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A retrospective controlled study of thiol disulfide homeostasis as a novel marker in Crimean Congo hemorrhagic fever

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Objectives: Crimean Congo hemorrhagic fever (CCHF) is the second most common hemorrhagic fever worldwide. This study aimed to evaluate the oxidant-antioxidant balance of patients with CCHF by detecting dynamic thiol disulfide homeostasis (TDH), which is a novel oxidative stress marker, and other molecules, including paraoxonase (PON), arylesterase (ARES), ceruloplasmin (CLP), myeloperoxidase (MPO), and catalase.

Methods: This retrospective, cross-sectional, controlled study, which involved patients with CCHF and healthy volunteers, measured dynamic TDH using a novel automated method developed by Erel.

Results: We recruited 69 adult patients with CCHF (31 females, 38 males, median age 46 years). The case fatality rate was 1.49% (1/69). Increased disulfide/native thiol and disulfide/total thiol ratios, decreased total antioxidant status (TAS), and increased total oxidant status (TOS) were found in patients with CCHF. TAS, PON, and ARES values were found to be positively correlated with both native and total thiol levels, whereas TOS and CLP were negatively correlated with both, at a significant level. MPO activity was similar in both groups.

Discussion: This is the first study in the literature to evaluate dynamic TDH in CCHF. TDH shifts to the oxidative side in patients with CCHF, leading to an increase in TOS.

Keywords: Oxidative stress, Ceruloplasmin, Catalase, Total oxidant status, Paraoxonase, Arylesterase

Introduction

Crimean Congo hemorrhagic fever (CCHF) is a tick-borne disease caused by the CCHF virus, a nairovirus from the Bunyaviridae family. Although the disease is tick-borne, transmission may occur through contact with infected human and animal blood, tissue, or body fluids.^{1,2} In recent years, the CCHF virus has caused several outbreaks in the Middle East, Southeast Europe, Asia, and Africa. In 2015, a study reported a total of 1721 geopositioned occurrences worldwide, making CCHF the second most widespread viral hemorrhagic fever after dengue fever.³ The case fatality rate varies between 3 and 30%, depending on region.¹⁻³ Signs and symptoms of CCHF include high fever, headache, diffuse myalgia,

nausea, vomiting, diarrhea, and bleeding through the skin and mucosal surfaces. The most important laboratory findings are thrombocytopenia, leucopenia, elevation of aspartate aminotransferase (AST), alanine aminotransferase (ALT), creatine phosphokinase, and lactate dehydrogenase (LDH) levels, and prolongation of activated partial thromboplastin time and prothrombin time.⁴ Radiologic findings include hepatomegaly, splenomegaly, paraceliac lymphadenopathy, and effusion in the perihepatic, perisplenic, pleural, and hepatorenal recesses of the subhepatic space as well as between the intestinal loops.^{4,5} Diagnosis of CCHF is based on detection of viral RNA by real time reverse transcriptase-polymerase chain reaction (RT-PCR) and/or the presence of specific IgM antibodies (viral nucleocapsid and glycoprotein precursor antibodies: N-IgM and GPC-IgM).⁶

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Although many studies have tried to clarify the pathogenesis of CCHF, advances have been limited due to the virulence of the disease, which complicates animal studies. Endothelial damage and capillary leakage seem to be the most important underlying pathologies found so far. Many cytokines and molecules, such as soluble intercellular adhesion molecule-1 (sICAM-1), soluble vascular cell adhesion molecule-1, sE-selectin, sP-selectin, sL-selectin, and vascular endothelial growth factor-A, have been shown to be involved in the pathogenesis of CCHF.^{7,8}

