ISSN: 0975-833X

Available online at http://www.journalcra.com

International Journal of Current Research
Vol. 8, Issue, 11, pp.42303-42307, November, 2016

INTERNATIONAL JOURNAL OF CURRENT RESEARCH

RESEARCH ARTICLE

ALKALINE SINGLE CELL GEL ELECTROPHORESIS: A RAPID TECHNIQUE TO EVALUATE DNA DAMAGE IN TYPE 2 DIABETES MELLITUS PATIENTS

***,[1]Priyanka Biswas, [2]Dr. Prasanta Bhattacharya and [1]Dr. Madhusnata De**

[1]Department of Genetics, Vivekananda Institute of Medical Science, Ramakrishna Mission Seva Pratishthan, Kolkata- 700026

[2]Department of Surgery, Ramakrishna Mission Seva Pratishthan, Vivekananda Institute of Medical Sciences, 99 Sarat Bose Road, Kolkata-700026

ARTICLE INFO

Article History:
Received 15th August, 2016
Received in revised form
28th September, 2016
Accepted 17th October, 2016
Published online 30th November, 2016

Key words:

Oxidative stress,
DNA damage,
Comet assay,
Genomic instability.

ABSTRACT

Type 2 Diabetes mellitus (T2DM) is a chronic progressive disease affecting a significant proportion of the population and becoming an epidemic worldwide. Increasing evidence in both experimental and clinical studies suggest oxidative stress (OS) plays a major role in the pathogenesis of both types of diabetes mellitus and its complications. OS leads to damage of cellular biomolecules such as lipids, proteins and DNA. OS enhance the extent of DNA damage. Our objective is to detect the extent of DNA damage and to investigate the relationship between DNA damage and oxidative stress in T2DM patients and healthy controls. A case-control study of forty seven patients with T2DM (n=47) and sixteen healthy control subjects (n=16) were screened from different areas of West Bengal. All procedures were done with the informed consent of participants. Comet assay was used to detect DNA damage. Hence, in the present study, increased DNA damage was observed in T2DM patients than healthy controls that may be due to increased oxidative stress leading to genomic instability, which in turn may lead to the progression of T2DM and its complications and contribute to an increased risk for cancer. Therefore this study will explore the role of oxidative stress for genomic instability and causing cancer.

Citation: Priyanka Biswas, Dr. Prasanta Bhattacharya and Dr. Madhusnata De, 2016. "Alkaline single cell gel electrophoresis: A rapid technique to evaluate DNA damage in type 2 diabetes mellitus patients", *International Journal of Current Research*, 8, (11), 42303-42307.

INTRODUCTION

Type 2 diabetes mellitus (T2DM), also known as non-insulin dependent diabetes mellitus (NIDDM), is a metabolic disorder characterized by high blood glucose in association with insulin resistance and relative insulin deficiency. It is becoming one of the most challenging health problems in the 21st century as it has reached epidemic proportions worldwide. 285 million people are known to be suffering from T2DM globally which is expected to increase to 438 million in 2030. India ranked first in the world for the prevalence of the disease, followed by China and USA (Wild *et al*., 2004). The high prevalence of T2DM in India specifically West Bengal is a serious public health concern because it is the third highest cause of mortality (Ibarra-Costilla *et al*., 2009). Patients with type 2 diabetes mellitus suffer from various macro and microvascular complications such as atherosclerosis, neuropathy, retinopathy and nephropathy which lead to a devastating effect on morbidity and mortality (Dandona *et al*., 1996). Increasing evidence in both experimental and clinical studies suggest hyperglycemia results in the production and manifestation of reactive oxygen species (ROS), ultimately leading to increased oxidative stress in a variety of tissues, and playing an important role in late diabetic complications (Chandie Shaw *et al*., 2006; Yfjord and Bodvarsdottir, 2005) Oxidative stress (OS) leads to protein, lipid, and DNA modifications that cause cellular dysfunction and progression of late complications. Patients with type 2 diabetes mellitus has been associated with elevated levels of DNA damage, increased susceptibility to mutagens, and a decreased efficacy of DNA repair, causing genomic instability and consequently cancer. Previous studies (Dandona *et al*., 1996; Pitozzi *et al*., 2003; Choi *et al*., 2005; Blasiak *et al*., 2004) have reported that leukocytes from T2DM patients present a significant increase in DNA damage, measured by the comet assay. During the last few years, there has been a great interest in developing rapid and simple tests to identify the effects of DNA damage in chronic diseases. One of these methods is the Single cell gel electrophoresis (SCGE) or comet assay, which is a rapid and sensitive technique to measure DNA breaks in individual cells and alkali labile sites. It has been widely used in studies on DNA repair, genetic toxicology,

