

## Selenium levels, selenoenzyme activities and oxidant/antioxidant parameters in H1N1-infected children

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Selenium (Se) is an essential trace element, and it shows its biological functions within low molecular Se compounds and Se-containing proteins, known as "selenoproteins". Glutathione peroxidases (GPxs) and thioredoxin reductases (TrxRs) are the most important selenoproteins functioning as antioxidant enzymes. These enzymes protect the body from the endogenous products of cellular metabolism that have been implicated in DNA damage, mutagenesis, and carcinogenesis. H1N1 virus is a subtype of the influenza A virus and was an endemic in humans in 2009 and 2010. Taking into account the high incidence of Se deficiency and the high mortality and morbidity rates in H1N1 infection, this study was designed to investigate the plasma and erythrocyte Se levels, selenoenzyme activities and other oxidant/antioxidant parameters in H1N1-infected children during the 2009-2010 pandemic. We observed a significant increase in C-reactive protein levels (245%) and marked decreases in both plasma and erythrocyte Se levels (11%, both) and in GPx1 (45%), GPx3 (16%) and TrxR (30%) activities in H1N1-infected children compared to the control group. In addition, significant decreases were observed in erythrocyte catalase (CAT) (38%), total superoxide dismutase (SOD) (42%) and glutathione S-transferase (GST) (19%) activities and in erythrocyte total glutathione (GSH) (18%) and plasma GSH (10%) concentrations, while marked increases were observed in plasma lipid peroxidation levels (27%). However, we did not find a significant difference in selenoprotein P (SePP) levels between the groups. Our findings show that Se-dependent and -independent blood redox systems are down-regulated in H1N1 influenza. These findings emphasized the critical role of Se as an effective redox regulator and the importance of Se status in infections, particularly in H1N1 influenza.

**Key words:** H1N1, selenium, selenoenzymes, influenza, antioxidant enzymes, lipid peroxidation.

The role of nutrition in several infectious diseases has long been linked to changes in the immune response of the nutritionally deficient host<sup>1-3</sup>. It has been shown that nutritionally deficient humans or animals are more susceptible to a wide variety of infections. This increase in susceptibility is thought to be the result of an impaired host immune response due to a deficient diet<sup>4</sup>.

Malnutrition as well as deficiency states create fertile soil for host oxidative stress and for mild pathogens to become highly virulent<sup>5</sup>. However, recent studies have demonstrated that not only is the host immune response affected by the deficient diet, but the viral pathogen itself can also be altered<sup>4</sup>. Dietary deficiency states [like selenium (Se) deficiency] that lead to oxidative stress in the host can cause alterations of a viral genome such that a normally benign or

mildly pathogenic virus becomes highly virulent in the deficient and oxidatively stressed host. Once the viral mutations occur, even hosts with normal nutriture can be affected by the newly pathogenic strain<sup>5</sup>.

Selenium is a vital essential dietary element, and it is required to regulate the cellular redox homeostasis. Se is the integral component of several selenoproteins, and these proteins allow several crucial biological processes to take place, like reactive oxygen species (ROS) elimination or specific enzyme modulation<sup>6</sup>. Selenoenzymes like the glutathione peroxidase (GPx) enzyme family and thioredoxin reductases (TrxRs) have been attracting researchers' attention mostly<sup>7</sup>. The heterotetrameric enzymes GPx1 and GPx3 contain selenocysteine in their active centers and degrade a variety of peroxides, including H<sub>2</sub>O<sub>2</sub>. GPx1 is found in most tissues and erythrocytes. GPx3 is expressed by several types of epithelia and is secreted into plasma by the kidney<sup>7</sup>. Catalase (CAT) also degrades H<sub>2</sub>O<sub>2</sub><sup>8</sup>.

Among several trace element deficiency states, Se deficiency is prominently studied in infectious disease conditions, as Se exerts a vital role in the immune system<sup>9</sup>. Se is essential for the functioning of neutrophils, macrophages and natural killer (NK) cells<sup>10</sup>. Moreover, Se is a key nutrient for prevention of virulence development, and Se deficiency is associated with an increase in several infectious conditions, like human immunodeficiency virus (HIV), hepatitis and polio<sup>11-13</sup>. When Se-deficient virus-infected hosts were supplemented with dietary Se, viral mutation rates diminished and immunocompetence improved<sup>13</sup>. Studies show that Se deficiency may be one of the underlying factors of the solemnity of different types of influenza infections<sup>13-15</sup>. The normal Se concentration in adult human serum samples is 70-150 µg/L. As evidenced by the results of studies performed in different regions of Turkey, Se status in our country was not usually in the optimal range, but rather a marginal status was noted. The first study determining the serum Se levels in 76 children (age range: 2 months-13 years) belonging to middle-upper class families living in Ankara province reported that the mean Se level was found as 88 ± 12 µg/L<sup>16</sup>. In another study performed on 218 subjects from different socioeconomic status, the researchers found that the Se levels were

69 ± 13 µg/L in infants aged 2-12 months, 77 ± 12 µg/L in children aged 12 months-16 years, and 74 ± 16 µg/L in adults aged 18-48 years<sup>17</sup>. A study performed on high school children living in an endemic goiter area reported that the mean plasma Se levels in goitrous and control groups were 67 ± 11 µg/L and 85 ± 14 µg/L, respectively<sup>18</sup>. In a study conducted on rural adult populations (n=153, age range: 22-51 years) from three distinct regions of Turkey, mean Se levels were found to range from 55-75 µg/L<sup>19</sup>.

Influenza virus is a segmented RNA virus in the *Orthomyxoviridae* family. These viruses are responsible for a great deal of morbidity and mortality each year. The elderly, children and patients with chronic diseases of the lung and/or heart comprise the highest risk groups. Influenza viruses have a propensity to alter their surface proteins in order to escape early detection by the immune system of an infected host<sup>20</sup>. H1N1 virus, a subtype of the influenza A virus, was an endemic in humans in 2009 and 2010. When a strain of this virus was becoming a "seasonal flu", it showed up in pigs as an endemic, and was given the name "swine flu"<sup>21</sup>. The virus presented the following symptoms in the pigs: fever, lethargy, sneezing, coughing, difficulty breathing, and a decrease in appetite. The transmission of the virus from pigs to humans occurred, and in 2009, a new strain of the H1N1 virus named as "H1N1/09" became a general worldwide pandemic. In early June 2009, the World Health Organization raised the pandemic alert level to Phase 6<sup>21</sup>. This strain is contagious, its infectious characteristic is higher than that of seasonal flu, and it can be transmitted between people like seasonal flu. H1N1/09 strain was the cause of "acute respiratory distress syndrome", and half the deaths are due to this cause<sup>22</sup>.