Endothelium is a site of metabolic activity. Endothelial damage due to CCHF virus infection causes the emergence of reactive oxygen species (ROS), and as a consequence, harmful oxidative reactions can occur. ROS consist of superoxide radical anions, hydroxyl radicals, and hydrogen peroxide. As the oxidant–antioxidant balance in an organism sustains homeostasis, the antioxidant response is extremely important. Oxidative stress occurs when the balance shifts to the oxidative side.⁹ Thiols are compounds that contain sulfur and are the most important part of the antioxidant defense system. They protect an organism by destroying ROS using enzymatic and non-enzymatic mechanisms.¹⁰ Sulfur is an essential element because of its interactions with biomolecules. Thiols are susceptible to oxidation, and can react with disulfides through reversible thiol disulfide exchange reactions. As these disulfide bonds are reversible, they can be reduced to thiols depending upon the organism's oxidant–antioxidant balance; therefore, a dynamic thiol disulfide homeostasis (TDH) exists.^{11,12}

The balance of thiols and oxidative stress in different diseases is topical at present. The role and importance of TDH have been investigated in several recent studies. One of these studies found significantly lower total oxidant status (TOS) and also, interestingly, lower oxidative stress index values in patients with CCHF when the study results were compared with healthy age- and sex-matched controls.¹³ In another study, Guner *et al.*¹⁴ reported a significant decrease in total serum thiol levels and considerably higher TOS levels in patients with CCHF. However, as the two previous studies were based only on total thiol levels, the mechanism of TDH in CCHF remained unclear. In 2014, Erel and Neselioglu¹² developed a method for detecting total TDH, which allowed us to take another step forward in understanding the pathogenesis of CCHF in the present study.

This study aimed to evaluate the oxidant–antioxidant balance of patients with CCHF by detecting dynamic TDH, which is a novel oxidative stress marker, as well as some other well-known oxidant and antioxidant molecules, including paraoxonase (PON), arylesterase (ARES), myeloperoxidase (MPO), ceruloplasmin (CLP), and catalase (CAT).

Methods

Setting and study design

This retrospective, cross-sectional, controlled study took place in a 550-bed tertiary care hospital. CCHF is endemic in the area, and the Infectious Diseases and Clinical Microbiology Department of the hospital is a referral center for patients with CCHF.

The study population consisted of 69 adult patients (31 women, 38 men) who were hospitalized with CCHF. Diagnosis was confirmed by detection of viral RNA with RT-PCR and/or the presence of specific IgM antibodies by the Public Health Institute of Turkey, Microbiology Reference Laboratories Department, National Arbovirus and Viral Zoonoses Reference and Research Laboratory. RT-PCR was performed with Applied Biosystems, LightCycler[®] 480 Instrument II (Roche Diagnostic, Turkey), Rotor-Gene[™] 3000/6000 (Corbett Research, Germany) in accordance with the manufacturer's instructions. All patient results had been kept in the departmental archives, so the serology and viral RNA results were obtained from those files.

Twenty-five healthy volunteers without any acute or chronic illnesses were also included. Written informed consent was obtained from all participants (including the control group) or their relatives. The study was approved by the local ethics committee (Ethics Committee of Ankara Ataturk Training and Research Hospital), and performed in accordance with the ethical standards of the Declaration of Helsinki.

Measures

Blood samples for hematological and biochemical analyses were taken from each patient at admission, in the morning, after a fasting period of 12 hours. The serum samples were separated from the cells by centrifugation at 950g for 10 minutes, and stored at -80°C until analysis.

Dynamic TDH was measured by a novel automated method developed by Erel *et al.*,¹² which is comprised of three principal stages: reduction of disulfide bonds with sodium borohydride to form free functional thiol groups, removal of unused sodium borohydride with formaldehyde, and finally, determination of all thiol groups including reduced and native thiols following reaction with 5,5'-dithiobis-(2-nitrobenzoic) acid. The amount of dynamic disulfide is half of the difference between total thiols and native thiols. Therefore, disulfide/total thiol (SS/SH + SS), disulfide/native thiol (SS/SH), and native thiol/total thiol (SH/SH + SS) ratios can all be calculated with this method.

