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Association between Haptoglobin Polymorphism and DNA Damage in Type 2 Diabetes

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Abstract: In the present study we aimed to estimate the Oxidative DNA damage in Type 2 diabetic patients in different haptoglobin polymorphism. Type 2 diabetes is associated with imbalance of antioxidant defense mechanism, causes alteration in various biomolecules, including DNA. The oxidative DNA damage and its repair is very crucial in diabetes; the defect in the repair process may lead to cancer among diabetics. Haptoglobin (Hp) is a polymorphic glycoprotein provides antioxidant function against heam- driven oxidative stress and has strong association with diabetes and its complication. We investigated the DNA damage quantification of lymphocytes by AP site (Apurine/Apyrimidine site) counting and that correlated with Hp polymorphism among type 2 diabetics. The Hp1-1 phenotype had the least DNA damage than HP 2-1 & HP 2-2; this indicates that Hp alleles differ in their protective effects against oxidative damage, and HP 1-1 was the most protective.

Key words: Antioxidant, AP site counting, DNA damage, haptoglobin phenotypes, oxidative stress, type 2 diabetes

INTRODUCTION

Diabetes mellitus is associated with increased oxidative stress that results in damage of several cellular biomolecules (Aldebasi *et al*., 2011). 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 (Shigenaga and Ames, 1991). Rehman *et al*. (1999) showed that the products generated by oxidative DNA damage are significantly elevated in type 2 diabetes mellitus (T2DM) and the pattern of modification was the same as one expected from the attack of the hydroxyl radical (OH•) upon DNA. Moreover, it has been shown that hydroxyl radical which is produced by the Fenton reaction in the presence of transition metal ions is responsible for DNA damage (Aruoma *et al*., 1989). Extracellular Hemoglobin (Hb) becomes highly toxic due to the oxidative capacity of its iron containing heme, which participates in the Fenton reaction to produce reactive oxygen species (Guéye *et al*., 2006; Langlois and Delanghe, 1996). Haptoglobin (Hp) is a polymorphic (namely HP 1-1, HP 2-1, HP 2-2) protein that binds with free Hb in circulation. Such formation of Hemoglobin-Haptoglobin (Hb-Hp) complex prevents the loss of iron and iron-driven oxidative damage (Asleh *et al*., 2005; Giblett, 1968). This protective effect of Hp against oxidative mechanism is a phenotype dependent.

Many reports have established a strong association between Hp phenotypes and the occurrence of diseases, namely, complications of diabetes (Asleh *et al*., 2005). Choi *et al*. (2005) reported that lymphocyte DNA damage was significantly higher in T2DM patients with both poor glycaemic controls. DNA damage can be assessed a number of different ways, including techniques to measure strand breakage and baseless sites such as single cell gel electrophoreses or Aldehyde Reactive Probe (ARP) assays. Singh *et al*. (1988) who first demonstrated the potential of the single cell gel electrophoresis assay (comet assay) as a measurement of DNA damage. The comet assay has gained much popularity throughout the past decade due to its ease and relative inexpensiveness. The comet assay usually employs alkaline conditions (pH>13) to denature DNA. The presence of alkali-labile sites (ALSs), such as AP sites, at high pH can lead to DNA strand cleavage. The resulting overestimation of Single Strand Break (SSB) formation compromises the reliability of data obtained by the comet assay and other alkaline-based SSB assays (Anderson *et al*., 1998; Burlinson *et al*., 2007; Luke *et al*., 2010)

Fewer studies have utilized ARP assay as a measurement of baseless sites in DNA. These are sites that have lost the purine or pyrimidine base. The aldehyde reactive probe binds to the aldehyde group present in the baseless site. The baseless site is then tagged and can then be quantified using an ELISA like assay. Mohsin *et al*.

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(2004) used the ARP assay to detect oxidative damage in calf thymus DNA and He La cells following irradiation.

In humans, higher levels of 8-hydroxy-2`-deoxyguanosine (8-OHdG) are observed in mononuclear cells from diabetic patients (Dandona *et al*., 1996). Blasiak *et al.* (2004) has observed that DNA repair slowness in diabetic lymphocytes may reflect overall poor antioxidant protection.

