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Serum paraoxonase activity, total thiols levels, and oxidative status in patients with acute brucellosis

Ramazan Esen · Mehmet Aslan · Mehmet Emin Kucukoglu · Aytekin Cıkman · Umit Yakan · Mahmut Sunnetcioglu · Sahbettin Selek

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Summary It is well known that paraoxonase-1 (PON1) activity may decrease during the course of infection and inflammation. The aim of this study was to investigate serum PON1 activity, oxidative status, and thiols levels in patients with acute brucellosis. In addition, we investigated the PON1 phenotype in patients with acute brucellosis. Thirty patients with acute brucellosis and 35 healthy controls were enrolled. Serum paraoxonase and arylesterase activities, thiols levels, lipid hydroperoxide levels, total antioxidant capacity (TAC), total oxidant status (TOS) and oxidative stress index (OSI) were determined. Serum basal and salt-stimulated paraoxonase–arylesterase activities, TAC levels and thiols levels were significantly lower in patients with acute brucellosis than controls (for all, $p < 0.05$), while LOOH levels, TOS levels, and OSI values were significantly higher (for all, $p < 0.05$). We concluded that oxidative stress is increased, while serum PON1 activity is decreased in patients with acute brucellosis. These results indicate that lower PON1 activity is associated with oxidant–antioxidant imbalance.

Dr. M. Aslan (✉) · R. Esen · M. E. Kucukoglu
Medical Faculty, Department of Internal Medicine,
Yuzuncu Yıl University,
65000 Van, Turkey
e-mail: m.aslan301@mynet.com

A. Cıkman
Medical Faculty, Department of Microbiology,
Yuzuncu Yıl University,
Van, Turkey

U. Yakan · M. Sunnetcioglu
Medical Faculty, Department of Infectious Disease and Clinical Microbiology, Yuzuncu Yıl University,
Van, Turkey

S. Selek
Medical Faculty, Department of Clinical Biochemistry,
Bezmialem Vakif University,
Istanbul, Turkey

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Introduction

Brucellosis is a zoonosis widely distributed in rural Mediterranean areas [1]. The vast majority of cases are attributed to the subtype Brucella melitensis [2]. It is a small gram-negative, facultative intracellular pathogenic bacterium which is able to surviving and replicate within the cells of the mononuclear phagocytic system [3]. In humans, brucellosis is a systemic infection, which may involve any organ or system of the body [1].

It has been demonstrated that in the many infectious diseases, a variety of inflammatory cells are activated, which lead to production of reactive oxygen species (ROS) to kill intracellular and extracellular parasites [4]. ROS are one of the crucial molecules that kill bacteria internalized into phagocytic cells, such as polymorphonuclear neutrophils and macrophages [5]. Brucellae invade macrophages during the early phase of infection, adapt to the acidic environment, and proliferate in the vacuolar compartments [6]. It has been stated that the oxidative killing pathways of host macrophages represent a primary mechanism utilized by these host phagocytes to control the intracellular replication of the brucellae [7]. Furthermore, it has been shown that brucellosis is related to increased free radical production and antioxidant depletion. Thus, oxidative stress has been implicated in the pathogenesis of brucellosis [8].

Paraoxonase 1 (PON1) is a 354 amino acid Ca^{2+}-dependent serum esterase that is synthesized in the liver. The enzyme has both arylesterase and paraoxonase activities [9]. Human serum PON1 is an enzyme associated with high-density lipoprotein (HDL) that prevents oxidative modification of low-density lipoprotein (LDL) [10].

Therefore, PON1 plays an important role in the protection against atherosclerosis by preventing oxidative modification of serum lipoproteins [11]. It has been suggested that decreased serum PON1 activity might be associated with increased oxidative stress in serum and macrophages [12]. Furthermore, oxidative stress has been shown to decrease PON1 activity and downregulate the serum expression of PON1 [13]. It has been suggested that low serum PON1 activity is significantly associated with a risk for atherosclerotic disease [14]. It is well known that PON1 activity may decrease during the course of infection and inflammation and acute phase HDL is unable to protect LDL against oxidation [15, 16].