A retrospective multi-center analysis of case records was conducted in Turkey involving H1N1 influenza-infected and hospitalized children in 2009. The analysis showed that a total of 821 children with 2009 pandemic H1N1 were hospitalized. The majority of admitted children (~57%) were younger than 5 years of age. Respiratory complications including wheezing, pneumonia, pneumothorax, pneumomediastinum, and hypoxemia were seen in 272 (~33%) children. Thirty-five children

(~4%) died and (~26%) of these were healthy before the H1N1 virus infection. However, the death rate was significantly higher in patients with chronic diseases, cancer and respiratory complications. The most common causes of mortality were pneumonia and sepsis. It was concluded that in Turkey, the 2009 H1N1 infection caused high mortality and pediatric intensive care unit (PICU) admission due to severe respiratory illness and complications, especially in children with an underlying condition<sup>23</sup>.

Therefore, taking into account the high incidence of Se deficiency and high mortality and morbidity rates from H1N1 both in Turkey and throughout the globe, this study was designed to investigate the Se levels, selenoenzyme activities and oxidant/antioxidant parameters in H1N1-infected children during the 2009-2010 pandemic.

## Material and Methods

### Study Groups

The study group was composed of H1N1-infected children (n=11; 4 girls, 7 boys; mean age:  $9.3 \pm 4.5$  years) who were hospitalized in Hacettepe University Pediatric Infectious Disease Unit. Twelve healthy children of comparable age and sex and with no infectious disease were used as the healthy control group (6 girls, 6 boys; mean age:  $10.8 \pm 2.6$  years). The blood samples were collected in winter 2009 from the patient and control groups. All subjects participated in the study voluntarily, and written consent was obtained from the parents of the children involved. The study was approved by the Ankara Numune Research and Training Hospital Ethical Committee.

### Chemicals and Reagents

All chemicals were purchased from Sigma-Aldrich (St. Louis, MO). Colorimetric assay kit for TrxR was also from Sigma-Aldrich. Tetramethylammonium hydroxide (TMAH solution) (25% w/v in water) was also purchased from Sigma-Aldrich. Rhodium (Rh) stock solution (1 g/L) was obtained from Perkin-Elmer (Perkin Elmer, Norwalk, CT). Tina-quant C-reactive protein (CRP) (latex) high-sensitive assay was obtained from Roche Diagnostics (Mannheim, Germany).

### Preparation of Plasma and Erythrocyte Samples

Venous blood samples were taken in heparinized and EDTA-containing tubes and were centrifuged at  $800 \times g$  for 15 minutes (min). Plasma was separated and erythrocytes were washed with 0.9% NaCl and centrifuged at 3000 rpm for 10 min. This procedure was repeated three times and erythrocyte packages were obtained. Both plasma and erythrocyte samples were aliquoted and kept at  $-80^{\circ}\text{C}$  until analysis. Heparinized plasma samples were used for the detection of CRP, Se and malondialdehyde (MDA) levels and GPx3 activity, while EDTA-containing plasma samples were used for selenoprotein P (SePP) analysis. Erythrocytes were used for the determinations of GPx1, TrxR, CAT, superoxide dismutase (SOD), and glutathione S-transferase (GST) activities.

### Determination of C-Reactive Protein Levels

C-reactive protein levels were measured by an immunoturbidimetric method (Tina-quant CRP detection method) performed on a Hitachi 717 automated analyzer (Mannheim, Germany).

### Determination of Erythrocyte and Plasma Selenium Levels

#### Sample Preparation

The plasma and erythrocyte samples (200  $\mu\text{l}$ ) were pipetted into conical tubes. Then, 500  $\mu\text{l}$  of 10% v/v TMAH solution was added to the samples and incubated at room temperature for 10 min, and then the volume was made up to 10 ml with a solution containing EDTA (0.05% w/v) and Triton X-100 (0.005% v/v).  $^{103}\text{Rh}$  was added as internal standard to obtain a final concentration of 10  $\mu\text{g/L}$ . After this procedure, samples were directly analyzed by inductively coupled plasma mass spectrometry (ICP-MS)<sup>24</sup>.

### Instrument Configuration and Measurement with Inductively Coupled Plasma Mass Spectrometry

Plasma and erythrocyte Se levels were measured using ICP-MS (Perkin Elmer, Norwalk, CT) with high-purity argon (99.99%), which used a Meinhard concentric nebulizer (Spectron/Glass Expansion, Ventura, CA) connected to a cyclonic spray chamber. A radiofrequency (RF) of 1100 W power was selected in pulse mode with auto lens one. Argon nebulizer gas flow rate was optimized daily from 0.5 to 0.9 L/min. Data were acquired in counts per second (cps).

The isotopes measured were  $^{103}\text{Rh}$  (internal standard) and  $^{82}\text{Se}$ . Three repeats were done per sample, and 10 repeats were done for the blank and a dwell time of 50 milliseconds (ms).

#### Determination of Oxidant/Antioxidant Parameters

All spectrophotometric measurements were performed by a Shimadzu UV-1601 spectrophotometer (Kyoto, Japan). Specific activities of erythrocyte TrxR, erythrocyte CAT and erythrocyte SOD were expressed as U/mg hemoglobin (Hb), while erythrocyte GPx1 activity was expressed as U/g Hb and erythrocyte GST activity as nmol/min/mg Hb. Plasma GPx3 activity was expressed as U/L.

#### Determination of Selenoenzyme Activities

GPx1 activity in erythrocytes and GPx3 activity in plasma were measured in a coupled reaction with glutathione reductase (GR) as described earlier<sup>25,26</sup>. The assay is based on the instant and continuous reduction of oxidized glutathione (GSSG) formed during GPx reaction by an excess of GR activity providing for a constant level of total glutathione (GSH). As a substrate, t-butyl hydroperoxide was used, and the concomitant oxidation of NADPH was monitored spectrophotometrically at 340 nm. One unit of enzyme was defined as the amount of GPx1 that transformed 1  $\mu\text{mol}$  of NADPH to NADP per min at 37°C.

Erythrocyte TrxR activity was determined colorimetrically using the Thioredoxin Reductase Assay kit. As described previously by Arnér et al.<sup>27</sup>, the method was based on the reduction of 5,5'-Dithiobis(2-nitrobenzoic acid (DTNB) with NADPH into 5-thio-2-nitrobenzoic acid (DTNB), the concentration of which was measured at 412 nm. One unit of TrxR activity was defined as the amount of enzyme that caused an increase in absorbance of 1.0 per min and per ml at pH 7.0 at 25°C.

