Original Article

Higher Body Mass Index is Associated with Increased Oxidative Stress in Patients of Type 2 Diabetes Mellitus

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Abstract

Introduction: Type 2 diabetes mellitus (T2DM) is closely associated with the obesity; however, certain proportion of T2DM patients is non-obese or lean (BMI < 18.5 kg/m^2). Obesity has long been associated with oxidative stress; however, there are no studies available documenting levels of oxidative stress in the lean patients of T2DM. Therefore, this study was done to compare the levels of makers of oxidative stress (TL, mtDNA, TAS) and their regulators (mRNA expression of *TERT, Nrf2* and *Nqo1*) in lean and obese patients of T2DM. **Methods:** 60 newly diagnosed patients (treatment naïve) of T2DM were recruited and divided into lean (BMI < 18.5 kg/m^2) and obese (BMI > 25 kg/m^2) groups. Relative telomere length (T/S) and mtDNA content were estimated via real-time PCR. Serum total antioxidant status (TAS) was measured using a commercially available kit. mRNA expression of *TERT, Nrf2* and *Nqo1* was measured by real-time PCR. **Results:** Mean T/S and mtDNA content were lower in the obese group compared to the lean group ($P = 0.16$ and $P = 0.06$, respectively). Mean serum TAS levels were higher in obese group compared to the lean group ($P = 0.001$). mRNA expression of *TERT* and *Nrf2* was increased in obese group compared to the lean group. mRNA expression of *Nqo1* was similar in both the groups. **Conclusion:** Obese patients of T2DM are exposed to a greater degree of OS compared to the lean patients of T2DM.

Keywords: Lean, mtDNA content, Nrf2-Nqo1, obese, oxidative stress, telomere length, type 2 diabetes mellitus

Introduction

Diabetes mellitus (DM) is a group of metabolic diseases characterized by hyperglycaemia, resulting from defects in insulin secretion and/or insulin action.[1] The prevalence of the disease continues to rise rapidly despite advances in medicine, and this increase can be attributed to the obesity pandemic in the developed world.[2]

Obesity-associated type 2 diabetes mellitus (T2DM) has long been known to be a pro-inflammatory and pro-oxidant condition giving rise to a condition known as oxidative stress (OS). OS is said to exist when the production of oxidants or free radicals overpowers the antioxidant defence mechanisms. Beta (β)-cells are very sensitive to OS, as they express minute amounts of antioxidant enzymes.[3] Hence, β-cells are at greater risk of oxidative damage than other tissues which have higher levels of antioxidant protection.[4] OS is believed to be involved in both the disease process and the development of complications[5] with studies documenting increased OS in patients of T2DM compared to healthy control population.[6,7]

Telomere attrition has long been linked to OS and its associated diseases.[8] Telomeres are TTAGGG repeats at the end of chromosomes which prevents loss of genetic material. TERT is the enzyme responsible for maintaining telomere length. Conditions associated with OS such as hyperglycaemia are associated with telomere attrition. Mitochondria apart from being the powerhouse of the cell are also the major site of free radical generation and decrease in mitochondrial DNA (mtDNA) content has been documented in conditions associated with OS so much so that decreased mtDNA content and telomere length is often used as a marker of OS.[8-10]

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Antioxidant mechanisms are in place as a defence mechanism against OS. The Nrf2-Keap1-Nqo1 system is the main antioxidant system in place responsible for cellular antioxidant defence by inducing antioxidant enzymes in cells, thereby combating the effects of free radicals.[11] Nuclear factor erythroid-2-related factor 2 (Nrf2) mediates the induction of a set of antioxidant enzymes, such as glutathione s-transferase and NAD(P)H: quinone oxidoreductase 1 (Nqo1) in the presence of OS via various mechanisms.[12] Nqo1 in addition to being an antioxidant enzyme has direct free radical scavenging property.[13]

A detailed literature search did not yield any studies on oxidative stress in lean patients (Body Mass Index (BMI) <18.5 kg/m^2) of T2DM. Therefore, the current study was conducted to compare the levels of OS in newly diagnosed and untreated lean and obese patients of T2DM.

Objectives

1. To compare the mRNA expression of regulators of oxidative stress (*TERT, Nrf2* and *Nqo1*) in whole blood in lean and obese patients with T2DM.
2. To compare the levels of biomarkers of oxidative stress (telomere length, mtDNA content) in whole blood and total antioxidant status (TAS) in serum in lean and obese patients with T2DM.

Material and Methods

This pilot study was conceptualized, designed and performed in the Department of Biochemistry and Department of Endocrinology at the University College of Medical Sciences and GTB Hospital, Delhi.

Given the low frequency of lean patients attending OPD and fixed study duration, a convenience sample of 30 patients per group was recruited, i.e., a total of sixty patients.

