Indian J Med Res 148, October 2018, pp 435-440
DOI: 10.4103/ijmr.IJMR_620_17

Quick Response Code:

Oxidative stress in metabolic syndrome & its association with DNA-strand break

Rinchen Doma Bhutia[1], Mingma Lhamu Sherpa[1], T.A. Singh[1] & Bidita Khandelwal[2]

Departments of [1]Biochemistry & [2]Medicine, Sikkim Manipal Institute of Medical Sciences, Gangtok, India

Received April 16, 2017

***Background & objectives*: Oxidative stress (OS) is associated with numerous components of metabolic syndrome (MetS). This study was aimed to investigate if hydrogen peroxide (H_2O_2) as the reactive oxygen species was capable of depicting OS in MetS, and If MetS patients showed DNA damage in the form of DNA strand breaks (DSB).**

***Methods*: A total of 160 participants (90 males, 70 females) ≥20 yr of age were categorized into four groups based on the number of MetS risk parameters (n=40 in each group). Sugar and lipid profile, H_2O_2 concentration in blood and DNA-strand breaks were measured.**

***Results*: DSB was significantly more in those with MetS (n=40) than those without (n=120) whereas H_2O_2 levels were the same in both the study groups. The number of DSB differed significantly between the control and 3 risk factor groups. DSB was also higher in groups with 2 and 1 risk factors compared to 0 risk but the difference was not significant. H_2O_2 level was higher in groups with 3, 2 and 1 risk factors compared to 0 risk group but the difference was not significant. The H_2O_2 level correlated positively with triglyceride values but not with other MetS risk parameters. There was no significant correlation between DSB and MetS risk parameters.**

***Interpretation & conclusions*: Our findings showed a cumulative and synergistic effect of the risk factors of MetS on DSB. Individuals with three risk parameters had a greater effect on DNA damage than in those with two or one risk parameter. Although plasma H_2O_2 level increased with an increase in the fat depots, use of H_2O_2 to depict OS in MetS should be coupled with an adjunct and estimation of DSB in peripheral blood lymphocytes may be used as indicator of OS in MetS patients.**

Key words DNA-strand break - hydrogen peroxide - metabolic syndrome - oxidative stress - ROS

DNA damage has been reported to be the primary cause of cancers including those of lung[1] colorectal[2], breast[3] and prostate[4]. There are both exogenous and endogenous sources that could induce DNA damage. Water, oxygen and base-pairing errors incorporated into DNA at the time of replication are the examples of spontaneous endogenous sources of damage. Hydrolytic DNA damage causes deamination of bases on DNA and/or removal of individual bases[5]. This loss of DNA bases, known as apurinic/apyrimidinic sites, can be mutagenic and if left unrepaired, can inhibit transcription. Another

cause of endogenous DNA damage is the oxidative stress (OS).

Cells use oxygen to produce ATP and water, and in the process also produce toxic by-products called reactive oxygen species (ROS) at low levels which are detoxified and removed with antioxidants. However, mitochondrial dysfunction during pathophysiological conditions impairs the electron transport mechanism and generates excess ROS such as the hydroxyl and superoxide radicals[6], and their targets are guanine and thymine in DNA. Exogenous DNA-damaging agents, for instance, the ionizing radiations can also lead to the production of excess ROS[5]. The ROS or hydroxyl radical can further induce DNA-strand break by targeting the sugar residue of the DNA backbone. Hence, if the generation of ROS due to any unfavourable situation exceeds the body's capacity to detoxify these through antioxidants it would result in increased damage to DNA. The increase in DNA damage enhances the cellular load to correct the mistake especially DNA-strand breaks. Fixing a break in the DNA strand is a complicated process, and it is more likely that the body will tend to make mistakes when attempting the repair[7]. Accumulation of such mistakes may lead to genomic instability[7]. An example of such genomic instability is the increased risks of cancers (colorectal, endometrial and breast) in adults with metabolic syndrome (MetS) especially in women[8].