Several studies have investigated a possible link between PON1 activity and brucellosis [17, 18]. However, the results of these studies are conflicting. The measurement of total antioxidant capacity (TAC) and total oxidant status (TOS) were useful tests for prediction of oxidative status [19]. The studies concerning oxidative status in patients with brucellosis are controversial [8, 17]. Therefore, we measured serum PON1 activity and oxidative status a long with thiols levels in patients with acute brucellosis. In addition, we investigated the PON1 phenotype in patients with acute brucellosis.

Materials and methods

Subjects

In this study, 30 patients with brucellosis (17 men, 13 women) and 35 controls (19 men, 16 women) were enrolled.

A diagnosis of acute Brucella infection was confirmed by the presence of specific IgM antibodies against Brucella as determined by ELISA. In addition, a diagnosis of brucellosis was made by isolation of the bacterium from blood sample and/or serum agglutination test with titers ≥ 1:160 in conjunction with a compatible clinical presentation [2]. Patients with acute brucellosis did not receive any treatment prior to the study.

The control group consisted of 35 healthy subjects (without a history of chronic or recurrent disease). The subjects in the control group were asymptomatic with an unremarkable medical history and a normal physical examination. None of the control subjects were receiving antioxidant vitamin supplements including vitamin E or C.

All patients and controls subjects were evaluated by standard physical examination, chest X-ray, baseline electrocardiography, and routine clinical laboratory tests, including liver and kidney function tests.

Exclusion criteria

The exclusion criteria included a history of alcohol abuse, habitual smoking, intravenous drug abuse, pregnancy, antioxidant supplements, hypertension, diabetes mellitus, liver or renal disease, rheumatoid arthritis, pulmonary disease, and coronary artery disease.

Blood samples

Blood samples were collected at 8:00 and 11:00 a.m. after an overnight fasting period. Blood samples were collected into empty tubes and immediately stored at 4 °C. The serum samples were then separated from the cells by centrifugation at 3000 rpm for 10 min; lipid parameters were measured immediately. The remaining serum portions were stored in plastic tubes at –80 °C for a time no longer than 6 months [20] and used for analyzing of PON1 activity, thiols levels, oxidative status parameters, and lipid hydroperoxide (LOOH) levels.

Measurement of serum total antioxidant capacity

Serum TAC was determined using an automated measurement method, developed by Erel [21]. 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 is mixed by hydrogen peroxide, which is present in the Reagent 2. The sequential 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 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 are expressed as mmol Trolox Eq/L.

Measurement of serum total oxidant status

Serum TOS was determined using a novel automated measurement method, developed by Erel [22]. 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 are expressed in terms of micromolar hydrogen peroxide equivalent per liter (μmol H_2O_2 Eq/L).

Determination of oxidative stress index

Percent ratio of TOS level to TAC level was accepted as oxidative stress index (OSI) [23]. OSI (Arbitrary unit) = TOS (μmol H_2O_2 Eq/L)/TAC (mmol Trolox Eq/L).

Measurement of seum paraoxonase and arylesterase activities

Paraoxonase and arylesterase activities were measured using commercially available kits (Relassay, Gaziantep, Turkey). The PON1 activity was assayed using two different substrates [24]. First, the rate of hydrolysis of paraoxon was measured by monitoring the increase in absorbance at 412 nm and 25 °C due to the formation of p-nitrophenol. PON1 activity assays were performed in the absence of basal activity and in the presence of NaCl (salt-stimulated activity). The serum sample to be tested was added to a cuvette containing 1.0 mmol paraoxon, 1.0 mmol $CaCl_2$, and 50 mmol glycine/NaOH buffer at pH 10.5. The amount of p-nitrophenol generated was calculated from the molar extinction coefficient at pH 10.5, which was 18,290/mol per centimeter. PON1 activity was expressed as U/L and defined as 1 mmol p-nitrophenol generated per minute under well-established conditions. Second, the PON1 activity was measured in an arylesterase assay using phenyl acetate as the substrate. The 10 mL aliquots of prediluted (1:3) serum samples were added to 1.5 mL reaction mixture containing 1.0 mmol phenylacetate, 9.0 mmol Tris-HCl buffer, and 0.9 mmol $CaCl_2$ at pH 8.0 and 25 °C. Enzymatic activity was calculated from the molar extinction coefficient, which was 1310/mol per centimeter. Arylesterase activity was expressed as kU/L and defined as 1 mmol of phenol generated per minute under the reaction conditions [25]. Blanks without enzyme were used to correct for the spontaneous hydrolysis of both substrates.

