Peripheral DNA Damage in Active Pulmonary Tuberculosis

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ABSTRACT: In pulmonary tuberculosis patients, little is known about peripheral DNA damage, although increased oxidative stress is a well documented entity. Therefore, we aimed to investigate DNA damage along with oxidative status parameters in pulmonary tuberculosis patients. Twenty-seven pulmonary tuberculosis patients and 26 controls were included. DNA damage was assessed by comet assay. Total oxidant and antioxidant status, and oxidative stress index were determined. DNA damage, total oxidant status and oxidative stress index were higher in pulmonary tuberculosis patients than controls (all $P < 0.05$), while total antioxidant status was lower ($P < 0.05$). DNA damage was correlated with total oxidant and antioxidant status, and oxidative stress index ($r = 0.69$, $P < 0.05$; $r = 0.48$, $P < 0.05$, $r = -0.47$, $P < 0.05$; respectively) in pulmonary tuberculosis patients. Oxidative stress and DNA damage are increased in pulmonary tuberculosis patients. Increased oxidative stress associated DNA damage may be one of the pathogenetic mechanisms involved in the disorders suggested to be associated with pulmonary tuberculosis. © 2011 Wiley Periodicals, Inc. Environ Toxicol 27: 380–384, 2012.

Keywords: pulmonary tuberculosis; total antioxidant status; total oxidant status; DNA damage

INTRODUCTION

Pulmonary tuberculosis (PTB) is caused by infection with *Mycobacterium tuberculosis*, and remains a major cause of morbidity and mortality around the world (Dye, 2006). Current estimates suggest that one third of the worlds' population has been exposed, with an estimated 8–9 million new cases occurring annually (Corbett et al., 2003).

Mycobacteria are intracellular pathogens which are internalized mainly by alveolar macrophages, phagocytes then undergo respiratory burst resulting in production of capacious amounts of reactive oxgen species (ROS) and reactive nitrogen intermediates (RNI). ROS and RNI are essential for the destruction of ingested microorganisms but can also contribute to injury of host tissues (Kwiatkowska et al., 2007).

Lymphocyte deoxyribonucleic acid (DNA) damage is caused mainly by oxidative stress (Ames, 1989; Halliwell and Aruoma, 1991) and inflammation (Dong et al., 2003). Many pathological conditions such as various cancers, cardiovascular and neurodegenerative diseases, inflammation/infection, and ageing are associated with genomic damage (Loft and Poulsen, 1999). The alkaline comet assay is one of the useful methods in quantifying DNA damage. Because of its simplicity and sensitivity, the comet assay has gained fast acceptance as a genotoxicity assay (Collins, 2004; Faust et al., 2004). In PTB patients, increase in oxidants and decrease in antioxidants have been reported

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previously (Madebo et al., 2003; Wiid et al., 2004; Kwiatkowska et al., 2007). However, to our knowledge, little is known about the peripheral DNA damage in patients with PTB (Liu et al., 2003).

Therefore, in the present study, we aimed to investigate the level of DNA damage detected by comet assay (single-cell gel electrophoresis, SCGE) along with measurement of total oxidant status (TOS) and total antioxidant status (TAS) in patients with PTB.

MATERIALS AND METHODS

Subjects

Twenty-seven (6 women and 21 men) newly diagnosed pulmonary tuberculosis patients and 26 healthy controls (9 women and 17 men) were enrolled in the present cross-sectional study. The diagnosis of PTB was based on the clinical symptoms, radiological findings and bacteriological data (acid-fast microscopy and culture). The study protocol was carried out in accordance with the Helsinki Declaration as revised in 1989 and approved by the local research committee for ethics. All subjects were informed about the study protocol and the written consent was obtained from each one.

Exclusion Criteria

Exclusion criteria were consisted of usage of supplemental vitamins, the presence of other respiratory disorders, human immune deficiency virus infection, smoking habits, diabetes mellitus, coronary artery disease, rheumatoid arthritis, malignancy, heart failure, hypertension, hyperlipidemia, acute or chronic liver diseases, and renal dysfunction.

Blood Sample Collection

Heparinized venous blood samples (total 6 mL) were obtained following an overnight fasting state. For comet assay, blood samples were collected into heparinized tubes, immediately stored in ice in a dark environment to prevent further DNA damage and processed within 2 h. The remaining blood samples were centrifuged at 1500 $\times$ *g* for 10 min to obtain the plasma, and the plasma samples were stored at -80°C until analysis of TOS and TAS.