DNA damage is closely associated with the risk parameters of MetS[9]. Elevated blood pressure, higher visceral fat and glycated haemoglobin are all known to be associated with OS[10], and OS, in turn, causes damages to DNA, lipid and protein. Patients with MetS have been noted to have decreased antioxidant protection, in the form of depressed vitamin C and α-tocopherol concentrations, decreased superoxide dismutase activity and elevated oxidative damage as evidenced by increased lipid peroxidation (LPx) products such as malondialdehyde levels and protein oxidative products such as protein carbonyl and xanthine oxidase activity[11]. Of the OS parameters, we studied hydrogen peroxide as the ROS in MetS, H_2O_2 is a more direct marker of OS. It is 'diffusible' and thus crosses the cell membranes through aquaporins. While inside the cell H_2O_2 is converted into another highly reactive hydroxyl radical, this radical attacks DNA at the sugar residue of the DNA backbone, that leads to single strand breaks, an indicator of increased oxidative stress[12]. This study was conducted to investigate if H_2O_2, a direct marker of OS indicated OS in individuals with MetS, and if MetS patients demonstrated DNA damage in the form of DNA-strand break.

Material & Methods

This was a hospital-based cross-sectional study conducted from March 2014 to December 2016 in the department of Biochemistry, Central Referral Hospital, Sikkim Manipal Institute of Medical Sciences, Gangtok, Sikkim, India. Sample selection and collection were performed in the hospital's phlebotomy laboratory after obtaining permission from the Institutional Ethics Committee [IEC/192/12-05(a)]. Patients attending medicine outpatients department (also including those who came for annual health check-ups) with a requisition for biochemical investigations such as fasting blood sugar and lipid profile were enrolled for the study after obtaining informed written consent. General information on age, sex, anthropometric measurements, ethnicity, smoking/alcohol habits, present medications and history of the past and present diseases was recorded. Blood pressure and waist circumference of all participants were measured using standardized procedures. Blood pressure was recorded by an auscultatory method using sphygmomanometer (Life line, India). After the patient was comfortably seated, an average of two readings was taken at an interval of two minutes. Waist circumference was measured using a non-stretchable tape at the umbilical scar level in between the lowest rib and iliac crest. A volume of 3 ml of fasting blood sample drawn by the hospital phlebotomists was used to measure the fasting blood sugar and lipid profile in an ERBA Manheim EM 200 full autoanalyzer, USA. The blood sample was also used for estimating hydrogen peroxide using the H_2O_2 colorimetric detection kit (Arbor Assay, USA) in a Lab Life ER 2007, Microplate Reader (India). Cell lymphocytes required for DNA damage study were separated using Histopaque-1077 (Sigma-Aldrich, USA); 1 ml of heparinized blood was layered over 1 ml histopaque and centrifuged at 2000 rpm (376 × *g* for Eppendorf centrifuge 5424R model, Germany) for 35 min. The buffy coat was aspirated into 1.5 ml of phosphate-buffered saline (PBS), *p*H 7.4 and centrifuged at 1800 rpm (211× *g*) for 15 min to pellet the lymphocytes. The pellet was suspended in 1 ml of PBS and counted over a haemocytometer (HBG Germany). Nearly 1×10^5 cells/ml were resuspended in ice-cold PBS. Cells were combined with agarose and processed as per the 'OxiSelect'- Comet assay kit protocol (OxiSelect, Cell Biolabs, USA). Estimation of DNA damage as DNA-strand breaks was thereafter

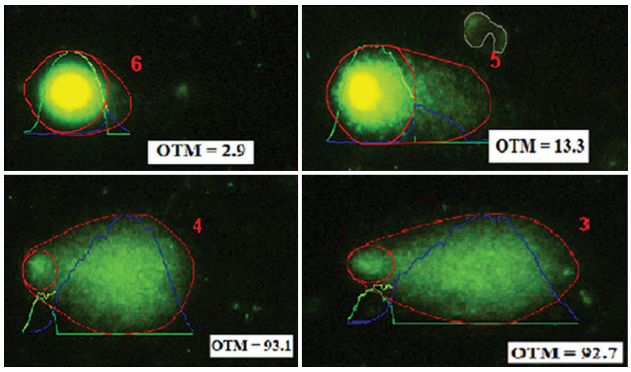

Fig. 1. Different levels of DNA damage as DNA-strand breaks (obtained from a single-cell suspension). Higher the olive tail moment value greater is the damage. The characteristic red profile corresponds to the comet border, green profile is the comet head, yellow profile is the comet tail and blue profile is the comet head and tail, as demarcated by the 'OpenComet' software.

