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Assessment of Genetic Damage in T2DM Patients

Shikha Jaggi[1] and Abhay Singh Yadav[2]

[1]Department of Zoology, Gandhi Memorial National College, Ambala Cantt., Haryana, India

[2]Department of Zoology, Kurukshetra University, Kurukshetra, Haryana, India

Corresponding Author Contact Information: Shikha Jaggi

Department of Zoology, Gandhi Memorial National College, Ambala Cantt., Haryana-133001, India. Ph no.: +91-7015853188, +91-8813862518 Email: sjzgmn@gmail.com

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Abstract

Background: Type 2 diabetes mellitus (T2DM) is a major health problem characterized by the development of insulin resistance or, sometimes, insufficient production of insulin. Genetic damage plays an important role in the pathogenesis of T2DM. In the current study, we have evaluated the levels of genetic damage in T2DM patients. The results of the two assays were also compared.

Methods: A total of 196 subjects were included in this study. Of these, 106 were T2DM patients and 90 were age- and gender-matched controls. Micronucleus assay and comet assay were used to assess the level of genetic damage in T2DM patients.

Results: A significant increase in nuclear anomalies and comet tail parameters, indicating genetic damage, was observed in T2DM patients. Significant correlations were observed for genetic damage parameters with age and duration of T2DM in patient group.

Conclusions: These results indicate the presence of significant genetic damage in patients with T2DM. DNA damage is significantly associated with the advancing age and duration of diabetes in T2DM patients.

Key Words: Comet assay; Genetic damage; Micronucleus assay; Type 2 diabetes mellitus.

Introduction

Type 2 diabetes mellitus (T2DM) is the most common form of diabetes, having 90-95% cases globally (Shettigar *et al*., 2012). It is characterized by inability to regulate blood glucose levels due to insulin resistance or sometimes due to insufficient production of insulin, resulting in hyperglycemia. Persons above 40 years of age possess a higher risk of T2DM. It affects individuals with significant morbidity of cardiovascular diseases, nephropathy, retinopathy and widespread diseases of peripheral and central nervous system.

The number of people with T2DM is growing rapidly worldwide. According to International Diabetes Federation, 537 million people were living with diabetes in 2021 across the world and this number is expected to increase to 783 million by the year 2045. India leads the South-East Asia region with the largest number of diabetic patients (74.2 million). In South-East Asia, Mauritius has the highest prevalence of diabetes among adults (22.6%) followed by India (9.6%) (International Diabetes Federation, 2021).

Usually T2DM remains undetected for quite long time because it may take years to recognize the symptoms, thereby causing cellular damage. Hyperglycemia induces glucose autooxidation, increased sorbitol and polyol pathway, non-enzymatic glycosylation of amino groups in proteins, lipids and nucleic acids and subsequently form advanced glycation end-products (Singh *et al*., 2001). Reactive oxygen species (ROS) are generated as a by-product in T2DM. Oxidative stress is referred to as an imbalance between pro-oxidants (free radicals, reactive oxygen species) and antioxidants. Excessive levels of pro-oxidants damage the structure of biomolecules such as DNA and cause DNA strand breaks (Shettigar *et al*., 2012). ROS are known to fuel diabetic vascular complications.

This has led to an increased interest in study of oxidative stress by the way of micronucleus and comet assay. Recently there has been an increased interest in oxidative stress by the way of micronucleus (MN) and comet assay. Micronucleus assay is one of the most preferred techniques to investigate chromosomal damage and genomic instability in humans exposed to environmental and occupational hazards. It is used to study the cytological effects due to lifestyle factors, nutritional deficiencies and diseases (Iarmarcovai *et al*., 2008). Proliferation of buccal epithelial cells in humans is very rapid, so these are quite suitable for MN assay (Yadav and Jaggi, 2015). The stem cells in the basal layer of buccal epithelium may carry