Corresponding author: Priyanka Biswas,
Department of Genetics, Vivekananda Institute of Medical Science, Ramakrishna Mission Seva Pratishthan, Kolkata- 700026.

radiation, pollution and ageing (Haines *et al*., 2002; Moller and Loft, 2002). This method was developed for the first time by Ostling and Johanson in 1984 and in 1988, Singh *et al*. introduced alkaline conditions to this technique (Östling and Johansson, 1984; Singh *et al*., 1988). Thus, our study aims to assess the DNA damage evaluated by comet assay (length of tail comet (LTC), tail extent moment (TEM), and olive tail moment (OTM) in patients with type 2 diabetes mellitus in population of West Bengal.

MATERIALS AND METHODS

Study setting and subjects

A sample of forty seven patients with T2DM (n=47) and sixteen healthy control subjects (n=16) were recruited from different areas of West Bengal. The patients were confirmed of having T2DM by impaired fasting glucose test (>126 mg/dl) and oral glucose tolerance test (>200 mg/dl). The controls had normal glucose metabolism and none had a family history of diabetes. Detailed personal histories were collected from the participants with the help of questionnaire for collection of demographic data and recording of relevant medical history and medications. All procedures were done with the informed consent of participants. We had excluded the patients who had fever, acute and chronic infections, malignancy, acute and chronic nephritis, cirrhosis, and congestive heart failure. All the patients were under stable conditions during assessment.

Collection of blood

Peripheral blood samples (5 ml) were withdrawn by venipuncture from both T2DM patients and healthy individuals into sterile vacutainer tubes (Becton Dickinson (BD), USA) containing K_3EDTA as anticoagulant. Blood was collected at a fixed time of the day (9.30-10.30 hours) to minimize diurnal variation.

Alkaline Single Cell Gel Electrophoresis (Comet assay) for the evaluation of DNA damage

Comet assay in Peripheral blood lymphocytes (PBL)

Analysis of DNA damage in PBL was done following the procedure of Singh *et al*. (1988) as modified by Zhu *et al*. (1999) (Singh *et al*., 1988; Zhu *et al*., 1999).

Lymphocyte Separation

EDTA-anticoagulated whole blood was centrifuged for 30 mins at 400 g at 20°C with Ficoll-Paque (Sigma Chem, USA) or Histopaque®-1077 following the instruction of the manufacturer. This procedure separated a buffy coat layer of lymphocyte by the density gradient centrifugation technique. The lymphocytes in the buffy coat were aspirated and washed twice by phosphate-buffered saline (PBS) at pH 7.4.

Preparation of cell microgels on slides

Cell microgels were prepared as layers. The first gel layer was prepared with 100 µl of 0.1% high-melting point agarose onto precleaned frosted microscopic slide and coverslipped gently. Thereafter the cover glass was removed after solidification of agarose at 4^0C. 70 µl of 0.6% low-melting point agarose was applied to the slide, covered with a cover glass and kept at 4^0C for another 5 mins to solidify. There after, 20 µl of the lymphocyte suspension was mixed with 80 µl of 0.8% low-melting point agarose. After gently removing the cover glass, 85 µl of this cell suspension was immediately added onto the agarose layer, spread with a cover slip, and again kept at 4^0C for 10 mins to solidify. Then the cover glass was removed and 60 µl of 0.8% low melting agarose was added and left to solidify at 4^0C. Then the coverslips were removed.

Lysis of cells, DNA unwinding, gel electrophoresis, DNA staining

The slides were immersed in freshly prepared lysis solution (2.5 mol/L NaCl, 100 mmol/L EDTA, 1% sodium hydroxide, 10 mmol/L Tris, 1% Triton X-100, 10% DMSO (pH 10)) for 1 hr at 4^0C. After lysis, the slides were washed with electrophoretic buffer and then placed on a horizontal electrophoresis tank filled with fresh electrophoresis solution to a level approximately 0.25 cm above the slides for 20 mins to allow the unwinding of the DNA and expression of alkali labile damage before electrophoresis. Gels were run with constant electric current of 25 V and 300 mA was applied for 30 min at 4^0C. All of these steps were conducted under dimmed light to prevent additional DNA damage. After electrophoresis, the microgels were gently washed to neutralize excess alkali by placing the slides horizontally and flooding them with neutralization buffer (0.4 M Trisma base at pH 7.5). The slides were stained with 25 µl of ethidium bromide (10 µg/mL) and visualized under a fluorescence microscope (Olympus, Model No.CX41).