calculated using the Comet assay software tool the OpenComet by Gyori *et al*[13] and represented in terms of Olive tail movement (OTM) (Fig. 1). Submarine electrophoretic chamber was from Bangalore Genei (India) and inverted routine microscope (ECLIPSE TS100) with Epi-fluorescence attachment was from Nikon Instruments Inc., America.

Participants (male and female ≥20 yr of age) were evaluated for MetS risk parameters following Harmonized International Diabetes Federation (IDF) definition[14]. MetS was diagnosed when the patient had three or more of the following five: fasting glucose ≥100 mg/dl (or receiving drug therapy for hyperglycaemia), blood pressure ≥130/85 mmHg (or receiving drug therapy for hypertension), triglycerides ≥150 mg/dl (or receiving drug therapy for hypertriglyceridaemia) and high-density lipoprotein-cholesterol (HDL-C) <40 mg/dl in men or <50 mg/dl in women (or receiving drug therapy for reduced HDL-C). Waist circumference is ethnic-specific: ≥90 cm in men or ≥80 cm in women for Asian Indians. Based on the presence of number of risk factors, the study participants were subdivided into four study groups. Group 1 was MetS diagnosed participants that had three or more risks, group 2 included those having two risk factors, for example, elevated blood pressure and raised fasting blood sugar, group 3 individuals had only one risk factor, and group 4 was the control group.

Participants ≥20 yr of age and not under long-term medication for any diseases other than for diabetes, hypertension and dyslipidaemia were included in the study. Pregnant women, smokers and alcohol users were excluded. Sample size for pairwise comparison was calculated according to Wang *et al*[15]. Assuming five per cent level of significance (α=0.05), power 80 per cent (β=0.84), mean difference (δ) of 67.5 and standard deviation (σ) of 60.2, a sample size (n)=12 was found in each group. However, 40 participants were enrolled under each group.

Statistical analysis: Comparison of DNA-strand break (DSB) and H_2O_2 concentration in the participants with and without MetS was determined using the Mann-Whitney U-test. These two parameters were also compared across those with 3, 2, 1 and 0 risk factors for MetS following the Kruskal-Wallis H test. Pairwise comparisons were performed using Dunn's procedure with a Bonferroni correction for multiple comparisons and for this statistical significance was accepted at P<0.012. Values were median scores unless otherwise stated. A Spearman's correlation coefficient was used to determine association between hydrogen peroxide concentration and the risk parameters of MetS, and between DNA-strand break and the risk parameters of MetS. Finally, to identify the parameters of MetS that contributed significantly to DNA-strand break and H_2O_2, a multiple regression analysis was conducted and a P<0.01 was considered significant. All statistical analysis was performed using SPSS 16 software package (SPSS Inc, Chicago, IL, USA).

Results

The median OTM for DSB was significantly higher in MetS (11.1) and lower in non-MetS (8.4), at P=0.001. The H_2O_2 level was 2.9 µg/ml in both the groups. This difference in observation for DSB and H_2O_2 between MetS and non-MetS groups was further examined and confirmed by comparing them across participants now categorized into 3 risk (n=40), 2 risk (n=40), 1 risk (n=40) and 0 risk (n=40). The H_2O_2 level was higher in those with 3, 2 and 1 risk factors when compared to control group (0 risk factor) but the difference was not significant (Fig. 2). DNA damage in the form of DSB differed significantly across 0-3 risk (P=0.001). DNA damage was also more in those with 2 and 1 risk factors when compared to control but this difference was not significant (Fig. 2).

