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Full Length Research Paper

Antioxidant and nitric oxide status in patients diagnosed with *Echinococcus granulosus*

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In the present study, we tried to investigate whether infection with cystic echinococcosis provokes oxidative stress in the host by measuring changes in plasma levels of anti-oxidants enzymes. 23 patients and 25 control individuals were included in the study. Plasma superoxide dismutase [Cu–Zn superoxide dismutases, cytoplasmic form/superoxide dismutase 1] and glutathione peroxidase [Cytoplasmic gluthatione peroxidase 1] activities and plasma nitrite levels were all determined based on the colorimetric methods. Statistically significant decreased cytoplasmic glutathione peroxidase and superoxide dismutase activities with decreased nitric oxide production, which produce superoxide radical was found in patients with cystic echinococcosis. Correlation analysis and statistical evaluation together showed that there was a significant negative correlation between glutathione peroxidase and superoxide dismutase activities ($p < 0.001$) and also significant negative correlation between glutathione peroxidase activity and nitric oxide level ($p < 0.001$). In addition, there was a significant negative correlation observed between superoxide dismutase activity and nitric oxide level ($p < 0.001$). These results clearly indicate a decline in the response to oxidative stresses. It may also be concluded that a decrease in the nitric oxide level can be associated with the low stimulation of the cell mediated immune system.

Key words: Cystic echinococcosis, superoxide dismutase, glutathione peroxidase, nitric oxide.

INTRODUCTION

Echinococcosis is often referred to as hydatid disease or echinococcal disease. Cystic echinococcosis; is transmitted from canidae to slaughter animals and accidently also transmitted to human. Therefore, it is a zoonotic disease that is important for human health and economy. The most common form found in humans is cystic echinococcosis (CE), which is caused by *Echinococcus granulosus*. Although Echinococcosis has been well-known, it was not until the past couple of hundred years that real progress was made in determining and describing its parasitic origin (Brunetti and Junghanss, 2009). Free radicals (any atom or molecule that has a single unpaired electron in an outer shell) interact with other molecules within cells. This can cause oxidative damage to proteins, membranes and genes. To counteract oxidative damage, the body produces antioxidant enzymes such as; superoxide dismutase (SOD, EC 1.15.1.1) and glutathione peroxidase (GPx, EC 1.11.1.9) to defend itself (Mishra et al., 1994; Afonso et al., 2007; Fattman et al., 2003).

SODs are a class of enzymes that catalyze the dismutation of superoxide into oxygen and hydrogen peroxide. As such, they are an important antioxidant defense in nearly all cells exposed to oxygen (Mishra et al., 1994; Afonso et al., 2007). Simply stated, SOD outcompetes damaging reactions of superoxide, thus protecting the cell from superoxide toxicity. In biological systems, this means its main reactions are with dismutation or with another biological radical such as nitric oxide (NO) (Fattman et al., 2003).

GPx is the general name of an enzyme family with

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peroxidase activity whose main biological role is to protect the organism from oxidative damage. GPx catalyzes is: $2GSH + H_2O_2 \rightarrow GS–SG + 2H_2O$. In other words, GPx, a key enzyme involved in the detoxification of many peroxides (Wilkinson and Kelly, 2003; Mei et al., 1996).

NO is produced by a number of different cell types in response to cytokine stimulation and thus has been found to play a role in immunologically mediated protection against a growing list of parasites (Khambu et al., 2007). NO also plays an important role in the immune mechanisms of human. It inhibits enzymes necessary for energy metabolism and cell growth. In addition, NO is produced by cells other than those involved in immune response, such as hepatocytes and endothelial cells which are significant in the life cycle of many parasites. The overproduction of NO is harmful for the cells and the surrounding tissues (Weinberg. et al., 2008; Sobolewski. et al., 2005; Zaki et al., 2005).