DNA Damage determination by Alkaline Comet Assay

Lymphocyte isolation for the comet assay was performed using Lymphoprep (Amerco, Axis-Shield, Oslo, Norway). An amount of 1 mL of heparinized blood was carefully layered over 1 mL of Lymphoprep and centrifuged for 35 min at 500 $\times$ *g* and 25°C. The interface band containing lymphocytes were washed with phosphate-buffered saline and then collected by 15 min centrifugation at 400 $\times$ *g*. The resulting pellets were resuspended in phosphate-buffered saline to obtain ~20 000 cells in 10 μL. The interface band containing lymphocytes were washed with 100 mM, pH 7.4 phosphate-buffered saline which also contains 8 g L^{-1} NaCI and 0.2 g L^{-1} KCI. Membrane integrity was assessed by means of the trypan blue exclusion method and revealed membrane integrity in 95% of cells.

The comet assay was performed according to Singh et al. (1988), with the following modifications. Ten microliters of lymphocyte suspension (around 20 000 cells) were mixed with 80 μL of 0.7% low-melting agarose in phosphate-buffered saline at 37°C. Subsequently, 80 μL of mixture was layered onto a slide precoated with thin layers of 1% normal melting point agarose, and immediately covered with a coverslip. Slides were left for 5 min at 4°C to allow the agarose to solidify. After removing the coverslips, the slides were submersed in freshly prepared cold (4°C) lysing solution (2.5 M NaCI, 100 mM ethylenediaminetetraacetic acid (EDTA)-disodium salt, 10 mM Tris-HCI, pH 10–10.5, 1% Triton X-100 and 10% dimethyl sulfoxide added just before use) for at least 1 h. Slides were then immersed in freshly prepared alkaline electrophoresis buffer (0.3 mol L^{-1} NaOH and 1 mol L^{-1} EDTA-disodium salt, pH $>$ 13) at 4°C for separation of DNA (40 min) and then electrophoresed (25 V/300 mA, 25 min). All the steps were carried out under minimal illumination. After electrophoresis, the slides were stained with ethidium bromide (2 μL mL^{-1} in distilled H_2O; 70 μL $slide^{-1}$), covered with a coverslip and analyzed using a fluorescence microscope (Nikon, Tokyo, Japan). Images of 100 randomly selected cells (50 cells from each of two replicate slides) were analyzed visually from each subject. Each image was classified according to the intensity of the fluorescence in the comet tail and was given a value of 0, 1, 2, 3, or 4 (from undamaged class 0 to maximally damaged class 4) (Fig. 1), so that the total score of slide could be between 0 and 400 arbitrary units (AU), (Collins et al., 1997; Collins, 2004). All procedures were completed by the same biochemistry staff and DNA damage was detected by a single observer who was not aware of subject's status.

Measurement of the Total Antioxidant Status

TAS of plasma was determined using a novel automated measurement method, developed by Erel (2004). In this method, hydroxyl radical, which is the most potent biological radical, is produced. In the assay, ferrous ion solution, which is present in the reagent 1 [*o*-dianisidine (10 mM), ferrous ion (45 AM) in the Clark and Lubs solution (75 mM, pH 1.8] is mixed by hydrogen peroxide, which is present in the reagent 2 [H_2O_2 (7.5 mM) in the Clark and Lubs solution]. The sequental produced radicals such as brown colored dianisidinyl radical cation, produced by the hydroxyl radical, are also potent radicals. Using this method, antioxidative effect of the sample against the