To the best of our knowledge, none of the previous studies have evaluated an association between Hp polymorphisms and DNA damage in T2DM. Therefore, we intended to estimate the lymphocytic DNA damage by AP Site counting among T2DM patients in correlation with different Hp phenotypes.

MATERIALS AND METHODS

The study was conducted in the Department of Medical laboratories, college of Applied Medical Sciences, Qassim University, Kingdom of Saudi Arabia. The study conducted between Nov 2010 to Nov 2011 Forty eight (24 diabetic subjects and 24 healthy donors) blood samples were collected in 5 mL heparinized tube from each subject under aseptic condition. Healthy donors comprised the control group, were normoglycemia without any known history of illness. Diabetic patients, comprised the case group, were diagnosed as T2DM. Sample preparation was done in dim light to prevent any further DNA damage. Ethical clearance was obtained from the Ethics & Research committee of Qassim University. Informed consent was taken from each subject. All fine chemicals and kits were obtained from Bio-Rad, Merck, Nucleospine and Sigma.

Haptoglobin phenotyping: Hp-Hb complex solution was prepared by adding 2-3 µL Hb solution to 10µL Plasma and mixing for 5 min at room temperature. Followed by addition of 10 µL sample buffer (50% v/v glycerol and 0.001 w/v bromophenol blue) to each sample prior to running on the gel. Native polyacrylamide gel electrophoresis (native-PAGE) was performed according to the Laemmli's method. The electrophoresis was performed using a protein vertical mini-gel electrophoresis system (Bio-Rad Mini protean III apparatus; USA) with a thickness of 0.75 mm. Total polyacrylamide concentrations of 7.0 and 4% were used respectively for separation and stacking gels of native-PAGE. On completion of electrophoresis, after 2 h under 140 V constant voltage condition, the gels were stained with peroxidase stain to visualize the different bands; Hp phenotypes were evaluated and documented by Photography.

DNA damage quantification (AP site counting): Blood samples from subjects were processed for lymphocytes separation using Percoll solution (density gradient1.077 g/mL, sigma) immediately after collection. Cells were counted by manual Neubaure hemocytometer using 1% HCL (and adjusted around 5000 cell/cumm in balance salt solution). Genomic DNA prepared by using NucleoSpin® Blood Quick Pure kit. Spectrophotometric determination was done for purity of Genomic DNA using Tris-EDTA buffer at 280/260 nm.

AP sites counting in the sample DNA was determined by colorimetric 96-well microplate assay based on biotin-avidin-peroxidase assay.

Statistical analysis: The data collected and analyzed using the Statistical Package for Social Sciences (SPSS) software (version 13). Results expressed as mean±SD or number (percentage) as appropriate. Comparison of variables between two groups performed with student t-test for continuous variables. The $p \leq 0.05$ were considered significant.

RESULTS

Characteristics of the study participants: Twenty four type 2 diabetic patients participated in this study; their gender ratio was 1.0 male: 2.4 female. The male: female ratio in healthy control group (n = 24) was 2.0: 1.0. Regarding age of participants, diabetic patients were found to be significantly (p = 0.000) older (51.5±10.7 years) than healthy subjects (40.75±7.9 years). The mean BMI of diabetic patients was 30.18±5.2 Kg/m^2 compared to 27.3±3.6 Kg/m^2 for healthy subjects; indicating that diabetic patient were obese. Moreover, plasma glucose level was significantly higher in diabetic patients (12.9±5.7 mmol/L) than healthy subjects (6.01±1.8 mmol/L). The mean duration of diabetes in diabetics was 8.54±6.3 years. No significant difference observed in glycaemic control (measured by HbA1c) between participants in the two groups. The demographic and clinical characteristics of all participants are shown in Table 1.

DNA damage quantification:

AP site counting: The data from this study didn`t document any significant difference (p = 0.067) in mean value of AP site counting in patients with type 2 diabetes compared to healthy donors (Table 2). However, in healthy donors, there was significant damage of DNA (based on AP site counting) in subjects with Hp 2-1 and Hp 2-2 phenotypes compared to subjects with Hp 1-1 (p = 0.025 and 0.001, respectively). Moreover, the same conclusion was observed among type 2 diabetics, in such that patients with Hp 2-1 and Hp 2-2 1phenotypes had shown significant damage of DNA (based on AP site counting) compared to patients with Hp 1-1phenotype (p = 0.000 and 0.021, respectively). Data shown in Table 3.