genetic damage as micronuclei during karyokinesis. These micronucleated cells migrate to keratinized layer at oral surface in 7-10 days (Ceppi *et al*., 2010; Holland *et al*., 2008). The various means of micronucleus formation and other nuclear anomalies and their molecular mechanisms have been dealt with in detail by a host of workers (Bonassi *et al*., 2011; Fenech *et al*., 2011; Huang *et al*., 2011 and Palmaro *et al*., 2007). The comet assay is one of the recent, simple, rapid, cheap, flexible methods established in order to detect different types of DNA damage i.e. single- and double-strand breaks at the level of individual cells (Collins *et al*., 2008; McKenna *et al*., 2008 and Tice *et al*., 2008). It is one of the highly sensitive techniques, applicable to fresh or frozen samples and can detect as few as 50-15000 breaks/cell (Piperakis, 2009). Lymphocytes are the cell types most frequently tested with the comet assay in human biomonitoring studies (Angerer *et al*., 2007; Faust *et al*., 2004 and Møller, 2006). The comet tail length, tail DNA (%) and olive tail moment were mostly used for investigating DNA damage (Ahuja and Saran, 1999; Armalyte and Zukas, 2002; Mohseni-Meybodi *et al*., 2009 and Tice *et al*., 1991). The percentage of DNA in tail is correlated to DNA break frequency, therefore, increase or decrease in DNA damage will correspondingly lead to increase in tail DNA (%), tail length, tail moment and olive moment (Collins *et al*., 1995 and Ross *et al*., 1994).

Growing index of research in the field of diabetes indicates that it is associated with increased risk of developing site-specific cancers of breast, endometrium, bladder, colorectum and pancreas, and decreases the risk of prostate cancer. Insulin resistance with secondary hyperinsulinemia is the most important assumption for causing cancers as it may have a mitogenic effect by activating insulin-like growth factor-1 receptor. Hyperglycemia may be another important factor (Noto *et al*., 2010). Evidence regarding the association between MN induction and the development of cancer has been collected (Murgia *et al*., 2008).

The aim of the present study was to assess the genetic damage using micronucleus assay and comet assay in T2DM patients and control subjects. The results of MN assay were also compared to those of the comet assay in T2DM patients and controls.

Materials and Methods

Study population

A total of 196 volunteers were enrolled in the present study. The subjects were divided into two groups: type 2 diabetes mellitus patients (n=106) and control subjects (n=90). T2DM patients were enrolled on the basis of impaired fasting blood glucose levels (>126 mg/dl) measured using ACCU-CHEK® blood glucose meter. Control subjects were matched with respect to age and gender. Exclusion criteria included acute illness, history of cancer, chronic liver or infectious disease. All the subjects answered to an epidemiological assessment evaluating gender, age, occupation, physical activity, family history of T2DM, smoking, drinking and dietary habits etc. Written informed consent was obtained from all the subjects and the study was approved by Institutional Ethics Committee, Kurukshetra University, Kurukshetra (vide letter number IEC/13/330 dated 27/4/2013).

Sample collection

Blood samples were collected in K2-EDTA containing tubes after overnight (10-12 h) fasting by venous puncture technique. Exfoliated oral mucosa cells were collected with help of stainless steel spatula on microscopic slide.

Cytological preparations

The study of exfoliated oral mucosa cells was performed by standard technique of Tolbert and co-workers (1992) with slight modifications. Exfoliated oral mucosa was obtained from right and left cheek with moistened stainless steel spatula after rinsing mouth with fresh tap water. Smears were prepared on pre-cleaned microscopic slides. Two slides were prepared for each subject. Slides were fixed with rectified spirit and air dried. Samples were transported to the laboratory on ice and hydrolyzed in 1N HCl at 60°C for 8 minutes and rinsed in water. Then they were stained with 2% aceto orcein (HIMEDIA, acetic acid RM 5564, orcein RM 277) and counterstained with 0.1% fast green solution (HIMEDIA, RM 4266). Coded slides were analyzed with Olympus CX 41 trinocular research microscope at 1000X magnification. A total of 1000 cells were scored for MN, BN, BE, KL and KH for each subject following the criteria of Tolbert and co-workers (1992). MN was identified by following criteria: (a) rounded or oval in shape; (b) less than one third the diameter of main nucleus; (c) same colour, texture and focal plane as the main nucleus; (d) clearly separated from main nucleus. Other nuclear anomalies studied are: (a) BN i.e. cell with two nuclei; (b) BE i.e. nuclei that is broken and connected by thin thread; (c) KL i.e.; nuclear dissolution (d) KH i.e. fragmented and disintegrated nucleus. Other processes like pycnosis and condensed chromatin were also observed but not have been counted in nuclear anomalies.