T2DM was diagnosed by WHO criteria.[14] 30 newly diagnosed lean patients (BMI < 18.5kg/m^2) in the age group of 20–65 years who weren't on any pharmacotherapy for T2DM were recruited first followed by 30 obese (BMI > 25kg/m^2) patients (age and sex-matched). WHO's Asia-Pacific guidelines were used for BMI stratification.[15] Exclusion criteria included the presence of any renal or hepatic disease, thyroid disorders, severe co-morbid diseases (cancer, chronic infections, smoking and chronic respiratory diseases), pregnant and lactating women and chronic alcoholism.

Percentage of body fat was analysed using the Body Composition Analyzer InBody 570 (InBody, S. Korea) based on the principle of bioelectric impedance. Biochemical investigations were processed on RANDOX RX Imola Autoanalyzer, (RANDOX, UK) as per the manufacturer's guidelines. HbA1c levels were estimated on BIO-RAD D-10 Autoanalyzer (BIO-RAD, USA) as per standard protocol.

Total antioxidant status estimation

Commercially available kit (Cayman Chemicals, USA) was used for the estimation of serum TAS [Assay Range: 0.044–0.330 mM; Precision: Intra-assay 3.4%, Inter-assay 3%] following the manufacturer guidelines. The values were expressed as μM Trolox Equivalents.

DNA isolation, TL and mtDNA content

QIAamp DNA Blood Mini DNA extraction kit (Qiagen, Germany) was used to extract DNA from whole blood. It was quantified using NanoDrop 2000c spectrophotometer (Thermo SCIENTIFIC, USA). 100 ng of DNA was used per reaction to measure telomere length as per the method described by Cawthon[16] with minor modifications using quantitative multiplex real-time PCR. Telomere (T) PCR and a single copy gene (S), i.e., β-globin gene PCR, were performed simultaneously using dye-based chemistry (DyNAmo ColorFlash SYBR Green qPCR kit, Thermo SCIENTIFIC) on CFX Connect™ Real-Time System (BIO-RAD, USA). The Ct values thus obtained were used to determine the T/S ratio which is a measure of relative telomere length.

The relative mtDNA content was estimated via real-time PCR[17] using dye-based chemistry (DyNAmo ColorFlash SYBR Green qPCR kit, Thermo SCIENTIFIC) on CFX Connect™ Real-Time System (BIO-RAD, USA)

RNA isolation and cDNA synthesis, real-time polymerase chain reaction

RNA was extracted from whole blood using RiboZol reagent (Amresco, USA) following the manufacturer's protocol. cDNA was synthesized from the extracted RNA using RevertAid First Strand cDNA Synthesis Kit (Thermo SCIENTIFIC, USA).

Gene expression was analysed using qPCR (dye-based chemistry) on CFX Connect™ Real-Time System (BIO-RAD, USA) using the ΔΔ Ct method and expressed as FC.[18] Fold change (FC) was determined as follows:FC = $2^{-\Delta\Delta Ct}$. Since FC was >1, true fold change = FC. Sequence of primers used is shown in Table 1.

Statistical analysis

Statistical analysis was carried out using SPSS v 26.0 (IBM Corporation, USA) software. Kolmogorov–Smirnov test was used to check for normality of data. Biochemical parameters, T/S, mtDNA content and TAS between the two groups were compared by the unpaired Student's *t*-test (for normally distributed data) or Mann–Whitney U test (for non-normally distributed data). The relative mRNA expression of *TERT, Nrf2* and *Nqo1,* was reported as FC. Correlation analysis was done using Spearman's rho correlation test. A *P* value of less than 0.05 was considered statistically significant.

Ethical aspects

The study was approved by the Institutional Ethics Committee-Human Research vide letter no IEC-HR/2019/41/25 on 16.10.2019. Written informed consent was obtained for participation in the study and use of the patient data for research and educational purposes. The procedures in the study follow the guidelines laid down in the Declaration of Helsinki.

Table 1: Sequence of primers used

Primer Code	5′ to 3′ Sequence (Forward)	5′ to 3′ Sequence (Reverse)
TERT	GCAAGTTGCAAAGCATTGGA	ACCTCTGCTTCCGACAGCTC
Nrf2	ACACGGTCCACAGCTCATC	TGTCAATCAAATCCATGTCCTG
Nqo1	GGCAGAAGAGCACTGATCGTA	TGATGGGATTGAAGTTCATGGC
18s	GTAACCCGTTGAACCCCATT	CCATCCAATCGGTAGTAGCG
GAPDH	TGACTTCAACAGCGACACCCA	CACCCTGTTGCTGTAGCCAAA
β2M	TAGCTGTGCTCGCGCTACT	TCTCTGCTGGATGACGTGAG