The aim of this study was to confirm whether infection with CE provokes oxidative stress in the host by measuring changes in plasma levels of antioxidant enzymes such as; SOD and GPx and oxidative agent of NO production.

MATERIALS AND METHODS

All chemicals used in this study were of analytical grade or the highest grade available. Informed consent was obtained from patients prior to the study. The study protocol and the procedures were approved by Erciyes University Ethical committee and were in accordance with the Helsinki Declaration. The participants in both patient and control groups were examined for the intestinal parasites and cyst hydatic using manual IHA (Indirect Hemaglutination Technique) and IFAT (Indirect Immunofluorescent Technique) methods. 23 patients and 25 control individuals were included in the study. A total of 23 patients with CE (mean age: 41.6 ± 18 years); and 25 age matched healthy subjects (mean age: 51 ± 20 years) were enrolled in the study as control group. None of them were smokers, had any known pathologies and taking steroids or medications such as iron for anaemia at the time of sampling. Plasma samples for control group were obtained from healthy people who have come to the different departments of Erciyes University, Medical Faculty for regular check-up and students or employees of the University. Repeat of all venous blood samples were taken between 8:00 and 9:00 a.m after 12 h of fasting and collected in polystyrene tubes and vacutainers containing heparin over several days. The tubes were centrifuged at 2000 × rpm for 10 min. The plasma was then removed and stored at -70°C until analysis.

Determination of SOD activity

SOD activity [Cu–Zn SOD, Cytoplasmic form/SOD 1] in plasma was measured according to the method of Sun et al. (1988). In this method, a xanthine–xanthine oxidase complex produces superoxide radicals, which react with nitrobluetetrazolium (NBT) to form the farmasone compound. The SOD activity is measured at 560 nm by detecting the inhibition of this reaction. One unit of SOD is defined as the activity that causes 50% inhibition of $NBTH_2$ reduction rate. SOD activity was expressed as U/dL.

Determination of GPx activity

GPx activity [Cytoplasmic gluthatione peroxidase 1] in plasma was measured according to the method of Paglia and Valentine (1967). Enzyme activity was determined from the oxidation of reduced NADPH in the presence of H_2O_2 used as substrate. The decrease in concentration of NADPH was monitored and recorded at 340 nm in a mixture containing reduced glutathione and glutathione reductase. Enzymes units were defined as the number of micromoles of NADPH oxidized per minute. Results were defined as U/L.

Determination of NO production

Plasma nitrite levels were determined with colorimetric method based on the Griess reaction in which nitrite is reacted with sulfanilamide and N-(1-naphthyl) ethylendiamine to produce an azo dye. NO is a labile compound, has a brief half-life and is rapidly converted to the stable end-products, nitrite and nitrate, in oxygenated aqueous solutions. Nitrite levels were measured after the enzymatic reduction of nitrate to nitrite with nitrate reductase (Bories and Bories, 1995). Sodium nitrite solution was used for standard measurements.

Nitrite assay

All plasma samples were deproteinized before assay. Briefly, for every 200 µl sample, 400 µl of 0.5 N sodium hydroxide and 400 µl of 10% zinc sulfate were added. The samples were then vortexed and centrifuged at 25 000 xg for 5 min at 4°C. Nitric oxide metabolites (nitrate and nitrite) were assayed by first reducing nitrate to nitrite. Nitrate reductase (0.05 unit/ml) along with reduced nicotinamide adenine dinucleotide (90 µmol/L) and flavin adenine dinucleotide (3.12 µmol/L) were added to each sample to convert nitrates to nitrites. Nitrite production was then determined with the spectrophotometric Greiss reaction. For this study, 100 µl of plasma or water blank was mixed with 50 µl of 0.32 mol/L potassium phosphate, pH 7.5 and 10 µl of nitrate reductase with cofactors. To the sample mixture, 40 µl of Greiss reagent (10% sulfanilamide and 1% naphtylethylenedlamine dihydrochloride in 85% phosphoric acid) was added. The mixture was incubated for 10 min at room temperature and the absorbance was read at 550 nm. Plasma nitrite levels were expressed in µmol/L.