The associations between H_2O_2 and DSB with the risk parameters of MetS were further analysed, for which the correlation coefficients are summarized in Tables I and II, respectively. H_2O_2 values correlated positively with parameters of waist circumference

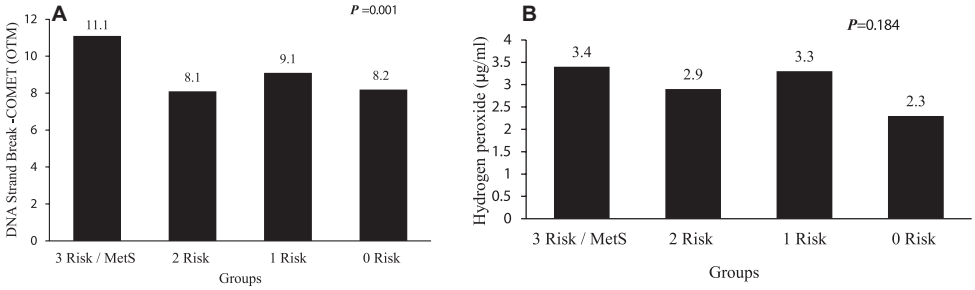

Fig. 2. Comparison of DNA-strand break and H_2O_2 levels in 3, 2, 1 and 0 risk groups of MetS (**A**) COMET; Olive tail movement (OTM) values significant at *P*=0.001. Pairwise *post hoc* analysis showed significant differences (*P*<0.012) between 0 and 3 risks at *P*=0.003, but not with 0-1 risk at *P*=0.145 and 0-2 risk at *P*=1.000. (**B**) Hydrogen peroxide (µg/ml) level compared between groups were not significant (*P*=0.184). H_2O_2, hydrogen peroxide; MetS, metabolic syndrome.

Table I. Spearman's correlation coefficient for association between hydrogen peroxide concentration and the five diagnostic risk parameters of metabolic syndrome

Risk parameters	r	*P*
Waist circumference	0.194	0.014
Systolic blood pressure	0.113	0.156
Diastolic blood pressure	0.093	0.244
Fasting blood sugar	0.010	0.895
Triglyceride	0.344	0.001
HDL-C	0.024	0.762

HDL-C, high-density lipoprotein-cholesterol

Table II. Spearman's correlation coefficients for association between DNA-strand breaks and the five diagnostic risk parameters of metabolic syndrome

Risk parameters	r	*P*
Waist circumference	0.093	0.241
Systolic blood pressure	0.181	0.053
Diastolic blood pressure	0.118	0.137
Fasting blood sugar	0.132	0.097
Triglyceride	0.017	0.142
HDL-C	−0.006	0.936

and triglyceride levels only. There was no significant correlation between DSB and MetS risk parameters.

To examine if the positive correlation between waist circumference and triglycerides with hydrogen peroxide concentration was independent of the confounders associated with MetS including age, gender, blood pressure, abdominal obesity and low HDL-C, a multiple regression analysis were performed (Table III). The multiple regression model significantly predicted hydrogen peroxide concentration, at *P*=0.001. Triglyceride concentration was the independent determinant of hydrogen peroxide concentration at *P*=0.001 whereas waist circumference was not an independent determinant at *P*=0.401.

Table III. Summary multiple regression analysis with hydrogen peroxide as the dependent variable

Variable	Regression coefficient	SE of the coefficient	Standardized coefficient	*P*
TG (mg/dl)	0.008	0.015	0.061	0.001
WC (cm)	0.012	0.001	0.432	0.401

SE, standard error; TG, triglyceride; WC, waist circumference

Discussion

ROS can cause damage to DNA, lipids and proteins[16]. OS is one of the chief characteristic features observed in MetS[17] and has been suggested to be an early-onset player in the pathophysiology of atherosclerosis, hypertension and type 2 diabetes mellitus[18]. In this study, the focus was on H_2O_2 as the ROS in MetS and its association with MetS risk parameters was investigated with special reference to DNA damage indicated by strand breaks in DNA.

