is reported that T-AOC is a possible new strategy in explaining the mechanism of biological aging and an innovative goal for human therapeutic intervention. Also the previous research shows a decrease in total antioxidant ability with ageing and suggests that this decrease could be correlated with free radical damage mechanisms to lipids, proteins and DNA throughout ageing [37]. The total antioxidant activity provides an illustration of all potential oxidants in a cell. Some reactive species have oxidative powers and various natural defences address the correct process of cytoplasmic oxidation antioxidant properties. The effect of antioxidants cannot be overemphasized as the production of free radicals facilitates the aging process [38]. Also these results are supported by previous study, the negative association between the total antioxidant potential and the oxidative stress level is triggered by certain pathophysiological modifications that represent cardiovascular disease, metabolic dysfunction, tissue damage, DNA and/or other macromolecules in the body [39]. Oxidative strain assumes a critical part inside the advancement old enough related illnesses like atherosclerosis and sort 2 diabetes mellitus [40].

8-OHdG elevated in normal aging and this result is consistence with the previous studies [41, 42]. The previous study proved that DNA harm has a tendency to boom with age, as repair features become much less efficient [43]. Similar reports have suggested that DNA damage by oxidative stress was a causal factor for aging [44]. 8-OHdG is a result of oxidative DNA harm and is a touchy marker of raised oxidative pressure [45]. The height of 8-OHdG mirrored the development of DNA harm with getting more established. The measure of eight-hydroxy-2′deoxy-guanosine (8-OHdG) in DNA outstandingly increased with age [46]. The expanded 8-OHdG level has been distinctive as a marker in the pathogenesis of some degenerative sicknesses, atherosclerosis and diabetes [47, 48]. Aging is trailed by the amassing of 8-OHdG in atomic DNA in different tissues. An age-related amassing of DNA harm may assume a part in the expanded rate of disease among matured individuals and creatures [49]. Likewise, an expansion in DNA harm can cause the combination of a repercussion of wrong proteins and subsequently weaken cell trademark [50].

The MDA level was increased by aging. In line with current findings, it has been shown that aging and stress increase the free radical production and development of ROS, thereby increasing lipid peroxidation and, consequently, MDA levels in blood and tissues during the aging process [51]. It is previously reported that damaging of peroxidants increased with aging process due to increased MDA and decreased antioxidants [52]. MDA was detected in the maximum level in diabetic aged group when compared to healthy aged groups. It is previously confirmed that an increase in MDA with Diabetic patients [53]. A past report revealed that Developing of malondialdehyde stages among a diabetic people may be ascribed to the height in peroxidative harm to lipids from oxidative pressure created all through diabetes [54]. MDA is oxidative stress end product that is developed in excessive amount as by product of the antioxidant defence mechanism in many pathophysiological disorders affecting the cardiovascular system, such as hypercholesterolemia, diabetes and hypertension [55, 56].

Mitochondrial genetic markers

The mtDNA copy number level showed significant decreasing with aging. The finding is consistent with aprevious study [57]. Also a preceding take a look at determined that the mtDNA feature decreased for the duration of ageing, together with a decline in TCA cycle enzymes, a reduction in breathing capacity and a rise in the improvement of reactive oxygen species (ROS) in each human and animal model. A previous take a look at recommended that a relative
deficiency of mitochondrial antioxidants might make a contribution to the big age-related decreasing in mitochondrial DNA molecules material in skeletal muscle, liver, and heart tissue [58]. In addition, such modifications can lead to DNA mutations, cell death, inflammation and decreased function of the stem cells, which contribute to tissue degeneration [59, 60]. The purpose why mtDNA reproduction quantity declined in aged people or animals is that one hypothesis assumed getting older as a result of the aggregation of damage to biomolecules because of the overproduction of highly poisonous reactive oxygen species (ROS) over their lifespan. This idea has been known as the mitochondrial aging theory because mitochondria are the principle wellspring of ROS in the cell [61]. This result is supported that ROS development may cause in decreasing of mtDNA molecules or loss of function in mtDNA through simple modification [62]. Such aging-related mitochondrial changes are added to the fact that age is the main cause for several disorders in the population [63]. An decrease in the level of T/S with aging is agreement with previous studies [64, 65] recorded that the level of T/S was decreased with aging and peoples with decreasing telomeres may be assumed to be at a higher risk of age-related pathologies, such as frailty, suggested as a clinical measure of biological age [66]. Take a look at first suggested an affiliation between shorter telomere and accelerated threat of loss of life in individuals aged ≥60 years [67]. According to this research, it also showed that the length of telomere was shorter for the age of more than 60 years [68]. At the same time as in agreement with several research, they have got now not determined a significant affiliation among lowering of telomere duration and age, a number of which have used small age group samples [69]. Also previous studies confirmed that decreasing of telomere length have a positive relationship with health complications like in type 2 diabetes patients [70].

4. CONCLUSION

This observe research possibly provide a therapeutic goal for aging and age-related disease. On the other hand, “check some molecular levels and oxidant status”, can be itself considered a hallmark of healthy ageing and diabetic aging. Glucose and hemoglobin A1c levels are increased with aging, and this increase was strongly associated with diabetic disease. While the level of the antioxidants were decreased and the level of oxidative markers were increased with aging and the effects of ageing were complicated significantly in diabetic aged elderly. The copy number of mtDNA and length of telomere were decreased with aging, and both are more associated with diabetic disease.

REFERENCE