Statistical analysis

Statistical evaluation was carried out with the SPSS 11.0 (Statistical Packages for Social Sciences; SPSS Inc, Chicago, Illinois, USA). Data obtained from the study groups were compared by the parametric student's *t* test; correlation analyses between variables were made by Pearson test; *p* value less than 0.05 was considered as statistically significant. All the results were expressed as means with their standard deviation (mean ± SD).

RESULTS

There was no statistically significant difference of age distribution between the control and CE patients ($p = 0.10$). Plasma GPx and SOD activities and NO level were lower in the patients group than the controls ($p < 0.001$, $p < 0.019$, $p < 0.001$, $p < 0.001$, respectively).

Plasma GPx and SOD activities and NO level of the

Table 1. Plasma GPx and SOD activities and NO level in patients with CE and controls.

Parameter	Controls (mean ± SD)	CE patients (mean ± SD)	p
Age (years)	51 ± 20	41,6 ± 18	= 0.10
GPx activity (U/L)	199,2 ± 42,1	101,3 ± 32,3	< 0.001
SOD activity (U/dl)	1,8 ± 0,3	0,7 ± 0,4	< 0.001
NO level (µmol/L)	69,7 ± 8,1	41.2 ± 7,5	< 0.001

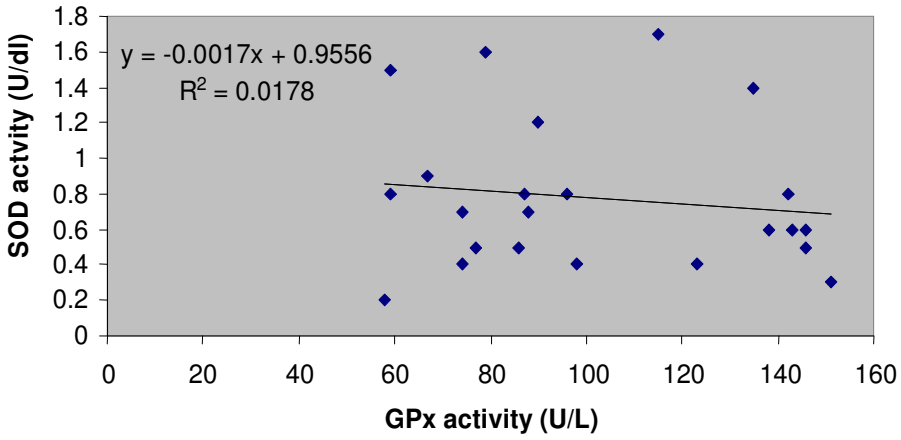

Figure 1. Correlation between GPx and SOD activities.

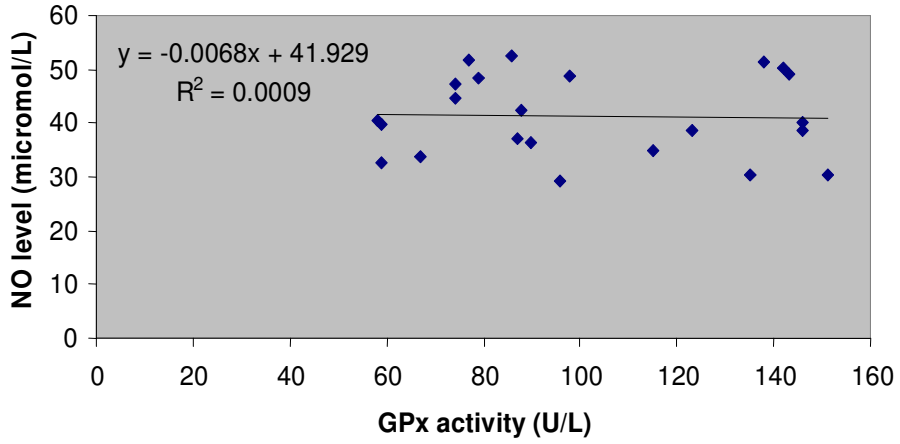

Figure 2. Correlation between GPx activity and NO level.

study groups have been presented in Table 1.