#### Determination of Catalase, Superoxide Dismutase and Glutathione S-Transferase Activities

Erythrocyte CAT activity was determined according to Aebi<sup>28</sup>. The enzymatic decomposition of  $\text{H}_2\text{O}_2$  was followed directly at 240 nm. One unit of CAT activity was defined as the amount of enzyme required to decompose 1  $\mu\text{mol}$   $\text{H}_2\text{O}_2$  in 1 min.

Erythrocyte total SOD activity was determined by monitoring the auto-oxidation of pyrogallol

at 420 nm<sup>29</sup>. One unit of total SOD activity was defined as the amount of enzyme required to inhibit the rate of pyrogallol auto-oxidation by 50%.

Erythrocyte GST activity was determined using 1-chloro-2,4 dinitrobenzene (CDNB) as a substrate, monitoring the rate of production of 2,4-diphenyl glutathione at 340 nm<sup>30</sup>.

#### Determination of Total Glutathione Levels

Both plasma and erythrocyte GSH levels were assessed by a kinetic assay in which catalytic amounts of GSH caused a continuous reduction of DTNB to TNB at 412 nm<sup>31</sup>. Quantification was achieved by parallel measurements of a standard curve of known GSH concentrations, and results of erythrocyte GSH levels were expressed pmol/g Hb, while plasma GSH levels were expressed in nmol/ml.

#### Determination of Lipid Peroxidation

As an indicator of lipid peroxidation, MDA levels were determined by high performance liquid chromatography (HPLC) after reaction with thiobarbituric acid, and the level of MDA was expressed as nmol/ml after quantification by parallel measurements of a standard curve of known MDA concentrations<sup>32</sup>.

#### Determination of Selenoprotein P Levels

A human-specific SePP ELISA based on polyclonal sheep antisera was established and ELISA was performed to measure SePP levels<sup>33</sup>.

#### Statistical Analyses

The results were expressed as mean  $\pm$  standard error of mean (SEM). The differences between the groups were evaluated by Student's t-test using the Statistical Package for the Social Sciences Program (SPSS) version 17.0. P values  $<0.05$  were considered as statistically significant.

## Results

The alterations in measured parameters of the H1N1 group vs. control are given in Table I.

#### C- Reactive Protein Levels

The immunoturbidimetric assay CRP (Latex) High Sensitive (HS) gives the normal values of CRP as 0.1-2.8 mg/L for children (2 months-15 years). The CRP level in the patient group was  $6.46 \pm 2.07$  mg/L, while the CRP level in the control group was  $1.87 \pm 0.74$  mg/L. An increase of 245% was observed in CRP levels in the



**Table I.** The Alterations in Measured Parameters H1N1 Group vs. Controls

Parameter	Control	H1N1	Change (%) in H1N1 vs. Controls
CRP (mg/L)	6.46 ± 2.07	1.87 ± 0.74	245% increase
Plasma Se (µg/L)	132.27 ± 5.47	117.54 ± 3.80*	11% decrease
Erythrocyte Se (µg/L)	599.72 ± 40.35	529.71 ± 38.62*	11% decrease
Erythrocyte GPx1 (U/g Hb)	18.80 ± 1.50	10.38 ± 1.54*	45% decrease
Plasma GPx3 (U/L)	836.00 ± 26.83	703.27 ± 25.75*	16% decrease
Erythrocyte TrxR (mU/mg Hb)	0.59 ± 0.04	0.41 ± 0.05*	30% decrease
Erythrocyte CAT (U/mg Hb)	339.90 ± 27.04	208.60 ± 21.14*	38% decrease
Erythrocyte SOD (U/mg Hb)	1.28 ± 0.14	0.75 ± 0.18*	42% decrease
Erythrocyte GST (nmol/min/mg Hb)	3.09 ± 0.2	2.42 ± 0.2*	19% decrease
Erythrocyte GSH (pmol/mg Hb)	12.07 ± 0.78	9.91 ± 0.71*	18% decrease
Plasma GSH (nmol/ml)	0.39 ± 0.03	0.36 ± 0.03*	10% decrease
Plasma MDA (nmol/L)	1299.80 ± 189.30	1646.10 ± 302.08*	27% increase
Plasma SePP (mg/ml)	2.74 ± 0.18	2.80 ± 0.36	no significant change

Values are given as mean±SEM. p<0.05 was considered statistically significant.

\*indicates H1N1 group is significantly different from the control group (p<0.05).

CRP: C-reactive protein. Se: Selenium. GPx1: Glutathione peroxidase 1. Hb: Hemoglobin. GPx3: Glutathione peroxidase 3. TrxR: Thioredoxin reductase. CAT: Catalase. SOD: Superoxide dismutase. GST: Glutathione S-transferase. GSH: Total glutathione. MDA: Malondialdehyde. SePP: Selenoprotein P.

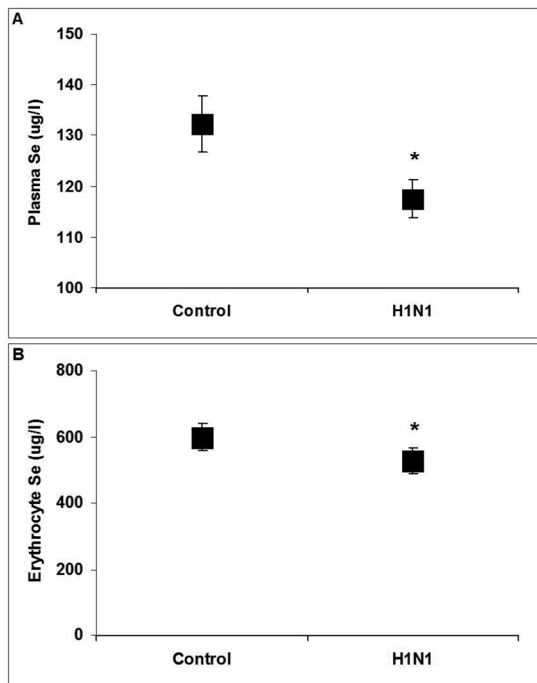


Fig. 1. Plasma and erythrocyte selenium levels in control and H1N1 groups.

A. Plasma selenium levels. B. Erythrocyte selenium levels. Both plasma and erythrocyte selenium levels are given in  $\mu\text{g/L}$ . Values are given as mean  $\pm$  SEM.  $p < 0.05$  was considered statistically significant. \*Indicates H1N1 group is significantly different from the control group ( $p < 0.05$ ).

patient group, and the difference between the groups was statistically significant ( $p < 0.05$ ).