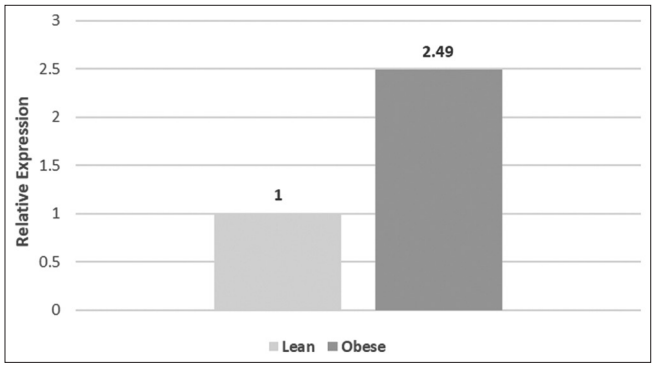

Figure 1: mRNA expression of *TERT* (fold change) in lean and obese patients with T2DM

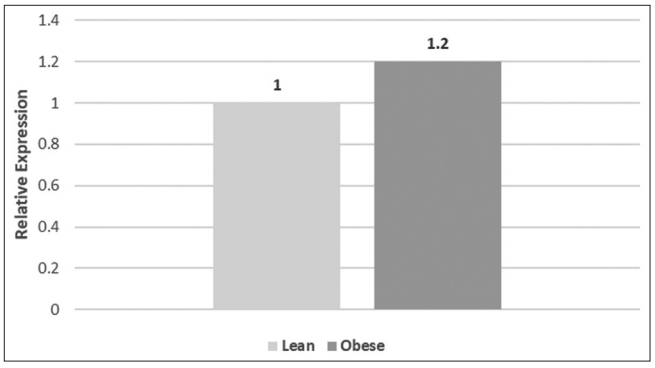

Figure 2: mRNA expression of *Nrf2* (fold change) in lean and obese patients with T2DM

Results

A total of 60 patients were recruited, i.e., 30 participants per group. The average age (mean ± SD) was 51.5 ± 10.40 years in the obese group and 52.10 ± 10.67 years in the lean group. Mean values of BMI, glucose profile, relative telomere length (T/S), mtDNA content and serum TAS levels are depicted in Table 2. FC in expression of *TERT* and *Nrf2* are depicted in Figures 1 and 2, respectively. There was no difference in mRNA expression of *Nqo1* in both the groups.

Correlation analysis was carried out by Spearman's rho analysis. A significant correlation was seen between mRNA expression of Nrf2 with telomere length ($r = 0.36$, $P = 0.047$) and with TAS ($r = 0.347$, $P = 0.007$) in both the groups.

The rest of the correlational analysis did not yield any significant association.

Discussion

OS-induced cell damage is associated with the pathogenesis of many diseases including T2DM. Free radicals due to inherent instability are highly reactive and react with cellular components (proteins, lipids, nucleic acids) thereby damaging them.[19]

The first parameter we analysed was telomere length, which is known to be affected by OS. Although studies[8-10] have reported a decreased telomere length in obese patients of T2DM compared to healthy control population, there are no studies comparing telomere length in lean and obese patients of T2DM or lean T2DM with healthy controls. In our study, obese patients had a decreased telomere length compared to the lean group suggesting a higher rate of telomere attrition. Decrease in telomere length in the obese group can be attributed to obesity and its resultant low-grade chronic inflammation. A study[20] has reported a correlation between body weight and telomere attrition. A study[21] has also reported a decrease in telomere length in pancreatic tissue of T2DM patients as compared to control population. Once the telomere undergoes shortening, it increases the risk of premature apoptosis of beta cells, leading to a decline in islet cell functioning and diabetes development and progression.[22,23]

The mRNA expression of TERT the enzyme responsible for the maintenance of telomere length was higher in the obese group compared to the lean group. *TERT* expression is most likely to be induced in the obese group due to shortened telomere length. While no study exists comparing *TERT* expression in lean and obese patients with T2DM, studies[24,25] have reported lower *TERT* expression in obese patients of T2DM. Another study[26] has reported comparable levels of protein expression of TERT in patients of T2DM and healthy controls. The contrasting results can be explained via the fact that in our study we have recruited newly diagnosed patients of T2DM. Since the disease is still in its early stages, the body is able to initiate mechanisms to restore the damages done. These mechanisms get exhausted on repeated and continuous insults which could explain the comparable and decreased expression of TERT seen in other studies who recruited chronic patients of T2DM. A study[26] has also suggested the role of insulin as a potentiator of TERT and could explain the increased expression of TERT in obese patients of T2DM who have a higher insulin level compared to the lean patients.