Lee *et al*[19] have reported that H_2O_2 has a great potential as a diagnostic biomarker of inflammatory responses. There have been attempts in developing strategies to detect H_2O_2 at physiological concentrations[17]. An imbalance in H_2O_2 production leads to OS and inflammation that leads to the onset and advancements of various life-threatening disorders[20]. Plasma H_2O_2 concentration was found to be higher in those having 3, 2 and 1 risk parameters for MetS when compared to those with 0 risk. A value of ≥50 µM H_2O_2 has been suggested cytotoxic to a wide range of animal, plant and bacterial cells in culture[21].

In our study, H_2O_2 level was 0.098 µM higher in the 3 risks group and 0.078 µM lower in those with the 0 risk group. Studies claim substantial levels of H_2O_2 up to ~35 µM in human blood plasma[22] while others have claimed levels to be very low[23]. Systemic H_2O_2 are known to have dichotomous effects; good and bad. Harmful effects have been attributed to H_2O_2 as the ROS while the beneficial effects include intracellular signalling molecule in vascular cell apoptosis[24], modulation of intracellular Ca^{2+} levels and cell proliferation and differentiation[25]. Furthermore, positive correlation of H_2O_2 with triglyceride levels suggested the possible role of fats in increasing the H_2O_2 concentration. Fatty acid supports the formation of H_2O_2[26]. Comparison of H_2O_2 as an OS biomarker by Bloomer *et al* in obese and non-obese mice also demonstrated a linear rise in H_2O_2 every 2 hourly in 6 h time following response to a high-fat meal[27].

Unlike H_2O_2, DNA damage in the form of strand breaks did not show a correlation with any of the five risk parameters of MetS (waist circumference, blood pressure, fasting blood sugar, triglyceride and HDL-C). DNA damage was significantly higher in those with MetS and less in non-MetS. Clustering of risk factors of MetS demonstrated increased DNA damage. Karaman *et al*[10] also showed increased DNA damage in lymphocytes of patients with MetS. Few reported that hyperglycaemia which is one of the classical risk factors of MetS, induced double-strand breaks in the DNA through OS[28,29]. Dyslipidaemia, yet another risk factor of MetS, has also been shown to be closely associated with lipid peroxidation and its consequent products including crotonaldehyde, acrolein, 4-hydroxynonenal (4-HNE) and malondialdehyde (MDA) which damages DNA by the formation of exocyclic adducts[30]. Such compounds are also known to disrupt the normal coding with the opposite DNA strand at the time of DNA replication[31]. Hence, if individual risk factors of MetS are capable of causing DNA damage, clustering of these risk factors (3 or more) will have a greater degree of DNA damage. However, the presence of DNA damage in those having a single risk factor of MetS should not be ignored.

Our study had a limitation. Although it was demonstrated that the clustering of risk factors of MetS increased the degree of DNA damage, we were unable to specify which risk factors combination expressed more DNA damage.

In conclusion, patients with MetS demonstrated increased DSBs. There was a cumulative and synergistic effect of the risk factors of MetS on DNA-strand break, clustering of three or more risk factors of MetS demonstrated a greater degree of DNA damage, which could be possibly due to OS generated by these parameters. Although H_2O_2 levels increased with an increase in the triglyceride level but the use of H_2O_2 to depict OS in MetS should be coupled with an adjunct and estimation of DSB in peripheral blood lymphocytes may be a good choice.

***Financial support & sponsorship*:** This study was a part of our research project [IEC/192/12-05(a)] on 'Metabolic Syndrome' (5/7/1099/2013-RCH) that was supported by the Indian Council of Medical Research, New Delhi, undertaken in the department of Biochemistry. Instruments utilized for the project were funded by the IL Biotech Hub Project North Eastern Region-Biotechnology Programme Management Cell, Department of Biotechnology, India.

***Conflicts of Interest*:** None.

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For correspondence: Dr Rinchen Doma Bhutia, Department of Biochemistry, Sikkim Manipal Institute of Medical Sciences, 5th Mile, Tadong, Gangtok 737 102, Sikkim, India
e-mail: nehcnir@yahoo.co.in