#### Erythrocyte and Plasma Selenium Levels

The ICP-MS measurements for erythrocyte and plasma Se levels are shown in Figure 1. Both erythrocyte and plasma Se levels showed marked decreases of 11% in H1N1 patients ( $p < 0.05$ ). For Se measurements, between-run precision was  $1.28 \pm 0.27\%$  (coefficient of variation [CV]) and within-day precision was  $3.01 \pm 0.62\%$  (CV). Determination of Se levels was also performed by a transversely heated graphite-furnace atomic absorption spectrophotometer (GFAAS, Model 800, Perkin Elmer, Waltham, MA) with a longitudinal Zeeman-effect background corrector according to Eckerlin et al.<sup>34</sup>. The correlation between ICP-MS and GFAAS in the plasma Se levels in patients was 90%, while it was 95% in the controls. The correlation between ICP-MS and GFAAS in the erythrocyte Se levels in patients was 98%, while it was 97% in the controls.

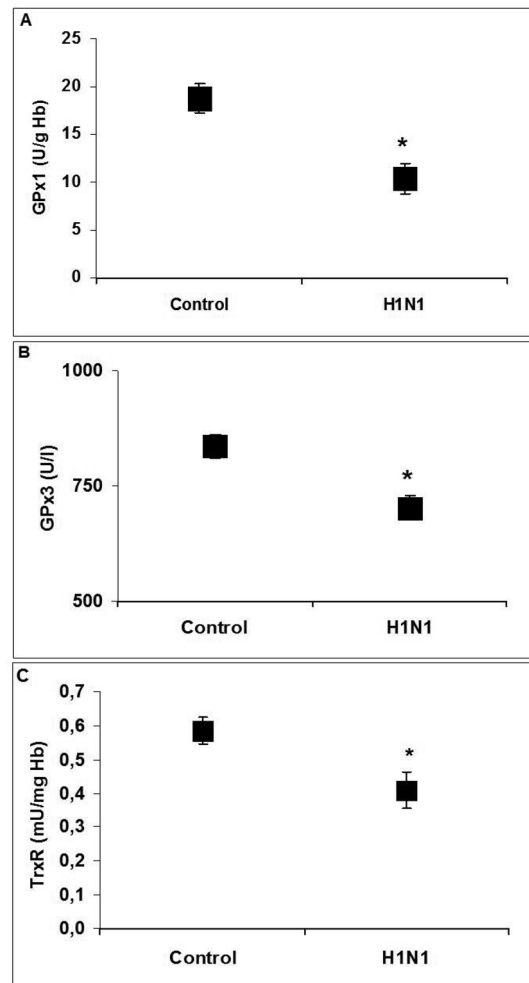


Fig. 2. Selenoenzyme activities in control and H1N1 groups. A. GPx1 activity.

B. GPx3 activity. C. Erythrocyte TrxR activity. GPx1 activity is expressed as U/mg Hb. GPx3 activity is expressed as U/L. Erythrocyte TrxR activity is expressed as mU/mg Hb. Values are given as mean  $\pm$  SEM.  $p < 0.05$  was considered statistically significant. \*Indicates H1N1 group is significantly different from the control group ( $p < 0.05$ ).

#### Oxidant/Antioxidant Parameters

The methods applied in this study for the measurement of selenoenzyme and other antioxidant activities have been used in our lab as well as in several other labs around the globe for many years.

These methods are trustworthy and have high precision.

#### Selenoenzyme Activities

Selenoenzyme activities are shown in Figure 2. Both GPx1 activity (45%) and GPx3 (16%) activity decreased markedly in the H1N1 group

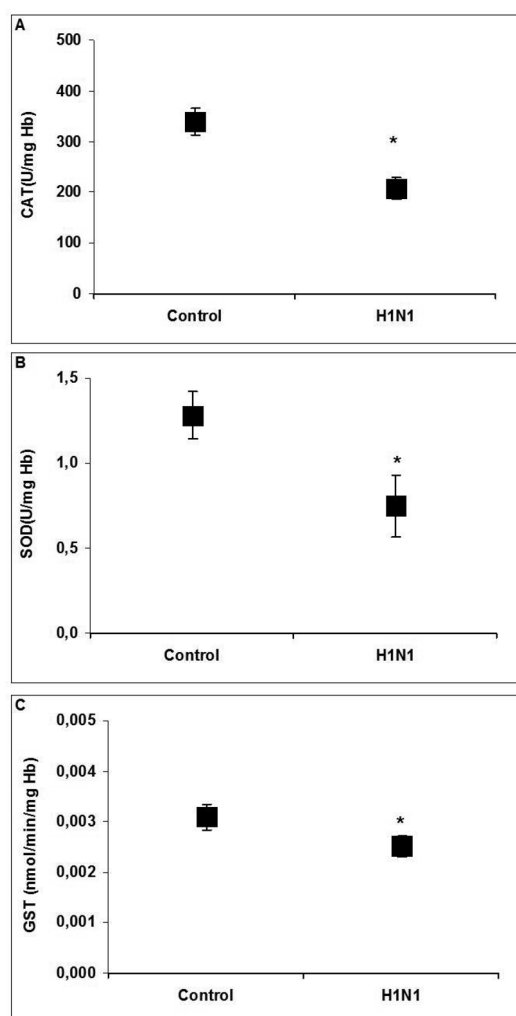


Fig. 3. Erythrocyte antioxidant enzyme activities in control and H1N1 groups.

A. Erythrocyte CAT activity. B. Erythrocyte SOD activity. C. Erythrocyte GST activity. CAT activity is expressed as U/mg Hb. SOD activity is expressed as U/mg Hb. GST activity is expressed as nmol/min/mg Hb. Values are given as mean ± SEM.  $p < 0.05$  was considered statistically significant. \*Indicates H1N1 group is significantly different from the control group ( $p < 0.05$ ).

vs. controls ( $p < 0.05$ ). Similarly, TrxR activity was significantly lower (30%) in the patient group compared to the control group ( $p < 0.05$ ).

#### Catalase, Superoxide Dismutase and Glutathione S-Transferase Activities

Erythrocyte CAT and SOD activities are shown in Figure 3. CAT activity showed a decrease of 38%, while SOD activity decreased 42% in the patient group compared to controls. GST activity was decreased markedly (19%) in the H1N1 group ( $p < 0.05$ , all).