Comet assay

The comet assay was carried out according to Ahuja and Saran (1999), but with slight modifications. Each microscopic frosted slide was covered with 150-200 µl of 1% normal melting point agarose and allowed to solidify at room temperature. A mixture of 20 µl whole blood and 100 µl low melting point agarose was added to slide and covered with coverslip immediately. The slides were refrigerated for 5-10 minutes until the agarose is solidified. After

that third layer of 180-200 µl of 1% normal melting point agarose was added and covered immediately with coverslip. After solidification step, coverslip was gently slid off and slides were placed in lysis solution overnight at 4˚C, and followed by electrophoresis at 25 V, 300 mA for 30 minutes. After electrophoresis, slides were coated with neutralization buffer 3 times each for about 10 minutes. The slides were then stained with ethidium bromide. All the steps were carried out in a dark room to avoid photolysis of DNA. Olympus trinocular research microscope CX-41 was used for comet visualization. Images of 100 cells (50 cells from each of the two replicate slides) were selected and analyzed using Lucia Comet Assay software (Nikon). DNA damage was evaluated by different comet tail parameters i.e. tail DNA (%), tail length (µm), tail moment and olive moment.

Statistical analysis

All data analysis was performed using SPSS statistical package, version 16.0. The continuous data was summarized as mean and standard error (S.E.). Comparisons between the groups were performed with independent sample t test. Pearson correlation was used to evaluate the relationship between variables.

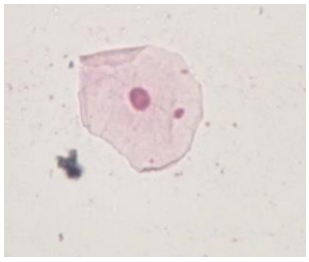
Results

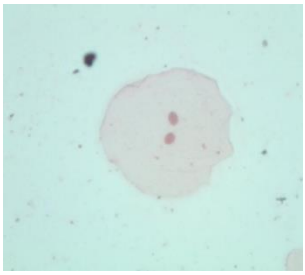
The general and clinical characteristics of the study groups are presented in Table 1. The mean duration of diabetes in T2DM patients was 7.504±0.612 years. There was no significant difference for average age and blood pressure between the two groups. However, T2DM patients had significantly ($P<0.001$) elevated fasting blood glucose than control subjects.

Table 1. General and clinical characteristics of control subjects and type 2 diabetes mellitus patients.

	Controls	T2DM
Sample size	90	106
Gender (Male/Female)	53/37	62/44
Average age (years)	53.667±1.049	55.972±1.060
Average duration of T2DM (years)	-	7.504±0.612
Fasting blood glucose (mg/dl)	89.978±0.544***	199.320±8.253***
Blood pressure (Diastolic/Systolic) (mmHg)	82.622±0.929/ 124.020±1.635	84.613±0.844/ 127.810±1.263

Data are expressed in means±S.E.; ***P<0.001.





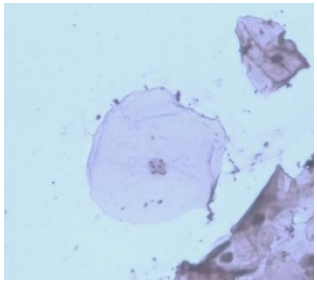


Figure 1. *Exfoliated buccal epithelial cells showing various nuclear anomalies at 1000X (A) Micronuclei; (B) Binucleated cell; (C) Karrorhexis.*