Table 1: Demographic and clinical characteristics of the study participants; comparing type 2 diabetic patients (n = 24) to healthy donors (n = 24)

Variable	Type 2 diabetic patients (n = 24)	Healthy donors (n = 24)	p-value
Gender (M/F)	7/17	16/8	
Age (year)	51.5±10.7	40.75±7.9	0.000*
Weight (kg)	77.13±11.3	73.08±12.4	0.245
Height (cm)	158.58±14.0	164.83±12.2	0.106
BMI (kg/m^2)	30.18±5.2	27.3±3.6	0.029*
Blood Glucose (mmol/L)	12.9±5.7	6.01±1.8	0.000*
HbA1c (%)	8.13±2.6	6.38±1.3	0.365
Duration of T2DM(year)	8.54±6.3	-	-

*: p-value<0.05; BMI: body mass index; HbA1c: glycosylated haemoglobin; M: male; F: female; T2DM: type 2 diabetes; Data presented as mean±SD for all variables

Table 2: DNA quantification by AP site counting (expressed as mean±SD) in healthy donors compared to type 2 diabetic patients

Subjects	AP site count mean±SD	p-value
Healthy donors	0.3643±0.247	0.067
Type 2 diabetics	0.5841±0.52	

DISCUSSION

It is well established that oxidative stress is produced under diabetic conditions through multiple sources causing an increase of hydroxyl radicals (Turko *et al*., 2001). A hydroxyl radical in turn produces a multiplicity of modifications in DNA. Oxidative attack by OH radical on the deoxyribose moiety of DNA will lead to the release of free bases from DNA, generating strand breaks with various sugar modifications, nucleotide modifications, particularly in sequences with high guanosine content (Hegde *et al*., 2008) and simple a basic (AP) sites. In fact, one of the major types of damage generated by Reactive Oxygen Species (ROS) is AP site, a site where a DNA base is lost (Kow, 1999). The oxidative DNA damage occurs in their peripheral blood lymphocytes (Dandona *et al*., 1996; Sardas *et al*., 2001) and the DNA damage in lymphocytes and leucocytes can be used as a marker of oxidative stress in diabetes (Collins *et al*., 1998; Pitozzi *et al*., 2003). Additionally, it has been demonstrated that DNA damage was significantly higher in the poorly controlled diabetic patients compared to well control subject, regardless of sex (Dinçer *et al*., 2002). Van Loon *et al*. (1992) showed significantly increased basal levels of DNA damage in whole blood but not lymphocytes from diabetic subjects compared to controls.

In the present study, we investigated the lymphocytic DNA damage by counting AP site (apurinic/Apyrimidine site) and found no significant difference between healthy control subjects and patients with T2DM. The detection of AP site (mean) is very much low near to detection limit (<1/100000 BP) of the assay. This result is very similar to previous study conducted by Mohsin *et al*. (2004) on gamma irradiated HeLa cell and calf thymus DNA. AP sites are generated due to spontaneous depurination and during the base excision repair. These steady low levels of AP sites may reflect an efficient in vivo removal of AP site. One of the major pathways to repair base lesions, AP sites and SSB is the Base Excision Repair (BER) pathway (Dianov *et al*., 2003; Dianov and Parsons, 2007),which minimizes the biological consequences of single lesions in cells. The BER pathway has evolved to repair single DNA lesions, such as those produced by endogenous ROS. Guido *et al*. (1994) reported that AP sites are very efficiently repaired by base-excision repair in mammalian cell; otherwise it could be lethal, as it is considered that AP sites are highly mutagenic, could lead to cancer. The association of cancer risk among diabetic very is old (Czyzyk and Szczepanik, 2000).