PON and ARES activities were measured using commercially available kits (Rel Assay Diagnostics, Gaziantep, Turkey). TOS and decreased total

antioxidant status (TAS) of plasma were measured by an automated colorimetric method, as previously described.¹⁵

MPO activity was determined by a modification of the o-dianisidine method.¹⁶ CLP levels were measured by an automated and colorimetric method, based on the enzymatic oxidation of ferrous ion to ferric ion.¹⁷ CAT activity was measured by Góth's method.¹⁸

Statistical analysis

Statistical analyses were performed using the Statistical Package for Social Sciences, version 15.0 for Windows (SPSS, Chicago, IL, USA). As the variables were not distributed normally, non-parametric tests were used. The Mann–Whitney *U*-test was used to compare the two groups. Values were presented as medians (interquartile ranges) and percentages. Relations between thiol disulfide and other parameters were evaluated using Spearman's correlation test. A *P*-value of less than 0.05 was defined as significant.

Results

The median age of the study patients was 46 (15–84) years. The mortality rate was 1.49% (1/69). All the patients had fever and fatigue on admission, while six (8.7%) had some kind of bleeding such as nose bleed. The laboratory findings [median (range)] were as follows: hemoglobin 13.6 g/dl (8.5–16.8), white blood cells 2980 K/ μ l (1150–12 000), platelets 83 K/ μ l (15–196), ALT 90 U/l (39–485), and AST 143 U/l (19–1079). Native and total thiol levels were decreased to a significant level in the CCHF patient group, while there was no significant difference in disulfide level between the two groups (Table 1). An increase in disulfide/native thiol and disulfide/total thiol ratios

was noted in the CCHF patient group. TAS was decreased and TOS increased in patients with CCHF, indicating that the oxidative–antioxidative balance had shifted towards the oxidative side in the patients with CCHF.

TAS, PON, and ARES values were positively correlated with both native and total thiol levels, whereas TOS and CLP were negatively correlated with native and total thiol levels to a significant level. Correlations between thiol parameters and TAS, TOS, CLP, PON, and ARES are shown in Table 2. There was no correlation between thiol parameters and bleeding to a significant level. In addition, there was no significant difference in terms of TDH between patients who had bleeding and patients who did not have bleeding.

Discussion

CCHF is endemic in a large geographic region consisting of Europe, Asia, and Africa. Therefore, research focusing on clarifying the pathogenesis of CCHF is of high importance. In this study, the oxidant–antioxidant balance of CCHF was evaluated using the following parameters: thiol, PON, ARES, CLP, CAT, MPO, and TDH, which is a novel oxidative stress marker.

Every cell in an organism has to maintain the balance between oxidative and antioxidative status to sustain its vitality. Any change to this balance in either direction results in cell damage.¹⁹ Thiols form the most important part of the antioxidant system. They are susceptible to oxidation and form disulfide bonds that may cause significant loss of biological activity. Disulfide bonds are reversible and can be reduced to thiols based on an organism's oxidant–antioxidant balance, which allows dynamic TDH.

Dynamic TDH status is important for antioxidant protection, detoxification, signal transduction, apoptosis, regulation of enzymatic activity and transcription factors, and cellular signaling mechanisms.^{19,20} Until recently, only one side of the TDH could be measured, using the Elman method defined in 1979. However, with the development of a new, low-cost, and easy method designed by Erel and Neselioglu in 2014, it has become possible to measure both sides of the disulfide homeostasis.¹²

Since the introduction of this novel detection method for TDH, several studies have evaluated its use in different diseases and shown promising results. Regarding CCHF, there are only a few studies in the literature concerning endothelial damage and oxidant–antioxidant status, and there are no studies on the subject of TDH. Guner *et al.*¹⁴ and Aydin *et al.*¹³ found a significant increase in TOS and oxidative stress index in patients with CCHF. An increase in oxidative stress can induce hepatic injury,²¹ which could