The mean frequency of MN was found to be significantly (P<0.001) higher in T2DM patients with respect to control subjects. Approximately, fivefold increase in BN frequency was observed in T2DM patients as compared to control subjects. The difference in mean frequency of BE in T2DM patients and control subjects was found to be highly significant (P<0.001). A significant (P<0.001) increase was observed in mean frequency of KL from control subjects to T2DM patients. Similarly, the mean frequency of KH in T2DM patients showed a significant difference (P<0.001) with respect to controls (Figure 1 and 2). Significant effect of age and duration of T2DM was found on MN and other nuclear anomalies in patients. Mean frequency of MN (P<0.01), BN (P<0.01), BE (P<0.001), KL (P<0.05) and KH (P<0.05) showed statistically significant positive correlation with age. Furthermore, all the nuclear anomalies showed a highly significant (P<0.001) positive correlation with duration of T2DM in patients (Table 2).

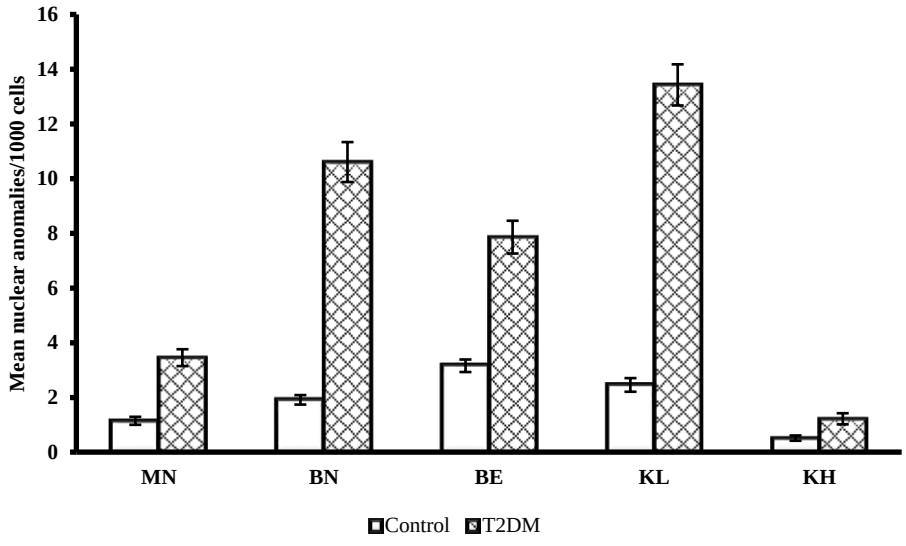


Figure 2. *Nuclear anomalies observed in control subjects and T2DM patients. Data are expressed as means±S.E. MN: Micronucleus; BN: Binucleated cell; BE: Broken egg; KL: Karyolysis; KH: Karyorrhexis.*

Comet tail parameters i.e. tail DNA (%), tail length (µm), tail moment and olive moment exhibited significantly (P<0.001) higher values in T2DM patients compared with control subjects (Figure 3 and 4). Tail DNA (%) increased up to 32.88% in T2DM patients in comparison to only 18.07% in controls (Figure 4). The extent of DNA damage as measured by the percent of DNA present in tail was positively correlated with age (P<0.001) and duration of T2DM (P<0.001) in T2DM patients (Table 2).

Figure 3. *DNA damage was visualized using comet assay in lymphocytes of type 2 diabetic patients.*