Phenotype distribution of PON1 activity

The phenotype distribution of PON1 activity was determined by the double-substrate method, which calculates the ratio of salt-stimulated paraoxonase activity and arylesterase activity, using paraoxon and phenylacetate as substrates [24]. The ratio of the salt-stimulated paraoxonase activity to the arylesterase activity was used to assign individuals to one of the three possible phenotypes [24]. The genetic polymorphism at codon 192 QYR is responsible for the presence of two isotypes: A (low activity) and B (high activity). The ratio of the hydrolysis of paraoxon in the presence of I M NaCl (salt-stimulated PON1 activity) to the hydrolysis of phenylacetate was used to assign individuals to one of the three possible (AA, AB, BB) phenotypes. AA represents low; AB, intermediate; and BB, high enzyme activity. Salt-stimulated paraoxonase/arylesterase activity ratio between 0.00 and 3.00 was classified as AA, 3.01–9.00 as AB, and 9.01–13.00 as BB phenotype [24]. All analyses were carried out in a blinded fashion.

Measurement of serum lipid hydroperoxide levels

Serum LOOH levels were measured with the ferrous ion oxidation-xylenol orange assay [26]. The principle of the assay is that it measures the oxidation of ferrous ion to ferric ion via various oxidants and the produced ferric ion is measured with xylenol orange. LOOHs are reduced by triphenyl phosphine (TPP), which is a specific reductant for lipids. The difference between measurements with and without TPP pretreatment gives the LOOH levels.

Measurement of serum total free sulfhydryl groups

The free sulfhydryl groups of serum samples were assayed according to the method of Ellman as modified by Hu et al. [27]. Briefly, 1 mL of buffer containing 0.1 M Tris, 10 mM EDTA at pH 8.2, and 50 µL serum was added to cuvettes followed by the addition of 50 µL of 10 mM DTNB in methanol. Blanks were run for each sample as a test, but there was no DTNB in the methanol. Following incubation for 15 min at room temperature, the sample absorbance was read at 412 nm on a Cecil 3000 spectrophotometer (Cecil Instruments, Cambridge, UK). Sample and reagent blanks were subtracted. The concentration of the sulfhydryl groups was calculated using reduced glutathione as the free sulfhydryl group standard, and the results were expressed as millimolar concentrations.

Other parameters

Fasting serum levels of triglycerides (TG), total cholesterol (TC), and HDL-cholesterol (HDL-C) were determined using commercially available assay kits (Abbott with an autoanalyzer (Aeroset®, Abbott Diagnostics, Germany)). Serum LDL-C levels were calculated with the Friedewald formula.

Statistical analysis

The results are expressed as the mean ± standard deviation. The comparisons of parameters of acute brucellosis and healthy controls were performed using Student's *t*-test. The correlation analyses were performed using Pearson's correlation test. The χ^2 test was used to compare PON1 phenotype distribution in patients and controls. The results were considered to be statistically significant when the p value was less than 0.05. The data were analyzed using the SPSS® for Windows computing program (version 11.0).

Results

The demographic characteristics of the patients and the controls are shown in Table 1. There were no significant differences between the groups with respect to age, gender, and body mass index ($p > 0.05$) (Table 1).