Correlation analysis and statistical evaluation together showed that there was a significant negative correlation between GPx and SOD activities ($p < 0.001$) (Figure 1) and also significant negative correlation between GPx activity and NO level ($p < 0.001$) (Figure 2). In addition, there was a significant negative correlation observed between SOD activity and NO level ($p < 0.001$) (Figure 3).

DISCUSSION

The parasites are noted to be an important health problem in Turkey as similarly reported in the globe (Brunetti and Junghanss, 2009; Torgerson and Deplazes, 2009). Oxidative stress and changes in antioxidant status have been already implicated in the pathogenesis of different parasites disease (Bertrand et al., 2008; Turrens, 2004).

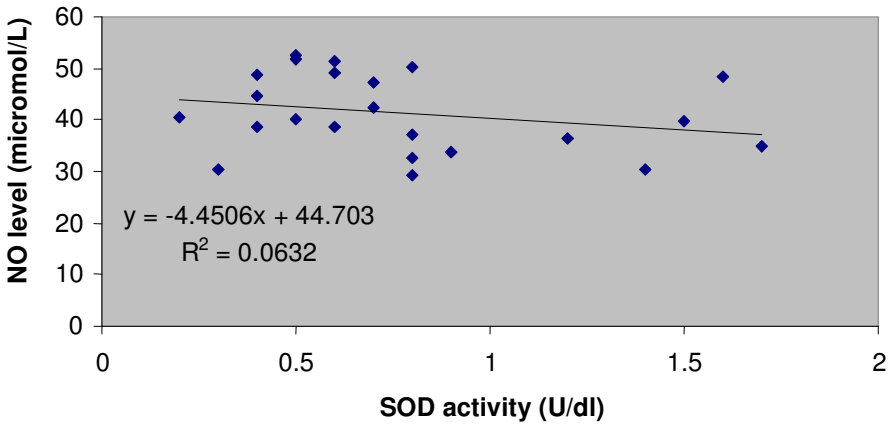

Figure 3. Correlation between SOD activity and NO level.

In this study, the results of decreased SOD and GPx activities in the plasma samples of CE patients were consistent with previous studies. However, plasma NO production has been found to be also decreased in contrast to some of the other similar studies (Serarslan et al., 2005; Atambay et al., 2008).

It can be suggested that the generation of O_2^-, H_2O_2 and OH species causes a decrease in SOD activity. It has been shown that in cases of oxidative stress GPx can be inactivated, as O_2^- can inhibit peroxide function (Blum and Fridovich, 1985). We could say that as a result of increased ROS (Reactive Oxygen Species) generation in CE patients (Ersayit et al., 2009), SOD and GPx are consumed during ROS scavenging and thus the activities of the enzyme has been decreased during CE.

GPx and SOD enzymes constitute the primary part of enzymatic antioxidant defense system against oxidative stress. SOD provides the efficient dismutation of O_2^- leading to the formation of H_2O_2 which is removed mainly by GPx.

SOD scavenges superoxide and inhibits the formation of peroxynitrite, thereby suppressing the resulting injury and regulating the bioavailability of NO. Therefore, it acts as a protective mechanism against tissue injury. Present results show that the disturbed metabolism of superoxide due to the decreased activities of SOD and GPx seems to be important in the pathogenesis of cysct hytatid. Treatment approaches that affect the antioxidant enzymes may be beneficial in patients with cystic echinococcosis.