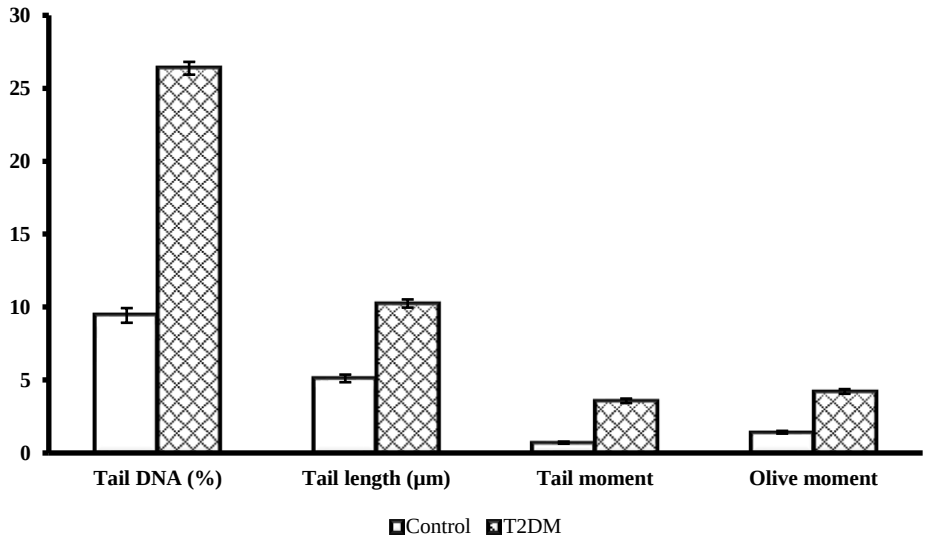


Figure 4. *Comet tail parameters observed in control subjects and T2DM patients. Data are expressed as means±S.E.*

Table 2. Pearson correlation coefficients of nuclear anomalies and tail DNA (%) according to age (years) and duration of T2DM (years) in T2DM patients.

Parameters	Age	Duration of T2DM
MN	0.278**	0.406***
BN	0.250**	0.333***
BE	0.334***	0.499***
KL	0.230*	0.381***
KH	0.202*	0.414***
Tail DNA (%)	0.465***	0.275**

MN: Micronucleus; BN: Binucleated cell; BE: Broken egg; KL: Karyolysis; KH: Karyorrhexis; *P<0.05; **P<0.01; ***P<0.001

The comparison of tail DNA (%) was done for T2DM patients and controls with lower nuclear anomalies (≤1) and higher nuclear anomalies (>1). Figure 5 shows the values of MN, BN, BE, KL and KH for the subgroups in comparison of T2DM patients and controls. The difference in tail DNA (%) between controls and T2DM patients was highly significant ($P<0.001$) in both the groups with lower and higher mean MN frequency. Similarly, tail DNA (%) showed highly significant ($P<0.001$) difference between control subjects and T2DM patients for different groups i.e. higher and lower mean frequency of BN, BE, KL and KH. Moreover, mean MN frequency is indicative of permanent DNA damage. While the comet assay parameters represents the DNA damage that still have the tendency to repair. Increased median value of tail DNA (%) in higher mean MN frequency group indicates the tail DNA (%) represents the permanent DNA damage in T2DM patients. Increased median value of tail DNA (%) was observed among the groups having higher mean frequency of BN and KH as compared to the groups having lower mean frequency of BN and KH, respectively. While the median value of tail DNA (%) was lower in groups having higher mean frequency of BE and KL as compared to groups having lower mean frequency of BE and KL, respectively.