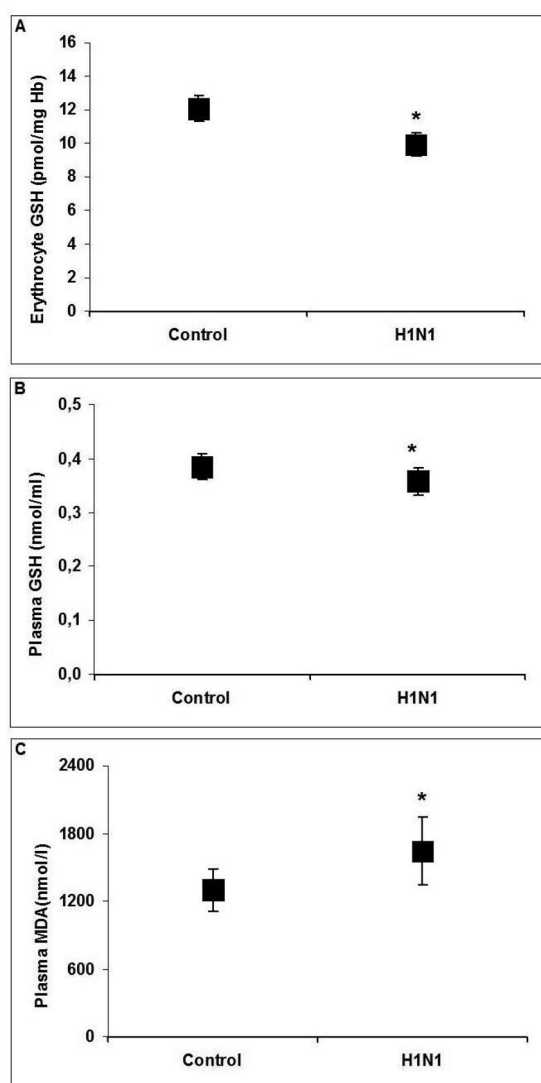


Fig. 4. Erythrocyte and plasma glutathione levels and plasma lipid peroxidation levels in control and H1N1 groups.

A. Erythrocyte GSH levels. B. Plasma GSH levels. C. Plasma MDA levels. Erythrocyte GSH levels are expressed as pmol/mg Hb. Plasma GSH levels are expressed as nmol/ml. Plasma MDA levels are expressed in nmol/L. Values are given as mean ± SEM.  $p < 0.05$  was considered statistically significant. \*Indicates H1N1 group is significantly different from the control group ( $p < 0.05$ ).

#### Total Glutathione and Lipid Peroxidation Levels

For MDA measurements, between-run precision was  $3.87 \pm 0.58\%$  (CV) and within-day precision was  $2.85 \pm 0.41\%$  (CV). For GSH determination, between-run precision was  $4.52 \pm 0.29\%$  (CV) and within-day precision was  $3.01 \pm 0.22\%$  (CV).

GSH and MDA levels are shown in Figure

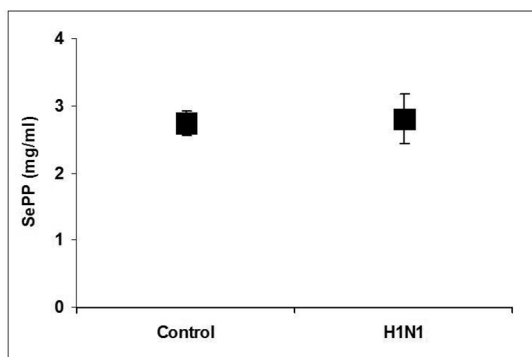


Fig. 5. Plasma selenoprotein P levels in control and H1N1 groups.

Plasma SePP levels are expressed in mg/ml. Values are given as mean  $\pm$  SEM.  $p < 0.05$  was considered statistically significant. \*Indicates H1N1 group is significantly different from the control group ( $p < 0.05$ ).

4. Both erythrocyte and plasma GSH levels decreased, 18% and 10%, respectively, in H1N1 patients when compared to controls (both,  $p < 0.05$ ). On the other hand, MDA levels showed a marked increase of 27% in the patient group vs. controls ( $p < 0.05$ ).

#### Selenoprotein P Levels

Selenoprotein P (SePP) levels are shown in Figure 5. SePP levels did not show any marked change in the patient group compared to controls.

#### Discussion

It has been known for several decades that nutritional deficiencies can cause increased susceptibility to infectious diseases<sup>35</sup>. Several viral infections are much more severe in malnourished hosts as compared with well-nourished hosts<sup>36</sup>. The association of poor host nutritional status with increased susceptibility to infectious disease has long been thought to be related to the host immune dysfunction, including impaired antibody responses, decreased macrophage activity and T cell dysfunction<sup>37</sup>. It has also been known that the viral pathogen itself may be affected by the nutritional deficiency. Several viruses have been shown to develop increased virulence due to changes in their genomes as a result of replicating in a nutritionally deficient host. The mechanism for the viral genomic changes is not well understood, although it appears to be related to increased oxidative stress in the

deficient host. Thus, both the host as well as the pathogen can be influenced by the nutritional status of the host<sup>38</sup>.

C-reactive protein, a marker for inflammation, is an acute-phase protein found in the blood, and its levels rise in response to inflammation<sup>39,40</sup>. In the present study, the CRP levels of the patient group were found to be significantly higher compared to control children. Several other studies performed on H1N1 patients also showed that CRP levels show substantial increases in this infection<sup>41,42</sup>.

Oxidative stress has been defined as “an imbalance between oxidants and antioxidants in favor of the oxidants”<sup>43,44</sup>. The ability of dietary antioxidants to affect oxidative stress status *in vivo* has been widely discussed, and the effect of dietary intake of these substances on human health is an area currently undergoing intensive investigation<sup>45</sup>. Se is a trace mineral that is believed to play an essential role in antioxidant protection due to its incorporation as selenocysteine into several antioxidant enzymes including GPx and TrxR<sup>46</sup>. Se also has an important role on an efficient immune system in both animals and humans<sup>10,47</sup>. It is reported that dietary Se is essential for an optimum immune response and that Se deficiency affects both cell-mediated and humoral components of immune response. However, the mechanisms underlying these effects are not fully understood<sup>47</sup>. Limited human data indicate that Se supplements can elevate immune response in the population with low Se status. Some human and animal studies suggest that Se intake can affect the progression of viral infections and that Se status can be associated with viral response<sup>5,48,49</sup>. There are some observations suggesting an association between host nutritional Se status and influenza virus<sup>37,50</sup>. The effect of low Se status on influenza virus genomic evolution is likely mediated via GPx1 deficiency<sup>4,14</sup>.