Table 1 Oxidant–antioxidant enzymes and biomolecules in patients with CCHF

	Patient <i>n</i> = 69	Control <i>n</i> = 25	<i>P</i> value
Native thiol, μ mol/l	156.5 (87.35)	294.7 (100.85)	<i>P</i> < 0.001
Total thiol, μ mol/l	191.9 (94.25)	325.4 (132.05)	<i>P</i> < 0.001
Disulfide, μ mol/l	17.55 (5.4)	19.2 (12.68)	<i>P</i> > 0.05
Disulfide/native thiol, %	11.25 (8.44)	6.41 (6.13)	<i>P</i> < 0.001
Disulfide/total thiol, %	9.18 (5.45)	5.68 (4.7)	<i>P</i> < 0.001
Native thiol/total thiol, %	81.62 (10.89)	88.63 (9.39)	<i>P</i> < 0.001
Total antioxidant status, mmol Trolox equiv./l	1.69 (0.47)	2.15 (0.36)	<i>P</i> < 0.001
Total oxidant status, μ mol H ₂ O ₂ equiv./l	8.68 (7.74)	1.67 (2.12)	<i>P</i> < 0.001
Paraoxonase, U/l	140.9 (96.8)	197.7 (162.35)	<i>P</i> = 0.007
Arylesterase, KU/l	143.85 (82.7)	186.9 (75.73)	<i>p</i> < 0.001
Ceruloplasmin, U/l	98.74 (31.79)	64.33 (20.83)	<i>P</i> < 0.001
Catalase, U/l	111 (155.63)	220.2 (216)	<i>P</i> = 0.001
Myeloperoxidase, U/l	95.51 (166.51)	117.3 (109.14)	<i>P</i> > 0.05

Table 2 Correlations between thiol parameters and other oxidant-antioxidant markers

<i>n</i> = 94	-SH	-SH + -S-S-	-S-S-	-S-S-/-SH	-S-S-/-SH + -S-S-	-SH/-SH + -S-S-
TAS	<i>r</i> = 0.386 <i>P</i> < 0.001	<i>r</i> = 0.408 <i>P</i> < 0.001	<i>r</i> = 0.161 <i>P</i> > 0.05	<i>r</i> = -0.219 <i>P</i> = 0.034	<i>r</i> = -0.219 <i>P</i> = 0.034	<i>r</i> = 0.219 <i>P</i> = 0.034
TOS	<i>r</i> = -0.346 <i>P</i> = 0.001	<i>r</i> = -0.331 <i>P</i> = 0.002	<i>r</i> = 0.248 <i>P</i> = 0.02	<i>r</i> = 0.449 <i>P</i> < 0.001	<i>r</i> = 0.449 <i>P</i> < 0.001	<i>r</i> = -0.449 <i>P</i> < 0.001
PON	<i>r</i> = 0.313 <i>P</i> = 0.002	<i>r</i> = 0.319 <i>P</i> = 0.002	<i>r</i> = 0.067 <i>P</i> > 0.05	<i>r</i> = -0.243 <i>P</i> = 0.019	<i>r</i> = -0.243 <i>P</i> = 0.019	<i>r</i> = 0.243 <i>P</i> = 0.019
ARES	<i>r</i> = 0.428 <i>P</i> < 0.001	<i>r</i> = 0.474 <i>P</i> < 0.001	<i>r</i> = 0.327 <i>P</i> = 0.001	<i>r</i> = -0.157 <i>P</i> > 0.05	<i>r</i> = -0.157 <i>P</i> > 0.05	<i>r</i> = 0.157 <i>P</i> > 0.05
CLP	<i>r</i> = -0.381 <i>P</i> < 0.001	<i>r</i> = -0.382 <i>P</i> < 0.001	<i>r</i> = 0.078 <i>P</i> > 0.05	<i>r</i> = 0.351 <i>P</i> = 0.001	<i>r</i> = 0.351 <i>P</i> = 0.001	<i>r</i> = -0.351 <i>P</i> = 0.001

-SH, native thiol; -S-S-, disulfide; -SH + -S-S-, total thiol.

The *r* value is the Spearman's rho correlation coefficient.

The *P* value is significance.

TAS: Total antioxidant status, TOS: Total oxidant status, PON: Paraoxonase, ARES: Arylesterase, CLP: Ceruloplasmin.

contribute towards the high levels of AST, ALT, alkaline phosphatase, gamma-glutamyl transferase, and LDH seen in patients with CCHF. Guner *et al.* showed a strong positive correlation between total thiol level and platelet count, which decreases in almost all CCHF cases.¹³ It is still unknown whether total thiol level decreases because of the pathogenesis of CCHF or high levels of total thiol are already protective against CCHF.