Decreased or increased SOD and GPx activities and NO production have been reported in different studies (Serarslan et al., 2005; Atambay et al., 2008; Blum and Fridovich, 1985; Ersayit et al., 2009; Karaman et al., 2008). The reasons for these dilemmas remain to be clarified. Oxidative stress, which may play a role in the pathogenesis of cystic echinococcosis, produces ROS and induces probably uncontrolled lipid peroxidation (Ersayit et al., 2009; Karaman et al., 2008). Cell membranes consist primarily of lipids, and therefore lipid peroxidation can cause cell injury and death. Cellular defense systems against this free-radical induced lipid peroxidation consist of antioxidative free-radical scavenging molecules such as SOD and GPx. If GPx levels decrease concomitantly with SOD, then the first O_2^- and second step H_2O_2 intermediate radicals accumulate. These oxygen free-radicals could undergo the Fenton's reaction, generating OH, which may lead to lipid peroxidation in cells (Ersayit et al., 2009; Baskol et al., 2007). The reason for increased lipid peroxidation in patients with cystic echinococcosis may be related to decreased antioxidant enzymes (Ersayit et al., 2009).

The potentially harmful effects of reactive oxygen species are controlled by the cellular antioxidant defense system. GPx is an important constituent of intracellular protective mechanisms against a number of noxious stimuli including oxidative stress. It maintains the sulfhydryl (-SH) groups in proteins in a reduced state and protects these groups against oxidation (Baskol et al., 2007). In the present study, the significant decrease found in GPx activity in the patient group can be explained with the oxidative stress caused by lipid peroxidation and depletion in the GPx level, which is an endogen antioxidant.

NO is an important biological mediator produced from L-arginine through the enzyme NO synthetase. It is also an important cytotoxic and cytostatic mediator for various intracellular parasites (Serarslan et al., 2005). NO has a complex role in immune functions. Immunologic and inflammatory stimuli induce the peroxidation of NO over long periods and NO exerts cytotoxic and cytostatic effects not only against invading cells, but also against healthy cells. Reduction of NO production is associated with increased platelet aggregation and leukocyte adhesion that contribute to vascular dysfunction and thrombosis (Sahin et al., 2006; Sendur et al., 2009). Physiological actions of NO are destroyed by the superoxide radical and stabilized by antioxidants such as

SOD and GPx. The short-lived NO and mildly reactive superoxide radical rapidly combine to form a potent and long lived oxidant, peroxynitrite which then breaks down to form a hydroxyl radical, thereby resulting in increased oxidative stress (Baskol et al., 2007). Other possible explanation of decreased plasma NO production might be increased free radical production with defective antioxidant defense mechanisms in patients infected with *E. granulosus*, which may directly induce endothelial cell damage, and therefore cause decreased production of NO by endothelial nitric oxide synthetase. The decreased levels of NO might also be due to its interaction with O_2^- yielding peroxynitrite molecule and hence being consumed. The difference especially between our study and that some of the other studies, showing that increasing amount of NO, might be due to the fact that the different numbers of subjects are included in the study. Further studies in this area in larger patient and control populations might clarify this issue.

There is no previous research which studied GPx, SOD activities and NO production, altogether in EC patients. A decrease of GPx and SOD activities in patients indicates mainly a decline in the response to oxidative stresses and a decrease in the NO level can be mainly associated with the low stimulation of the cell mediated immune system. Taking together our study results and all known information relating to the SOD, GPx and NO, we could be concluded and suggested that overproduction of ROS by activated neutrophils and macrophages results in oxidative stress and the acceleration of lipid peroxidation (Ersayit et al., 2009) associated disorders in these patients; all of these effects are consequences of altered enzymatic antioxidant activities. In other words, increased oxidative stress in patients with CE may result in a pro-oxidation environment, which in turn could result in decreased antioxidant enzyme activities and NO levels. Therefore effective antioxidant therapy to inhibit oxidative stress is necessary and agents to increase antioxidant enzyme may be a therapeutic option in patients with CE.

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