GPx enzyme activity can be a crucial factor in the course of infectious diseases. It was suggested that this malfunctioning in neutrophils is related to low activity of GPx1 in the neutrophils, and neutrophils are destroyed directly by free radicals<sup>51,52</sup>. In the present study, we observed marked decreases in both erythrocyte and plasma Se levels, supporting the importance of Se status in viral infections. In



addition, both GPx1 and GPx3 activities showed substantial decreases in the H1N1 patients. TrxR activity also decreased significantly in the patient group. The augmenting effect of low levels of Se on the course of H1N1 was also determined by marked decreases in erythrocyte and plasma GSH levels, as well as marked increases in plasma MDA levels, in H1N1-infected children vs. controls. These changes point out that lower Se levels make the patients more prone to oxidative stress. A study conducted on mice infected with H3N2 determined that the activity of lung GPx1 of the infected mice was lower than in controls. Further, GSH levels showed a decrease for the first five days of the infection<sup>14</sup>. Mice infected with influenza A/Puerto Rico/8/34 (PR8) virus showed a vigorous drop in the activity of liver GPx1, as well as GSH<sup>49</sup>. An *in vitro* study performed on epithelial cells showed that Se deficiency alters epithelial cell morphology with substantial decreases in GPx1 activity<sup>53</sup>. On the other hand, studies utilizing both Se-deficient mice as well as GPx1 knockout mice demonstrated both Se and GPx1 activity as providing a unique role in preventing enhanced virulence of Coxsackie virus. In addition to having an effect on the immune system of the host, a deficiency in Se and/or a deficiency in GPx1 activity can lead to enhanced virulence of a viral pathogen due to genetic changes in the viral pathogen itself. Once these changes have occurred, even hosts with normal Se status and GPx activity are susceptible to its newly virulent properties<sup>54</sup>. Se deficiency and low GPx3 activity were reported in patients with systemic inflammatory response syndrome<sup>55</sup>.

Selenoprotein P is the most common selenoprotein carrying most Se in the plasma. It is thought to have antioxidant potential<sup>56,57</sup>. SePP decreases were shown as a potential marker for septic shock, sepsis and related syndromes<sup>58-60</sup>. However, in the present study, we did not find any difference in plasma SePP levels between the patient group and the control group. Other than SePP, several selenoproteins like selenoprotein W (SelW), 15-kDa selenoprotein (Sep 15) and glutathione peroxidase 4 (GPx4) were also suggested to play a role in the outcome of influenza infections and several other disease conditions; however, their possible roles are not well-identified<sup>14</sup>.

We determined significant decreases in CAT activity in the patient group vs. controls. In a study by Shi et al.<sup>61</sup>, mice were infected with influenza virus H1N1 (FM1 strain) and treated with recombinant human CAT by inhalation. The survival time and survival rates of H1N1-induced pneumonic mice were increased by treatment with recombinant human CAT. The protective efficacy of CAT was also observed in lung histology, antioxidant parameters, pulmonary pathology, and influenza viral titer in mice lungs. In an earlier study by Choi et al.<sup>62</sup>, it was found that CAT mRNA was not induced by H1N1 infection.

We observed a marked decrease in erythrocyte total SOD activity in the patient group compared to controls. In a study conducted by Case et al.<sup>63</sup>, conditional loss of manganese (Mn)-SOD led to increased superoxide, apoptosis, and developmental defects in the T cell population, resulting in immunodeficiency and susceptibility to the influenza A virus H1N1. On the other hand, Mn-SOD administration to H1N1-infected mice provided increases in the survival rate of the animals compared to the placebo group<sup>64</sup>. It was found that Mn-SOD protein expression was increased by H1N1 infection in mice; however, the enzyme activity did not increase. Furthermore, no changes were observed in copper (Cu), zinc (Zn)-SOD expression and activity<sup>62</sup>. In a study by Jaspers et al.<sup>53</sup> in primary human bronchial epithelial cells, Se deficiency caused an insignificant decrease in total SOD activity, while leading to a marked decrease in CAT activity.

Glutathione is the major free thiol in most living cells. It is a substrate for the GPxs and GSTs and is the key antioxidant in animal tissues<sup>31</sup>. GSH displays anti-influenza activity both *in vitro* and *in vivo*<sup>65</sup>. In human small airway epithelial cells and in Madin-Darby canine kidney (MDCK) cells infected with A/X-31 strain influenza virus, GSH supplementation inhibited expression of viral matrix protein and virally induced caspase activation and Fas upregulation. In BALB/c mice, inclusion of GSH in the drinking water decreased the viral titer in both lung and trachea homogenates four days after intranasal inoculation with a mouse-adapted influenza strain A/X-31. Oxidative stress or other conditions that deplete GSH in the epithelium of the oral,

nasal, and upper airway may, therefore, enhance susceptibility to influenza infection<sup>65</sup>. On the other hand, N-acetyl-L-cysteine (NAC), the precursor of GSH, was shown to inhibit the proliferation of influenza virus and scavenge superoxide anion in mammalian epithelial cells and in A549 cells<sup>66,67</sup>. Further, in mice infected with influenza virus, NAC supplementation markedly decreased the mortality<sup>68</sup>. In humans, administration of NAC appears to reduce both the incidence and severity of influenza-like episodes, and the length of time confined to bed in H1N1 Singapore 6/86 influenza virus infection<sup>69</sup>. In a study by Lai et al.<sup>70</sup>, the researchers reported that a patient with viral pneumonia caused by the novel influenza A (H1N1) virus 2009 infection and septic shock improved rapidly after continuous intravenous infusion of high-dose NAC at 100 mg/kg combined with oseltamivir. In the present study, we observed marked decreases in both plasma and erythrocyte GSH levels in H1N1 group vs. controls ( $p < 0.05$ ), indicating that an oxidative stress exists in H1N1 patients.

Among the molecular targets of ROS, lipids constitute an important class of biomolecules. Peroxidation of lipids can greatly alter the physicochemical properties of membrane lipid bilayers, resulting in severe cellular dysfunction. It is well established that end-products of lipid peroxidation, such as MDA, can lead to protein oxidation<sup>71</sup>. In the present study, we observed a significant increase in lipid peroxidation in the patient group vs. controls. Mileva et al.<sup>72</sup> also observed a marked increase in lipid peroxidation in mice infected with influenza virus H3N2 (A/Aichi/2/68). Vitamin E supplementation significantly reduced the lipid peroxidation (conjugated dienes and MDA). He et al.<sup>73</sup> also observed an increase in lipid peroxidation in H1N1-infected mice, and apple phenol extract significantly reduced MDA levels and increased survival time.

To our knowledge, this is the first study that suggests a relationship between H1N1, low Se levels and oxidative stress in pediatric patients. However, our study has a small number of subjects. Nevertheless, for such studies, relatively few patients are employed due to several problems, including consent from the patients' parents. In spite of the limitations, the data are consistent in demonstrating the

presence of oxidative stress in pediatric H1N1 influenza and that low Se levels might be a triggering factor in the emerging of the disease.

In conclusion, overall results suggest the importance of low Se status and the presence of oxidant/antioxidant imbalance in H1N1 infection. Se deficiency and the changes in the antioxidant defense system, particularly decreased selenoenzyme activities, might have a role in the viral infection progression in H1N1 influenza-infected children. More clinical studies including multi-center approaches and larger study groups are needed to clarify the roles of Se in the influenza infections, particularly in H1N1.