The serum TG, TC, and LDL-C levels were significantly higher in the patients with brucellosis compared with the

Table 1 Demographic characteristics of the two groups in this study

Parameters	Patients ($n=30$)	Controls ($n=35$)	p
Age (years)	39.9 ± 6.7	38.1 ± 3.6 ns	ns
Sex (female/male)	17/13	19/16 ns	ns
Body mass index (kg/m^2)	23.1 ± 1.2	22.4 ± 2.2 ns	ns
TG (mg/dL)	206.36 ± 88.81	137.53 ± 57.44	< 0.01
TC (mg/dL)	207.26 ± 55.34	168.25 ± 22.17	< 0.01
HDL-C (mg/dL)	31.93 ± 8.36	43.52 ± 14.72	< 0.01
LDL-C (mg/dL)	134.06 ± 46.13	96.63 ± 13.85	< 0.01

TC total cholesterol, *TG* triglyceride, *HDL-C* high-density lipoprotein-cholesterol, *LDL-C* low-density lipoprotein-cholesterol, *ns* nonsignificant
Values are mean ± SD

Table 2 PON1 activity, total thiols levels and oxidative status levels of the two groups

Parameters	Patients ($n=30$)	Controls ($n=35$)	p
Total antioxidant status (mmol Trolox Eq/L)	1.30 ± 0.28	1.51 ± 0.18	< 0.05
Total oxidant status (µmol H_2O_2 Eq/L)	18.01 ± 5.56	7.93 ± 3.07	< 0.05
Oxidative stress index (arbitrary unit)	1.941 ± 0.53	0,564 ± 0.30	< 0.05
Paraoxonase (U/L)	120.49 ± 58.22	193.19 ± 52.58	< 0.05
Salt-stimulated paraoxonase (U/L)	276.21 ± 145.03	438.26 ± 226.15	< 0.05
Arylesterase (kU/L)	137.13 ± 41.29	186.31 ± 56.29	< 0.05
LOOH (µmol/L)	9.70 ± 3.03	6.13 ± 1.53	< 0.05
-SH (mmol L^{-1})	0.37 ± 0.17	0.51 ± 0.07	< 0.05

LOOH lipid hydroperoxide, *-SH* free sulfhydryl groups (total thiol)
Values are mean ± SD

controls (all $p<0.01$), while the HDL-C levels were significantly lower ($p<0.01$) (Table 2).

Serum basal salt-stimulated paraoxonase and arylesterase activities, TAC levels and thiols levels were significantly lower in patients than controls (for all, $p<0.05$), while LOOH levels, TOS levels, and OSI values were significantly higher (for all, $p<0.05$) (Table 2).

In acute brucellosis patients, serum TAC levels were inversely correlated with TOS levels ($r=-0.329$, $p<0.001$) and OSI values ($r=-0.572$, $p<0.05$). However, no correlation was found between TAC levels and LOOH levels ($p>0.05$).

In acute brucellosis patients, serum TOS levels, LOOH levels, and OSI values were inversely correlated with thiols levels ($r=-0.387$, $p<0.001$; $r=-0.241$, $p<0.01$; and $r=-0.525$, $p<0.05$; respectively), arylesterase activity ($r=-0.295$, $p<0.001$; $r=-0.213$, $p<0.01$ and $r=-0.349$, $p<0.01$; respectively), and paraoxonase activity ($r=-0.395$, $p<0.001$; $r=-0.284$, $p<0.05$; and $r=-0.485$, $p<0.05$; respectively).

In acute brucellosis patients, serum TC and LDL-C levels were inversely correlated with TAC levels ($r=-0.358$, $p<0.01$; $r=-0.308$, $p<0.01$; respectively), thiols levels ($r=-0.461$, $p<0.05$; $r=-0.482$, $p<0.05$; respectively), paraoxonase activity ($r=-0.335$, $p<0.05$; $r=-0.363$, $p<0.01$; respectively). In additon, serum HDL-C levels were positively correlated with paraoxonase activity ($r=0.355$, $p<0.01$), TAC ($r=0.352$, $p<0.01$), thiols levels ($r=0.368$, $p<0.01$), and negatively correlated with TOS levels ($r=-0.610$, $p<0.05$), OSI values ($r=-0.587$, $p<0.05$), and LOOH levels ($r=-0.393$, $p<0.01$). However, no correlation was found between serum TG levels and paraoxonase, arylesterase, TAC levels, thiols levels, TOS levels, OSI values and LOOH levels ($p>0.05$).