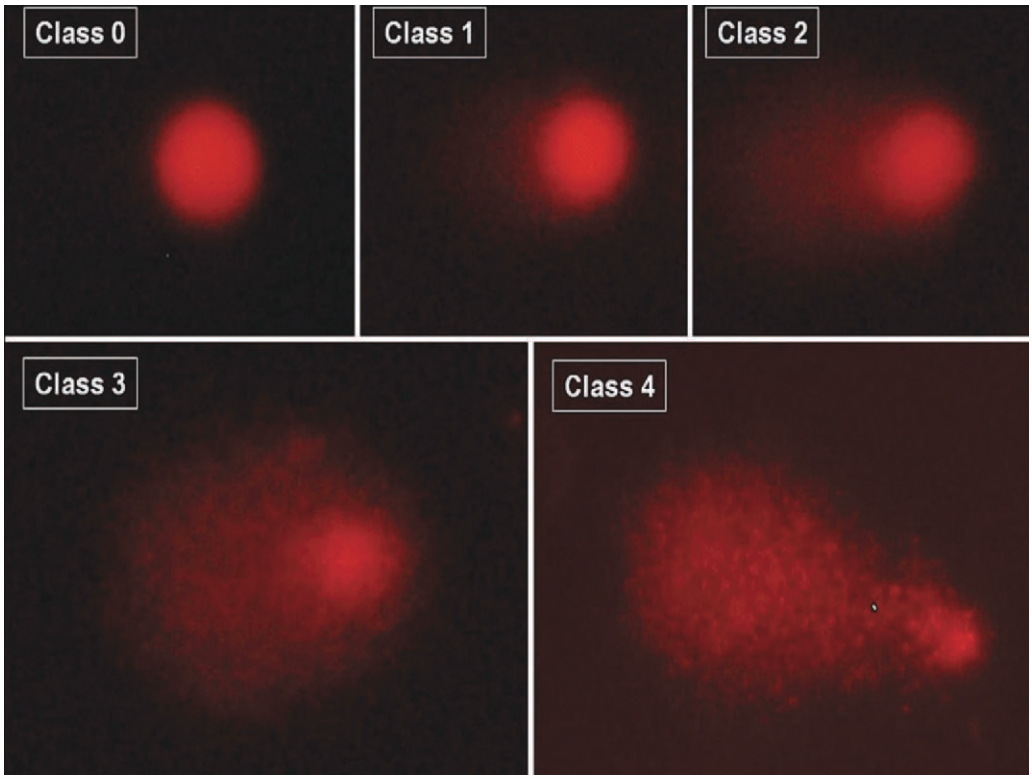

Fig. 1. Photomicrographs showing the different images of comet classes (class 0, undamaged; class 4, maximally damaged). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

potent free radical reactions, which is initiated by the produced hydroxyl radical, is measured. The assay has got excellent precision values lower than 3%. The results were expressed as mmol Trolox Equiv. L^{-1}.

Measurement of Total Oxidant Status

TOS of plasma was determined using a novel automated measurement method, developed by Erel (2005). Oxidants present in the sample oxidize the ferrous ion-*o*-dianisidine complex to ferric ion. The oxidation reaction is enhanced by glycerol molecules, which are abundantly present in the reaction medium. The ferric ion makes a colored complex with xylenol orange in an acidic medium. The color intensity, which can be measured spectrophotometrically, is related to the total amount of oxidant molecules present in the sample. The assay is calibrated with hydrogen peroxide and the results were expressed in terms of micromolar hydrogen peroxide equivalent per liter (μmol H_2O_2 Equiv. L^{-1}).

Oxidative Stress Index

The ratio percentage of TOS level to TAS level gave the OSI, an indicator of the degree of oxidative stress (Selek et al., 2007).

Statistical Analysis

All data were expressed as mean ± standard deviation. Qualitative variables were assessed by Chi-square test. Continuous variables were compared using Student's *t* test. Pearson correlation analysis was used to find out the correlation between DNA damage level, and TOS, TAS, and OSI. Differences were regarded as significant at $P < 0.05$. Data were analyzed using the SPSS® for Windows computing program (Version 11.0).

RESULTS

Demographic, clinical, and laboratory parameters of the subjects are shown in Table I. There were no significant differences between PTB patients and controls in respect to age, gender and body mass index (BMI) (all $P > 0.05$).

Peripheral DNA damage, TOS, and OSI were significantly higher in patients with PTB than controls (all $P < 0.05$), while TAS was significantly lower ($P < 0.05$; Table I).

In Pearson correlation analysis, DNA damage level was found to be significantly correlated with TOS, TAS, and OSI levels ($r = 0.69$, $P < 0.05$; $r = 0.48$, $P < 0.05$, $r = -0.47$, $P < 0.05$; respectively) in patients with PTB, while no association was observed in controls (all $P > 0.05$).