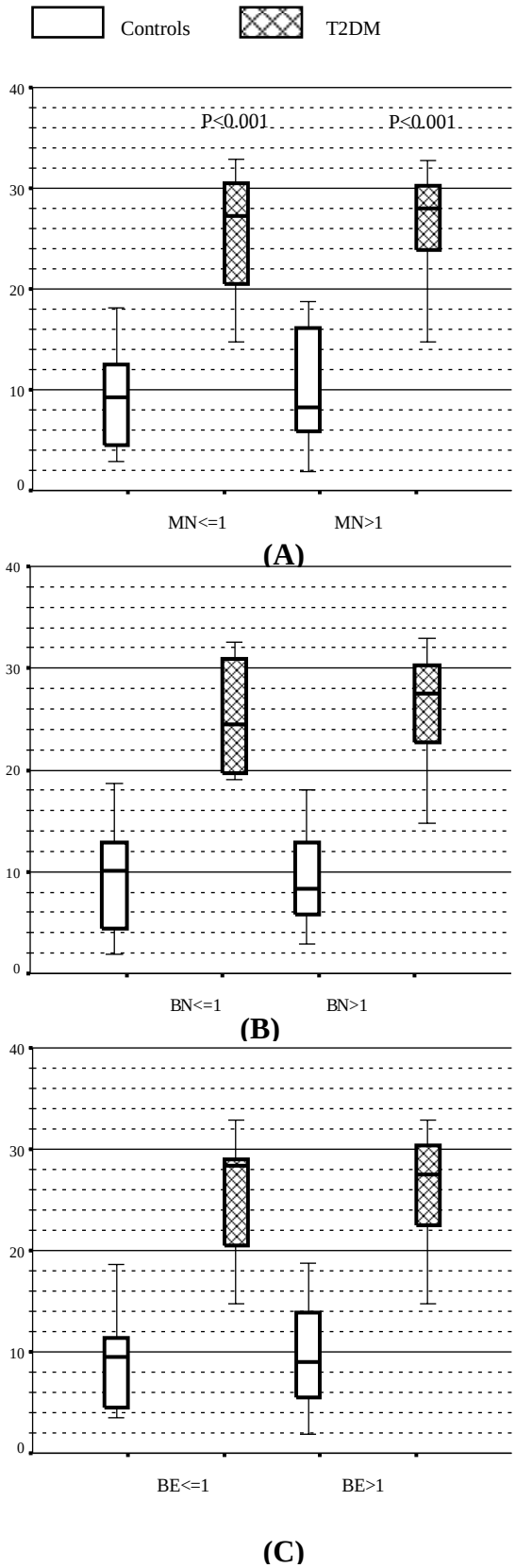


Figure 5. *Tail DNA (%) according to mean frequency of (A) micronuclei (MN), (B) binucleated cell (BN), (C) broken egg (BE), (D) karyolysis (KL) and (E) karyorrhexis (KH). Internal horizontal lines represent the median in each groups; boxes and whiskers show the interquartiles and lowest/highest ranges of individual values.*

Discussion

Long regarded as a condition with little bearing on global health, diabetes mellitus is becoming one of the biggest threats of the twenty-first century. The global number of diabetics has increased so dramatically during the last 20 years. There is often a protracted asymptomatic phase in T2DM. T2DM is typically not detected until difficulties arise, and around one-third of all people with the disease may go untreated. The chance of acquiring diabetes complications can be reduced by early diabetes screening and treatment.

In the present study, we have found a significantly increased frequency of all the nuclear anomalies in T2DM patients. Other studies with glycosylated hemoglobin and type 2 diabetes revealed similar findings, indicating an increased micronuclei frequency (Shettigar *et al.*, 2012). A prior study that used glimepride and pioglitazone together found that patients with diabetes mellitus had a higher frequency of micronuclei (Shaik *et al.*, 2010). An increase in MN frequency in T2DM Mexicans was also documented by Martinez-Perez and co-workers (2007). An increase in MN frequency in buccal cells has been reported in diabetes mellitus patients with twice the genetic damage in comparison to controls (Zuniga-Gonzalez *et al.*, 2007). The high frequency of nuclear anomalies in exfoliated buccal mucosa cells of T2DM patients could be due to an elevated level of products of oxidative stress, i.e., reactive oxygen species, which can cause protein, lipid, and DNA damage via oxidation (Jlyon *et al.*, 1998; Krapfenbauer *et al.*, 1998 and Mantena *et al.*, 2008). In previous studies with cultured human blood lymphocytes, genomic instability in patients with T2DM on hemodialysis using the CBMN test and comet assay has been reported (Palazzo *et al.*, 2012). According to research studies, premature children born to women with diabetes and arterial hypertension had higher MN formation (Batista-Gonzalez *et*

al., 2006). A few studies found a significant increase in sister chromatid exchange frequency in T2DM patients as compared with healthy controls (Sheth *et al.*, 2006).