There is one CCHF study that showed contrasting results to the two studies described above. Karadag-Oncel *et al.*²² reported significantly lower TOS, oxidative stress index, and total thiol levels in patients with CCHF. However, the authors of this study stated that their results were 'paradoxical' and 'difficult to explain.' To our knowledge, the three previously mentioned studies are the only studies in the literature to have investigated TOS, thiol, and CCHF.

Other molecules and enzyme activities also provide insights into the oxidant-antioxidant balance of patients with CCHF, including PON, ARES, MPO, CLP, and CAT. PON1 enzyme has an antioxidant effect.²³ Several studies have demonstrated a negative correlation between ROS and both PON and ARES.^{24,25} Other studies have shown that TAS, PON, and ARES were all decreased in patients with CCHF, while CAT remained stable.^{13,26} In the current study, a strong positive correlation was found between PON and both native and total thiol levels, and significantly lower levels of PON, ARES, and CAT were found in the CCHF patient group. CAT is an important enzyme that protects against the cell damage caused by ROS.²⁶ The oxidant-antioxidant balance in the current study shifted toward TOS in the patients with CCHF, which may correlate with CAT activity. Unfortunately, there are no previous studies that have focused on reduced CAT activity and the severity of CCHF.

CLP is another molecule with oxidase activity. Although CLP was once studied in relation to the

severity of CCHF, no difference was found between fatal and non-fatal cases in terms of CLP levels.²⁷ Significantly higher CLP levels were found among patients with CCHF compared with the control patients in the current study.

MPO activity generally increases in viral infections, and this has been shown to be the case in CCHF in a previous study.²⁸ However, the CCHF patient sample size in that study was small (*n* = 47) compared with the current study (*n* = 69), and the difference in MPO activity was not as noticeable when compared with the controls (57.6 vs. 44.8). Although MPO is an indicator for oxidative stress and endothelial dysfunction, especially in chronic diseases, it does not seem to be significantly involved in the pathogenesis of CCHF, which is not a chronic disease.

There are several limitations to this study. Pyrogens may induce oxidative stress by increasing oxygen radical formation.²⁹ The control group consisted of healthy individuals, and a third group with fever of other origin was not included; therefore it is not clear whether the results are influenced also by the fever. Another limitation is that the onset of fever could influence the results. The samples were all taken at admission, when the patient was febrile, but the onset of fever and follow-up samples were not studied.

Endothelium is very important in the pathogenesis of hemorrhagic fevers. Endothelial pathologies (which include activation of the endothelial cells, release of vasoactive substances, endothelial apoptosis, and increased mitochondrial ROS) are implicated in the hypovolemic shock that is seen in hemorrhagic fevers such as dengue fever and Ebola hemorrhagic fever.³⁰ Dengue fever, being the most frequent hemorrhagic fever, has drawn most research attention so far, and many studies have been published about its pathogenesis. In their study, Olganier *et al.*³¹ emphasized the importance of oxidative stress response in dengue fever. Increased ROS and changes in the redox

homeostasis were described in many viral infections and maintaining the redox balance is very important. Unfortunately, there has been no study published about thiol homeostasis and dengue fever.

To our knowledge, the present study is the first study in the literature to have evaluated dynamic TDH in CCHF. We found significant decreases in both total and native thiol levels in patients with CCHF, while disulfide levels remained stable.

In conclusion, TDH shifts to the oxidative side in patients with CCHF, leading to an increase in TOS. Lower levels of PON, ARES, and CAT, and higher levels of CLP also correlate with these findings. No further research is required into the oxidant-antioxidant balance in CCHF. Other enzymes and molecules that cause vascular damage may be worthwhile subjects for future research aimed at increasing understanding of CCHF pathogenesis.

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Disclaimer statements

Contributors All authors contributed equally.

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Conflicts of interest None.

Ethics approval All investigators agreed to obey the ethical standards as described in the Declaration of Helsinki and the study was approved by the Local Ethics Committee (December 2014).

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