Table 2: Comparison of physical and biochemical parameters among study groups

Variables*	Lean (*n*=30)	Obese (*n*=30)	*P*
BMI (kg/m^2)	17.9±0.9	27.2±2.7	-
Percentage Body Fat (%)	22.11±5.92	35.97±5.76	-
Fasting Plasma Glucose (mg/dL)	254.2±63.1	207.3±73.9	0.01**
2-hour Post-Prandial Plasma Glucose (mg/dL)	361.2±76.6	329.2±88.1	0.131
HbA1c (%)	11.5±2.6	9.4±2.1	0.001***
Fasting serum insulin (μIU/mL)	16.1±8.4	27.1±4.9	0.001***
T/S	945.7±533.7	716.31±397.9	0.16
mtDNA content	332.8±147.1	300.82±169.7	0.06
TAS (μM Trolox Equivalents)	3.8±3.3	5.39±2.3	0.001***

*values are expressed as mean±SD.** Significant (P<0.05). ***highly significant

This study reports a decrease in mtDNA content in the obese group compared to the lean group. Studies[27,28] have documented a decreased in mtDNA content in obese patients with T2DM compared to healthy control population. A study[29] has also reported a reduction of mtDNA content to precede the development of T2DM. Decrease in mtDNA content in obese probably a result of OS-induced damage and subsequent mitophagy of damaged mitochondria. Decrease in number or damage to mitochondria can lead to disruption of cellular homeostasis as mitochondria are the site of energy production in a cell and leakage of cytochrome c from damaged mitochondria can trigger the intrinsic pathway of apoptosis.

In this study, we report a higher TAS in the obese group compared to the lean group of newly diagnosed patients of T2DM. This is in line with the findings of Kharroubi *et al*.[30] the only difference being that TAS levels were compared between patients with normal BMI and obese patients of T2DM, the same study also reported a higher TAS levels in diabetics compared to healthy population. The increase in TAS could be explained as a counter-regulatory mechanism, increasing antioxidants, in response to the increased OS. mRNA expression of Nrf2 was slightly increased in the obese group and that of Nqo1 was similar in both the groups. The Nrf2-Nqo1 pathway is the major cytoprotective pathway against OS. Increased mRNA expression of Nrf2 in the obese group supports the fact that OS in obese patients is higher compared to the lean groups. While there are no studies on Nqo1 expression in lean and obese patients of T2DM, studies[31,32] have reported a decreased expression of Nrf2 in patients of T2DM compared to healthy controls. This contrast can also be explained due to the fact that we recruited newly diagnosed cases of T2DM who have the capacity to try to compensate for the increase in OS.

Another interesting finding was that although the hyperglycaemia was worse in the lean group, the obese group was found to have higher OS. This suggests that obesity and adipokines contribute to OS to a much greater extent than hyperglycaemia in patients with T2DM.

Limitations and future prospects

The present study was done with a limited sample size due to constraints of time and resources. Non-diabetic subjects weren't recruited in the study. Visceral fat was not measured in the participants. The study did not include dietary history of participants and antioxidants present in diet can contribute to TAS. We plan to extend this study to include a larger sample size to further confirm the above-mentioned findings, for better statistical accuracy.

Clinical implications

This is the first study that has been carried out to compare OS in lean (BMI < 18.5 kg/m^2) and obese (BMI > 25 kg/m^2) patients of T2DM. Thus, it has described important baseline details and highlighted the difference in the disease process which might warrant different strategies of management in these two groups (antioxidant supplementation in obese patients in addition to anti-hyperglycaemic medications).

Conclusion

Obese patients of T2DM have a higher degree of OS leading to an upregulation of antioxidant mechanism. Based on our findings and available scientific literature, we may postulate that obese patients of T2DM are more prone to OS-induced cellular damage (receptors, membranes, organelles and nucleic acid) compared to their lean counterparts. Increased OS in obese patients may be attributed to obesity-induced chronic low-grade inflammation and effect of various adipokines. This suggests that different therapeutic approaches (antioxidant supplementation in obese patients) to these groups might be of greater benefit in the treatment of T2DM.

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Author contributions

EAA: Concept, design, definition of intellectual content, literature search, clinical studies, data acquisition, data analysis, statistical analysis, manuscript preparation, manuscript editing. MM Concept, design, definition of intellectual content, literature search, clinical studies, data acquisition, data analysis, statistical analysis, manuscript preparation, manuscript editing and manuscript review. SVM: Concept, design, definition of intellectual content, literature search, clinical studies, data acquisition, data analysis and manuscript review. RK: Concept, design, definition of intellectual content, clinical studies, data analysis, statistical analysis, manuscript editing and manuscript review.

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Conflicts of interest

There are no conflicts of interest.

Data availability

The datasets generated during and/or analysed during the current study are not publicly available due to administrative reasons but are available from the corresponding author on reasonable request.

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