In acute brucellosis patients, no significant correlation was found between serum agglutination test with titers and paraoxonase and arylesterase activities, thiols levels, TAC levels, TOS levels, OSI values and LOOH levels ($p>0.05$).

The PON1 phenotype distribution was calculated in two groups. The resulting ratio was used to identify subject phenotypes: homozygous AA (PON1192QQ), heterozygous AB (PON1192QR), and homozygous BB (PON1192RR). PON1 phenotype distribution of the subjects was not significantly different between these three groups ($p>0.05$).

Discussion

In the present study, we measured the enzymatic activities of PON1 (paraoxonase and arylesterase) in patients with brucellosis. We observed that brucellosis subjects had decreased paraoxonase and arylesterase activities along with increased LOOH levels. In the present study, we found a negative correlation between LOOH levels, and paraoxonase and arylesterase activities in brucellosis patients.

Although previous studies have been suggested a relationship between oxidative stress and brucellosis, there is limited and conflicted information about oxidative status in patients with brucellosis [8, 17]. Serefhanoglu et al. [8] found decreased TAC levels along with increased TOS levels and OSI values in patients with brucellosis. Conversely, more recently, Demirpençe et al. [17] observed increased TAC levels and decreased OSI values in patient with brucellosis when compared with the control group. In the present study, we observed that brucellosis subjects had decreased TAC levels along with increased TOS levels and OSI values. Our results were in accordance with previous report [8].

Brucella melitensis is a facultative intracellular pathogen which is able to survive and replicate within phagocytic cells. Therefore, it has to adapt to a range of different hostile environments. The oxidative killing by polymorphonuclear leukocytes and macrophages plays a primary role in the elimination of intracellular brucellae [28]. Infection and inflammation are associated with a decrease in HDL-C levels. Several studies have indicated that chronic infection was closely associated with alterations of lipid metabolism [29], being also associated with an atherogenic lipid profile [30]. Induction of changes in lipoproteins by cytokines indirectly predisposes patients to atherosclerosis [31]. The decrease in cholesterol levels in various infections is well known, but the underlying mechanisms remain unclear. Several studies showed a reduction in cholesterol levels in patients with various infections [32, 33]. In the present study, we found that serum TG, TC, and LDL-C levels were significantly higher in patients with brucellosis compared with controls, while HDL-C levels were significantly lower.

Blood contains many antioxidant molecules that prevent and/or inhibit harmful free radical reactions. Free sulfhydryl groups serve as main antioxidant component of serum by several mechanisms [21]. Sulfhydril groups prevent tissue damage through reacting with free oxygen radicals and lipid peroxides and neutralization of these molecules [21]. Therefore, the measurement of thiol levels is a good reflection of excess free radical generation [27]. In the present study, we found that total serum thiol levels were significantly lower in brucellosis patients than controls group. In addition, serum TOS levels, LOOH levels, and OSI values were inversely correlated with serum thiols levels. Decreased total serum thiol levels in brucellosis may reflect its decreasing effect against oxidative stress. Moreover, serum thiol levels were positively correlated with TAC levels and PON1 activity. Therefore, it is possible to say that, in the inactivation of TAC and PON, the thiol groups can have a role.

PON1 activity may be altered as part of the inflammatory response [15, 16]. Reduced PON1 activity has been reported in various diseases such as diabetes mellitus [34], atherosclerotic heart disease [35], and hypercholesterolemia [36] who are under increased oxidative stress [37, 38]. PON1 is also one of the antioxidants which act as an enzymatic defense against lipid hydroperoxides [39]. Moreover, oxidative stress has been reported to affect the expression and activities of PON1 [13]. Reduced serum PON1 activity has been reported to be associated with some diseases under oxidative stress and inflammation conditions [40, 41].