#### REFERENCES

1. Bendich A. Antioxidant vitamins and human immune responses. *Vitam Horm* 1996; 52: 35-62.
2. Scrimshaw NS. Nutrition and infection. *Prog Food Nutr Sci* 1975; 1: 393-420.
3. Semba RD. Vitamin A, immunity, and infection. *Clin Infect Dis* 1994; 19: 489-499.
4. Beck MA, Levander OA. Dietary oxidative stress and the potentiation of viral infection. *Annu Rev Nutr* 1998; 18: 93-116.
5. Beck MA, Levander OA, Handy J. Selenium deficiency and viral infection. *J Nutr* 2003; 133: 1463S-1467S.
6. Flohé L. The glutathione peroxidase reaction: molecular basis of the antioxidant function of selenium in mammals. *Curr Top Cell Regul* 1985; 27: 473-478.
7. Avissar N, Whitin JC, Allen PZ, et al. Plasma selenium-dependent glutathione peroxidase. Cell of origin and secretion. *J Biol Chem* 1989; 264: 15850-15855.
8. Deisseroth A, Dounce AL. Catalase: physical and chemical properties, mechanism of catalysis, and physiological role. *Physiol Rev* 1970; 50: 319-375.
9. Spallholz JE, Boylan LM, Larsen HS. Advances in understanding selenium's role in the immune system. *Ann N Y Acad Sci* 1990; 587: 123-139.
10. Arthur JR, McKenzie RC, Beckett GJ. Selenium in the immune system. *J Nutr* 2003; 133: 1457S-1459S.
11. Baum MK, Shor-Posner G, Lai S, et al. High risk of HIV-related mortality is associated with selenium deficiency. *J Acquir Immune Defic Syndr Hum Retrovirol* 1997; 15: 370-374.
12. Dworkin BM. Selenium deficiency in HIV infection and the acquired immunodeficiency syndrome (AIDS). *Chem Biol Interact* 1994; 91: 181-186.
13. Harthill M. Review: micronutrient selenium deficiency influences evolution of some viral infectious diseases. *Biol Trace Elem Res* 2011; 143: 1325-1336.
14. Sheridan PA, Zhong N, Carlson BA, et al. Decreased selenoprotein expression alters the immune response during influenza virus infection in mice. *J Nutr* 2007; 137: 1466-1471.

15. Kiremidjian-Schumacher L, Stotzky G. Selenium and immune responses. *Environ Res* 1987; 42: 277-303.
16. Hincal F, Yetgin S, Ataçeri N. Selenium status in Turkey I. Serum selenium levels in infants and children in Ankara. *Biol Trace Elem Res* 1989; 20: 161-167.
17. Hincal F, Basaran N, Yetgin S, Gokmen O. Selenium status in Turkey I. Serum selenium concentration in healthy residents of different ages of Ankara. *J Trace Elem Electrolytes Health Dis* 1994; 8: 9-12.
18. Giray B, Hincal F, Teziç T, Okten A, Gedik Y. Status of selenium and antioxidant enzymes of goitrous children is lower than healthy controls and nongoitrous children with high iodine deficiency. *Biol Trace Elem Res* 2001; 82: 35-52.
19. Giray B, Hincal F. Plasma selenium levels in healthy adults living in rural areas of Turkey. In: Hincal F, Cavdar A, Giray B (eds). *Selenium in Health and Disease (Proceedings of the International Symposium in Health and Disease, October 12-13, 2006)*. Ankara: Pozitif Press; 2006: 51-65.
20. Bender BS, Small PA Jr. Influenza: pathogenesis and host defense. *Semin Respir Infect* 1992; 7: 38-45.
21. Chan M. World now at the start of 2009 influenza pandemic. Statement to the press by WHO Director-General Dr Margaret Chan. Available at: [http://who.int/mediacentre/news/statements/2009/h1n1\\_pandemic\\_phase6\\_20090611/en/index.html](http://who.int/mediacentre/news/statements/2009/h1n1_pandemic_phase6_20090611/en/index.html).
22. Ramsey C, Kumar A. H1N1: viral pneumonia as a cause of acute respiratory distress syndrome. *Curr Opin Crit Care* 2011; 17: 64-71.
23. Çiftçi E, Tuygun N, Özdemir H, et al. Clinical and epidemiological features of Turkish children with 2009 pandemic influenza A (H1N1) infection: experience from multiple tertiary paediatric centres in Turkey. *Scand J Infect Dis* 2011; 43: 923-929.
24. Nunes JA, Batista BL, Rodrigues JL, et al. A simple method based on ICP-MS for estimation of background levels of arsenic, cadmium, copper, manganese, nickel, lead, and selenium in blood of the Brazilian population. *J Toxicol Environ Health A* 2010; 73: 878-887.
25. Flohé L, Günzler WA. Assays of glutathione peroxidase. *Methods Enzymol* 1984; 105: 114-121.
26. Günzler WA, Kremers H, Flohé L. An improved coupled test procedure for glutathione peroxidase (EC 1-11-1-9-) in blood. *Z Klin Chem Klin Biochem* 1974; 12: 444-448.
27. Arnér ES, Zhong L, Holmgren A. Preparation and assay of mammalian thioredoxin and thioredoxin reductase. *Methods Enzymol* 1999; 300: 226-239.
28. Aebi H. Catalase. In: Bergmeyer HU (ed). *Methods of Enzymatic Analysis*. New York: Academic Press; 1974: 673-677.
29. Marklund S, Marklund G. Involvement of the superoxide anion radical in the autoxidation of pyrogallol and a convenient assay for superoxide dismutase. *Eur J Biochem* 1974; 47: 469-474.
30. Habig WH, Pabst MJ, Jakoby WB. Glutathione S-transferases. The first enzymatic step in mercapturic acid formation. *J Biol Chem* 1974; 249: 7130-7139.
31. Akerboom TP, Sies H. Assay of glutathione, glutathione disulfide, and glutathione mixed disulfides in biological samples. *Methods Enzymol* 1981; 77: 373-382.
32. Templar J, Kon SP, Milligan TP, et al. Increased plasma malondialdehyde levels in glomerular disease as determined by a fully validated HPLC method. *Nephrol Dial Transplant* 1999; 14: 946-951.
33. Hollenbach B, Morgenthaler NG, Struck J, et al. New assay for the measurement of selenoprotein P as a sepsis biomarker from serum. *J Trace Elem Med Biol* 2008; 22: 24-32.
34. Eckerlin RH, Hoult DW, Carnrick GR. Selenium determination in animal whole blood using stabilized temperature platform furnace and Zeeman background correction. *At Spectrosc* 1987; 8: 64-66.
35. Scrimshaw NS. Nutrition and infection. *Prog Food Nutr Sci* 1975; 1: 393-420.
36. Semba RD. Vitamin A, immunity, and infection. *Clin Infect Dis* 1994; 19: 489-499.
37. Bendich A. Physiological role of antioxidants in the immune system. *J Dairy Sci* 1993; 76: 2789-2794.
38. Beck MA. Antioxidants and viral infections: host immune response and viral pathogenicity. *J Am Coll Nutr* 2001; 20: 384S-388S.
39. Thompson D, Pepys MB, Wood SP. The physiological structure of human C-reactive protein and its complex with phosphocholine. *Structure* 1999; 7: 169-177.
40. Pepys MB, Hirschfield GM. C-reactive protein: a critical update. *J Clin Invest* 2003; 111: 1805-1812.
41. Ni Z, Guo Z, Chen X, et al. Cardiac injury in patients with pandemic 2009 influenza A (H1N1) infection. *Acta Cardiol* 2011; 66: 427-432.
42. Song JY, Cheong HJ, Heo JY, et al. Clinical, laboratory and radiologic characteristics of 2009 pandemic influenza A/H1N1 pneumonia: primary influenza pneumonia versus concomitant/secondary bacterial pneumonia. *Influenza Other Respi Viruses* 2011; 5: e535-e543.
43. Sies H. Oxidative stress: oxidants and antioxidants. *Exp Physiol* 1997; 82: 291-295.
44. Davies KJ. Oxidative stress: the paradox of aerobic life. *Biochem Soc Symp* 1995; 61: 1-31.
45. Halliwell B. Oxidative stress, nutrition and health. Experimental strategies for optimization of nutritional antioxidant intake in humans. *Free Radic Res* 1996; 25: 57-74.
46. Stadtman TC. Selenium biochemistry. Mammalian selenoenzymes. *Ann N Y Acad Sci* 2000; 899: 399-402.
47. Fairweather-Tait SJ, Bao Y, Broadley MR, et al. Selenium in human health and disease. *Antioxid Redox Signal* 2011; 14: 1337-1383.
48. Broome CS, McArdle F, Kyle JA, et al. An increase in selenium intake improves immune function and poliovirus handling in adults with marginal selenium status. *Am J Clin Nutr* 2004; 80: 154-162.
49. Reid ME, Duffield-Lillico AJ, Sunga A, et al. Selenium supplementation and colorectal adenomas: an analysis of the nutritional prevention of cancer trial. *Int J Cancer* 2006; 118: 1777-1781.