Observation and scoring

For each slide, 100 chosen cells were scored randomly under a fluorescence microscope. A computerized image analysis system (Komet version 5.5, Kinetic Imaging Ltd., Liverpool, UK) was equipped to measure various comet parameters. Damaged cells were analysed by the "comet appearance", with a bright fluorescent head and a tail formed in one side by the DNA containing strand breaks that were moved away during electrophoresis.

RESULTS

The clinical characteristics of the study participants are listed in Table 1. The mean age of theT2DM patients was 63.2±15.8 years ranging between 40 - 77 years, with a mean duration of the diabetes of 8.7±6.4 years (range: 1–25 years). Thirty eight patients (80.85%) had a family history of T2DM in our study. Diabetic individuals had higher BMI (basal metabolic rate) than the healthy controls. Impaired fasting glucose, Oral glucose tolerance test and HbA1C were significantly higher in T2DM patients than the healthy controls. The study participants included both men and women along with personal history of education, smoking, addiction of alcohol, betel quid or antioxidant supplement consumption, or the practice of exercise. Analysis of DNA damage in lymphocytes of patients with T2DM and the healthy control evaluated by comet assay are summarized in Table II. Most cells (79%) in T2DM patients contained DNA strand breaks, which was higher (Fig. 1) whereas, lymphocytes with DNA strand breaks were very few or almost absent in the control group (Fig. 2). The comet tail length was higher in T2DM patients (19.2 ± 5.7µm) compared to healthy controls (11.1 ± 6.6µm).

Table I. General characteristics of the study participants

	Controls	T2DM patients
Number of subjects	16	47
Gender (males/females)	9/7	31/16
Age (years) (mean ± SD)	57.6±10.1	63.2±15.8
Duration of diabetes (years) (mean ± SD)	-	8.7±6.4
Impaired fasting glucose test(mean ± SD)	97.2±15.8	149.3±27.2
Oral glucose test tolerance(mean ± SD)	140.1±22.9	226.9±48.8
HbA1C(%)(mean ± SD)	4.2±0.47	7.54±2.1
BMI(kg/m^2) (mean ± SD)	23.4±3.89	30.6±3.8
Addiction (Tobacco/ Alcohol/ Betel Quid)	(5/3/5)	(12/5/4)
Habit (Exercise)	7	24
Education (None/ Elementary/ Bachelor)	(3/9/4)	(9/25/13)
Antioxidant Supplementation	2%	9%

(N.B.: SD: standard deviation, BMI: basal metabolic rate).

Table II: Comet Parameters of the study participants

COMET PARAMETERS	T2DM n=47	Healthy Control n=16
Comet tail DNA (%)		
Mean ± SD	25.7 ± 7.6	20.8 ± 7.4
Median (range)	25.1 (35.7 - 14.33)	19.3 (37.4 – 11.3)
Tail length (µm)		
Mean ± SD	19.2 ± 5.7	11.1 ± 6.6
Median (range)	17.2 (27.52 - 14.92)	10.4 (36.56 – 3.0)
Olive tail moment (in arbitrary unit)		
Mean ± SD	2.86 ± 1.37	2.23 ± 1.05
Median (range)	3.48 (4.46-1.41)	2.35 (3.58-1.19)