Free radical mediated DNA damage and impaired antioxidant defense have also been implicated as contributors to the development of cancer. Recent evidence indicates that T2DM is associated with increased incidence and mortality from a number of cancers, including those of the colon, breast, endometrial, liver, bladder and pancreas (Giovannucci and Michaud, 2007; Saydah *et al*., 2003). Although the DNA repair defect is present in all cell types, cancer is limited to certain tissues, underscoring the notion that a deficiency in a particular repair gene in itself is not sufficient to cause cancer. Additional major factors are involved, including the level of DNA damage generated in a particular tissue, and the activity of other protection mechanisms at both the molecular and cellular levels, such as alternative DNA repair pathways, apoptosis, and the immune system (Paz-Elizur *et al*., 2008). Measuring the steady state of DNA

Table 3: DNA quantification by AP site counting (expressed as mean±SD) based on haptoglobin phenotypes in healthy donors compared to type 2 diabetic patients

	AP site counting based on Hp phenotypes				
HP phenotype	HD	p-value	HP phenotype	T2DM	p-value
Hp1-1 (n = 4)	0.1346±0.05		Hp1-1 (n = 7)	0.2368±0.03	
Hp2-1 (n = 13)	0.2715±0.10	0.025*[a]	Hp2-1(n = 11)	0.4727±0.06	0.000*[c]
Hp2-2 (n = 7)	0.6678±0.22	0.001*[b]	Hp2-2(n = 6)	0.9051±0.68	0.021*[d]

*: p≤0.05 significantly different; a: p-value compared subjects with Hp 2-1 to subjects with Hp 1-1; b: p-value compared subjects with Hp 2-2 to subjects with Hp 1-1in healthy donors; c: p-value compared diabetics with Hp 2-1 to diabetics with Hp 1-1; d: p-value compared diabetics with Hp

damage reflects the sum of the rates of its formation and repair. The advantage of this method is that it integrates exposure and repair, and thereby offers a 'bottom-line' of the amount of damage in DNA (Farmer, 2004; Sorensen *et al*., 2003; Bartsch *et al*., 2002; Vineis *et al*., 2001; Marnett, 2000). 2-2 to diabetics with Hp 1-1

Ayumi *et al*. (1998) concluded that ARP assay has been modified to improve its feasibility and sensitivity, "the improved method enabled us to quantitate the extremely small number of AP sites in mammalian cells. Furthermore, the data from this study showed that the DNA damage by AP site counting in both the control group and T2DM was significantly elevated in different Hp phenotypes. Subjects with HP 2-2 has more AP site than HP 2-1 and HP 1-1 (HP 2-2>HP 2-1>HP1-1).

The first study was conducted by *et al*., Moreira (2009) with a young population group of Brasilia to evaluate the genetic polymorphism of Hp and its ability to protect DNA in human leukocytes against oxidative damage induced by exposure to hydrogen peroxide. The findings of DNA damage were found to be dependent on Hp phenotypes indicated that among Hp1-1 was the most protective. In the second study by Akimoto *et al*. (2010) carried out with runners, reported that the level of exercise-induced oxidative stress and DNA damage was Hp phenotype dependent, indicating that Hp 1-1 genotype can be more protective in situations that impose oxidative stress. Since it is well known that polymorphisms in genes involved in xenobiotic metabolism represent a substantial component of individual susceptibility to environmental mutagens and carcinogens (Testa *et al*., 2007).

Miranda-Vilela *et al*. (2010) concluded from their results that Hp1-1 could be more protective against oxidative damages to DNA induced by hydrogen peroxide in different age group. The possible explanation for least protection for DNA damage among diabetics may be stated. In diabetes HP 2-2 polymorphism has impaired iron cargo, Free iron can participate in a Fenton type reaction, in which H_2O_2 is reduced to OH^- and •OH. Alternatively, H_2O_2 can participate in a Haber-Weiss reaction ($O_2^{\bullet-} + H_2O_2 \rightarrow O_2 + {}^{\bullet}OH + OH^-$), that combines a Fenton reaction and the reduction of Fe (III) by $O_2^{\bullet-}$, yielding Fe (II) and oxygen (Fe (III) $+O_2^{\bullet-} \rightarrow$ Fe(II) O_2) that rapidly generates ${}^{\bullet}OH$, the generally assumed critical reactive species directly attacking DNA (Asleh and Levy, 2005).

CONCLUSION

Hp1-1 phenotype had greater antioxidant properties than the other phenotypes, which resulted in more efficient protection of lymphocytes against oxidative DNA damage.

RECOMMENDATIONS

Since the number of participants, both patients and controls, enrolled in the present study was limited; further studies on larger sample size are required to determine the consistency of these observations among various levels of glycemic control and various complications of diabetes.

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