It is well known that PON1 activity may decrease during the course of infection and inflammation and acute phase HDL is unable to protect LDL against oxidation [15–17]. Furthermore, previous investigators have shown that PON1 activity was decreased in patients with hepatitis C [42] and human immunodeficiency virus [43]. Aslan et al. [33] reported reduced levels of PON1 activity in the serum of *Helicobacter pylori* infected patients. Several investigators have assessed the role of PON1 enzyme activity in patients with brucellosis. However, these studies are limited and conflicting [17, 18]. Recently, Demirpence et al. [17] assessed the effect of PON1 activity in patients with brucellosis. In that study, there was no significant difference in the PON1 activity of patients with brucellosis compared with the healthy controls. Apostolou et al. [18] reported that PON1 activity was increased after treatment in patients with acute brucellosis. In the present study, we found decreased serum PON1 activity in patients with acute brucellosis compared with the healthy controls.

The mechanism of the observed decrease in serum PON1 activity in brucellosis patients remains unclear. This decrease could be related to enhanced lipid peroxidation, since oxidized lipids are reported to inhibit PON1 activity. Increased inactivation of PON1 according to increased generation of ROS in brucellosis can explain the decrease in serum PON1 activity [44]. Furthermore, PON1 is an HDL-associated enzyme and it has been suggested that reduced serum PON1 activity might be associated with decreased HDL-C levels [45]. Also, in accordance with a previous report [46], we found a positive correlation between PON1 activity and HDL-C levels.

Although PON1 activity and concentration are determined genetically, several factors—such as diet, lifestyle, and environmental factors—can influence PON1 activity and/or concentration. Degraded cooking oil has been reported to lower serum PON1 levels in humans [47]. Van der Gaag et al. [48] reported that daily moderate alcohol intake and dietary polyphenols can increase PON1 activity. Smoking has also been shown to decrease serum PON1 activity [49]. On the other hand, PON1 activity is positively correlated with the intake of vitamins C and E in the diet [50].

The PON1 activity varies among individuals and populations and this difference is chiefly related to the position 192 polymorphism of the PON1 gene [51]. PON1 has two common coding region polymorphisms, a methionine (M) to leucine (L) substitution at position 55 and a glutamine (Q) to arginine (R) substitution at position 192. The position 192 polymorphism is the major determinant of PON1 activity polymorphisms [52]. However, the position 55 polymorphisms also exert a smaller, but significant, effect on activity. Both polymorphisms may be associated with cardiovascular disease development [53]. The ratio of salt-stimulated PON1 activity to arylesterase activity is used for the definition of phenotypes [54]. In the present study, we wanted to investigate whether phenotypic variations in PON1 could play a role in the reduction of serum PON1 activity observed in brucellosis patients. However, we found no significant difference between PON1 192 Q and R polymorphism distribution in brucellosis patients and control group. To the best of our knowledge, this is the first study on PON1 192 Q and R polymorphism distribution in patients with brucellosis.

There were several limitations in the present study. First, this study is a cross-sectional study. Second, the number of patients with brucellosis that were enrolled in the study was relatively small. However, a large sample would have increased the power to detect serum PON1 activity in patients with brucellosis. Third, PON1 genotype was not determined in the study population; however, it was reported that serum PON1 activity is a better predictor of the risk for cardiovascular diseases than PON1 genotype [55].

We concluded that oxidative stress is increased, while serum PON1 activity is decreased in patients with acute brucellosis. These results indicate that lower PON1 activity is associated with oxidant–antioxidant imbalance. Therefore, measurement of serum PON1 activity may be usefull in acute brucellosis. Thus, since antioxidants increase the activity of PON1, it may be possible to conclude that addition of antioxidant vitamins such as vitamins C and E may be useful for these patients. Further studies with larger populations are needed to explore the results in acute brucellosis.

Ethical statement

The study protocol was conducted in accordance with the Helsinki Declaration as revised in 1989 and was approved by the local ethics committe. All subjects were informed about the study and the written consent was obtained from each one.

Conflict of interest

The authors do not report any conflict of interest regarding this work.

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