Analysis of the results regarding the comet assay indicated substantial DNA damage in T2DM patients as cells exhibited a distorted appearance. Contrarily, the control group's compact DNA and intact circular nucleus showed no signs of comet formation, indicating that T2DM patients had more DNA damage than the control group. These findings concur with others who found that T2DM patients had greater levels of DNA damage in the alkaline comet assay than controls (Arif *et al.*, 2010; Dinçer *et al.*, 2002 and Tatsch *et al.*, 2012). Lodovici and co-workers (2008) observed that the tail DNA (%) in leukocytes of T2DM patients was significantly ($P<0.05$) higher compared to healthy subjects. Peripheral blood leukocytes from diabetic patients had more DNA strand breaks than those from healthy controls, according to Comet analysis conducted on a total of 28 T2DM patients and 25 controls (El-Wassef *et al.*, 2012). Nevertheless, some research has indicated that there is no correlation between diabetes and increased DNA damage levels (Anderson *et al.*, 1998 and Ibarra-Costilla *et al.*, 2010). A study involving tumors discovered that elevated glucose levels exacerbate DNA synthesis and trigger the formation of free radicals, which have the potential to damage DNA and enzymes involved in gene repair (Suba and Ujpal, 2006). Patients with type 2 diabetes may have weakened antioxidant defenses, including lower antioxidant levels and decreased activities of catalase, glutathione peroxidase, and superoxide dismutase (Kennel and McGee, 1979; Lean *et al.*, 1999 and Memisogullari *et al.*, 2003). Gene polymorphisms of antioxidant DNA-repairing genes are also reported to influence DNA damage (Hernández *et al.*, 2002).

According to our research, the frequency of micronuclei and other nuclear abnormalities in the buccal epithelial cells of T2DM patients is strongly influenced by their age and the length

of their diabetes. Moreover, an increased incidence of diabetes has been observed with age, which might be due to lower beta cell proliferation potential and increased beta cell apoptotic rates with advancing age in diabetes (Gunasekaran and Gannon, 2011). In contrast to the results of the current investigation, Milosevic-Djordjevic and co-workers (2002) reported a decrease in MN frequencies in the older group, which might be due to a decline in the proliferation capacity of cells with an increasing age.

Age and duration of diabetes are also associated with DNA damage observed by tail DNA (%), as demonstrated by a previous study demonstrating that older T2DM patients had far higher levels of DNA damage (Møller, 2006). Age is frequently connected to a variety of physiological issues, including a decrease in DNA repair capacity. Fenech and Morley (1985) discovered that 80-year-olds had four times more micronuclei than younger people. They found that the expression of micronuclei correlated positively ($P<0.001$) with advancing age. Ganguly (1993) observed that the aged males and females showed a larger number of aberrations, and the correlation of micronucleus formation with the donor's age is highly significant in the peripheral lymphocytes of 127 healthy individuals. Bakou and co-workers (2002) found that whole chromosomes containing micronuclei in cultured binucleated lymphocytes predominated in older females, and micronuclei containing acentric chromosome fragments were also enhanced with advancing age. On the other hand, others reported that DNA damage was not affected by age (Palazzo *et al.*, 2012 and Thakar and Jain, 2010). The researchers Tybetg and co-workers (2002) discovered a favorable correlation between the length of diabetes and the expression of DNA glycosylase 8-oxoG DNA glycosylase (Ogg I), the primary repair enzyme responsible for defense against the build-up of 8-hydroxy-2'-deoxyguanosine (8-OH-dG) adducts. Ogg I upregulation can be explained as a reaction to elevated 8-OH-dG DNA levels and the lesion that

it heals. El-Wassef and co-workers (2012) used peripheral blood leukocytes from 28 T2DM patients and 25 healthy volunteers and reported no connection between DNA damage and length of diabetes.

Patients with type 2 diabetes had statistically higher levels of genetic damage than control subjects when tail DNA (%) was compared between T2DM patients and control participants for various nuclear abnormalities. The findings of the current study suggested that the greater the mean frequency of MN, the higher the lesion in DNA that has been misrepaired. Hence, an increase in tail DNA (%) corresponding to the mean MN frequency revealed permanent DNA damage in T2DM patients.

In summary, the results of the present study demonstrate that T2DM patients are manifested by increased mean frequencies of all the nuclear anomalies and tail DNA (%) as compared to age- and gender-matched control subjects. In T2DM patients, DNA damage is substantially correlated with both the length of diabetes and increasing age. Hence, T2DM results in DNA damage in patients.

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Disclosure

The authors declare no conflict of interest.

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