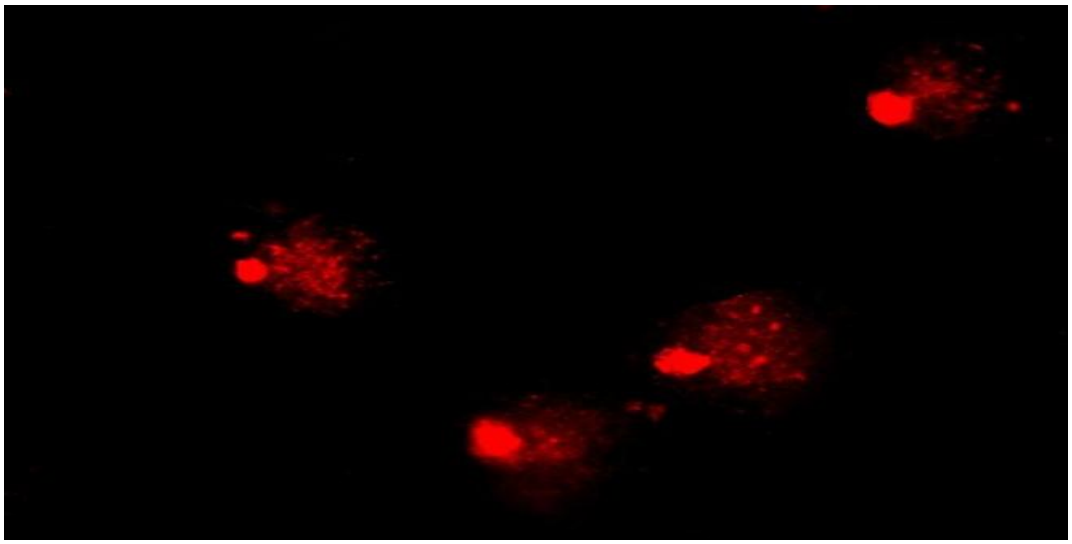

Fig. 1. Comet image of peripheral blood lymphocytes showing DNA strand breaks in patients with T2DM

Fig. 2. Comet image of peripheral blood lymphocytes showing DNA strand breaks in healthy controls

DISCUSSION

Type 2 Diabetes mellitus is associated with OS that leads to protein, lipid and DNA modifications causing cellular dysfunction. This can enhance the teratogenic or carcinogenic consequences (Jee *et al*., 2005). There are increasing evidences obtained from both experimental and clinical studies suggesting that a close association has been found among hyperglycemia, OS and diabetic complications. It has also been reported that OS contributes to the pathological processes of late diabetic complications (Mehrotra *et al*., 2011). The production of ROS are increased in T2DM patients that enhance the extent of DNA damage. ROS may play an important role in the pathogenesis of T2DM and its complications. The presence of an increased OS in diabetic patients is a well established fact. Under conditions of OS, damage to cellular biomolecules such as lipids, proteins and DNA occurs. Oxygen-free radicals induce a variety of lesions in DNA, including oxidized bases, abasic sites, DNA strand breaks and formation of cross-links between DNA and proteins. Many of these lesions are cytotoxic and mutagenic (Dandona *et al*., 1996). It is also well documented in previous studies that diabetes enhances the risk of cancer but till date the underlying mechanism is not clearly understood. Although there are many risk factors that are shared between cancers and diabetic patients, it is well established that an increased OS is responsible which contribute to genomic instability (Limoli and Erich, 2003; Sudhaa Anand *et al*., 2014) directly or indirectly. Several epidemiologic studies reported higher significant risk of cancer in diabetic patients and were 20% more in diabetic patients (Hyun *et al*., 2013; Zendehdel *et al*., 2003). Diabetics are known to have an increased risk of renal cell carcinoma by 40% in both male and females. In the present study, we attempted to use the comet assay to measure the extent of DNA strand-break damage induced by T2DM, as the technique is mostly reliable and less susceptible to artefacts. It is also a highly sensitive, simple method to detect very low levels of damage (Collins *et al*., 2004). The current study revealed that the number of DNA strand breaks in peripheral blood lymphocytes was significantly increased in patients with T2DM than the healthy controls. Increased DNA damage in T2DM patients may be due to increased OS leading to oxidative damage that results in genomic instability, which in turn may contribute to an increased risk for cancer. Previous studies relating DNA damage and diabetes showed contradictory results. Several studies revealed an increased extent of DNA damage in T2DM patients compared to controls (Choi *et al*., 2005; Pitozzi *et al*., 2003; Blasiak *et al*., 2004). The variation between different studies may be due to difference in duration of diabetes, glycemic control or the type of cell used in the comet assay (Blasiak *et al*., 2004). In conclusion, diabetic patients have more severe DNA damage than the healthy controls. Extent of DNA damage is increased in T2DM patients, depicting that increased OS may be associated with the progression of T2DM and its complications. Assessment of oxidative stress in diabetic patients may be important in predicting and preventing oxidative stress-related complications.

Abbreviations: T2DM, NIDDM, BMI, HbA1C, ROS, OS.