TABLE I. Demographic, clinical and laboratory parameters in patients with PTB and controls

Parameters	PTB patients ($n = 27$)	Controls ($n = 26$)
Age (years)	37 ± 9	36 ± 12
Sex (M/F)	21/6	17/9
BMI (kg m^{-2})	21.4 ± 2.1	22.6 ± 1.8
DNA damage (arbitrary unit)	52.16 ± 18.65*	29.81 ± 11.79
TAS (mmol trolox Eqv. L^{-1})	1.27 ± 0.51	1.86 ± 0.50**
TOS (μmol H_2O_2 Eqv. L^{-1})	18.55 ± 7.23*	13.12 ± 5.62
OSI (arbitrary unit)	1.82 ± 0.98*	1.01 ± 0.86

Data were presented as mean ± SD.

PTB, pulmonary tunerculosis; BMI, body mass index; TAS, total antioxidant status; TOS, total oxidant status; OSI, oxidative stress index.

*$P < 0.05$ vs. controls.

**$P < 0.05$ vs. pulmonary tuberculosis patients.

DISCUSSION

The key findings of the present study were increased DNA damage detected by comet assay and its significant association with increased oxidative stress in patients with PTB.

Infection and inflammation activate a variety of inflammatory cells that induce and activate various oxidant-generating enzymes that produce high concentrations of various different free radicals and oxidants including superoxide anion, nitric oxide, nitroxyl, nitrogen dioxide, hydrogen peroxide, and hypochlorous acid. These oxidants react with each other to produce other reactive species that are much more harmful than the oxidants themselves (Beckman and Koppenol, 1996). ROS and RNS are produced primarily to attack invading infectious agents and foreign bodies. However, excess amounts of these reactive species in inflamed tissues can induce oxidative stress (Dizdaroglu, 1994).

In the present study, in accordance with the previous reports (Madebo et al., 2003; Wiid et al., 2004; Kwiatkowska et al., 2007), we observed an increase in oxidative stress in patients with PTB. It is well known that subjecting cells to oxidative stress can result in severe metabolic dysfunctions, including peroxidation of membrane lipids, depletion of nicotinamide nucleotides, rises in intracellular free Ca^{+2} ions, cytoskeletal disruption, and DNA damage (Halliwell and Aruoma, 1991). In several *in vitro* cell culture studies, *M. tuberculosis* has been shown to cause DNA fragmentation and promotes human alveolar macrophage apoptosis (Keane et al., 1997; O'Sullivan et al., 2007). However, to our knowledge, peripheral DNA damage along with oxidative stress parameters in patients with tuberculous pleurisy was reported only in study Liu et al. study (Liu et al., 2003). In accordance with our results, they also reported that DNA damage was increased and significantly correlated with the degree of oxidative stress in patients with tuberculous pleurisy.

It is well known that accumulation of DNA damage with time can lead to gene modification in cells that may be mutagenic or carcinogenic (Valko et al., 2004). Indeed, in many papers, DNA damage, as well as DNA damage repair defect has been implicated in the pathogenesis of many cancer types, as well as lung cancer (Caliskan-Can et al., 2008; Danoy et al., 2008; Paz-Elizur et al., 2008). In a review of epidemiological studies on concurrent pulmonary tuberculosis and lung cancer conducted by Aoki, it has been stated that patients with active pulmonary tuberculosis had a higher risk of dying from lung cancer or other malignancies, despite the high mortality from tuberculosis (Aoki, 1993). This observation was supported by subsequent studies, especially on the association between lung cancer and PTB (Wu et al., 1995, Ko et al., 1997). Although the precise mechanisms for a direct role of tuberculosis on the development of lung cancer is still unclear, chronic inflammatory process has been suggested to enhance the effects of other carcinogenic exposures and stimulate cell proliferation and growth (Zheng et al., 1987). In addition, in a review of Ohshima and Bartsch, they have stated that DNA damage produced by reactive oxygen species could contribute to the process of carcinogenesis which might be associated with chronic inflammatory processes (Ohshima and Bartsch, 1994). Similar mechanism can be applied for the lung cancer suggested to be associated with PTB infection. Therefore, on the basis of the findings of the present study and findings of Liu et al. study (Liu et al., 2003), it can be suggested that increased oxidative stress associated DNA damage, may, in part have a role in the pathogenesis of lung cancer suggested to be associated with PTB.

In the lightening of the findings of the present study, it can be suggested that oxidative stress and DNA damage are increased in patients with PTB. Increased oxidative stress associated DNA damage may be one of the pathogenetic mechanisms which might be involved in the pathogenesis of the development of lung cancer suggested to be associated with PTB. Further large scale prospective studies are needed to clarify the association between PTB associated DNA damage and lung cancer.

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