Acknowledgment

I would like to acknowledge our Secretary, Ramakrishna Mission Seva Pratishthan, DST, West Bengal, colleagues of our department and all the patients who participated in this study.

Author Statements

Ethical approval: This study was approved by the institutional ethical committee on 10th May, 2016. Reg No. ECR/62/Inst/WB/2013 issued under Rule 122DD of the Drugs & Cosmetics Rules 1945.

Funding: DST, West Bengal.

Conflict of interest: None declared.

REFERENCES

Blasiak J, Arabski M, Krupa R, *et al*. 2004. "DNA damage and repair in type 2 diabetes mellitus," *Mutat Res*., 554:297–304.

Chandie Shaw P, Baboe F, van Es L, *et al*. 2006. "SouthAsian type 2 diabetic patients have higher incidence and faster progression of renal disease compared with Dutch-European diabetic patients," *Diabetes Care*, 29(6):1383-1385.

Choi S, Benzie I, Lam C. 2005. "Inter-relationships between DNA damage, ascorbic acid and glycaemic control in type 2 diabetes mellitus," *Diabet Med*., 22:1347-1353.

Collins A, Cadet J, Moller L, *et al*. 2004. "Are we sure we know how to measure 8-oxo-7, 8-dihydroguanine in DNA from human cells?," *Arch Biochem Biophys*, 423:57-65.

Dandona P, Thusu K, Cook S, *et al*. 1996. Oxidative damage to DNA in diabetes mellitus. *Lancet*, 347:444-445.

Haines G, Hendry J, Daniel C, *et al*. 2002. "Germ cell and dose-dependent DNA damage measured by the comet assay in murine spermatozoa after testicular X-irradiation," *BioReprod*, 67(3):854-861.

Hyun HC, Jun SM, Ji SY *et al*. 2013. "The relationship between metformin patients with type 2 diabetes," *Diabetes Metabolism J*, 37, 125-31.

Ibarra-Costilla E, Cerda-Flore R, Da'vilaRodriguez M, *et al*. 2009. " DNA damage evaluated by comet assay in Mexican patients with type 2 diabetes mellitus," *Acta Diabetol*, DOI 10.1007/s00592-009-0149-9.

Jee S, Ohrr H, Yun J, *et al*. 2005. "Fasting serum glucose level and cancer risk in Korean men and women," *JAMA*, 293(2):194-202.

Limoli C, Erich G. 2003. "Induction of chromosomal oxidative stress," Neoplasia, 4, 339-46.

Mehrotra S, Ling K L, Bekele Y, *et al*. 2001. "Lipid hydroperoxide and markers of renal disease susceptibility in African-Caribbean and Caucasian patients with Type 2 diabetes mellitus," *Diabet Med*., 18(2): 109-115.

Moller P, Loft S, 2002. "Oxidative DNA damage in human white blood cells in dietary antioxidant intervention studies," *Am J Clin Nutr*., 76(2):303-310.

Ōstling O, Johansson K. 1984. "Microelectrophoretic study of radiation induced DNA damages in individual mammalian cells," *Biochem Biophys Res Commun*., 123:291-298.

Pitozzi V, Giovannelli L, Bardini G, Rotella CM, Dolara P, 2003. "Oxidative DNA damage in peripheral blood cells in type 2 diabetes mellitus: higher vulnerability of polymorphonuclear leukocytes," *Mutat Res*., 529:129–133.

Singh N, McCoy M, Tice R, *et al*. 1988. "A simple technique for quantitation of low levels of DNA damage in individual cells," *Exp Cell Res*., 175:184-191.

Sudhaa Anand *et al*. 2014. "Diabetes - Increased Risk for Cancers through Chromosomal Aberrations?," *APJCP*, 15(11): 4571-4573.

Yfjord J, Bodvarsdottir S. 2005. "Genomic instability and cancer: networks involved in response to DNA damage," *Mutat Res*., 592:18-28.

Zendehdel K, Nyren O, Ostenson CG, *et al*. 2003. "Cancer in patients with type 1 diabetes mellitus: a population-based cohort study in Sweden," *J Natl Cancer Inst*., 95:1797-800.

Zhu CQ, Lam TH, Jiang CQ, Wei BX, Lou X, Liu WW, Lao XQ, Chen YH, 1999. "Lymphocyte DNA damage in cigarette factory workers measured by the Comet Assay," *Mutat Res*., 444: 1-6.

****