50. Nelson HK, Shi Q, Van Dael P, et al. Host nutritional selenium status as a driving force for influenza virus mutations. *FASEB J* 2001; 15: 1846-1848.
51. Boyne R, Arthur JR, Wilson AB. An in vivo and in vitro study of selenium deficiency and infection in rats. *J Comp Pathol* 1986; 96: 379-386.
52. Li W, Beck MA. Selenium deficiency induced an altered immune response and increased survival following influenza A/Puerto Rico/8/34 infection. *Exp Biol Med* (Maywood) 2007; 232: 412-419.
53. Jaspers I, Zhang W, Brighton LE, et al. Selenium deficiency alters epithelial cell morphology and responses to influenza. *Free Radic Biol Med* 2007; 42: 1826-1837.
54. Beck MA. Nutritionally induced oxidative stress: effect on viral disease. *Am J Clin Nutr* 2000; 71: 1676S-1681S.
55. Manzanares W, Biestro A, Galusso F, et al. Serum selenium and infection in rats. *J Comp Pathol* 1986; 96: 379-386.
56. Greenman E, Phillipich MJ, Meyer CJ, et al. The effect of selenium glutathione peroxidase-3 activity: biomarkers of systemic inflammation in the critically ill? *Intensive Care Med* 2009; 35: 882-889.
57. Arteel GE, Klotz LO, Buchczyk DP, et al. Selenoprotein P. *Methods Enzymol* 2002; 347: 121-125.
58. Saito Y, Takahashi K. Characterization of selenoprotein P as a selenium supply protein. *Eur J Biochem* 2002; 269: 5746-5751.
59. Forceville X, Mostert V, Pierantoni A, et al. Selenoprotein P, rather than glutathione peroxidase, as a potential marker of septic shock and related syndromes. *Eur Surg Res* 2009; 43: 338-347.
60. Hollenbach B, Morgenthaler NG, Struck J, et al. New assay for the measurement of selenoprotein P as a sepsis biomarker from serum. *J Trace Elem Med Biol* 2008; 22: 24-32.
61. Shi XL, Shi ZH, Huang H, et al. Therapeutic effect of recombinant human catalase on H1N1 influenza-induced pneumonia in mice. *Inflammation* 2010; 33: 166-172.
62. Choi AM, Knobil K, Otterbein SL, et al. Oxidant stress responses in influenza virus pneumonia: gene expression and transcription factor activation. *Am J Physiol* 1996; 271: L383-L391.
63. Case AJ, McGill JL, Tygrett LT, et al. Elevated mitochondrial superoxide disrupts normal T cell development, impairing adaptive immune responses to an influenza challenge. *Free Radic Biol Med* 2011; 50: 448-458.
64. Sidwell RW, Huffman JH, Bailey KW, et al. Inhibitory effects of recombinant manganese superoxide dismutase on influenza virus infections in mice. *Antimicrob Agents Chemother* 1996; 40: 2626-2631.
65. Cai J, Chen Y, Seth S, et al. Inhibition of influenza infection by glutathione. *Free Radic Biol Med* 2003; 34: 928-936.
66. Uchide N, Toyoda H. Antioxidant therapy as a potential approach to severe influenza-associated complications. *Molecules* 2011; 16: 2032-2052.
67. Geiler J, Michaelis M, Naczki P, et al. N-acetyl-L-cysteine (NAC) inhibits virus replication and expression of pro-inflammatory molecules in A549 cells infected with highly pathogenic H5N1 influenza A virus. *Biochem Pharmacol* 2010; 79: 413-420.
68. Ungheri D, Pisani C, Sanson G, et al. Protective effect of n-acetylcysteine in a model of influenza infection in mice. *Int J Immunopathol Pharmacol* 2000; 13: 123-128.
69. De Flora S, Grassi C, Carati L. Attenuation of influenza-like symptomatology and improvement of cell-mediated immunity with long-term N-acetylcysteine treatment. *Eur Respir J* 1997; 10: 1535-1541.
70. Lai KY, Ng WY, Osburga Chan PK, et al. High-dose N-acetylcysteine therapy for novel H1N1 influenza pneumonia. *Ann Intern Med* 2010; 152: 687-688.
71. Catalá A. Lipid peroxidation of membrane phospholipids generates hydroxy-alkenals and oxidized phospholipids active in physiological and/or pathological conditions. *Chem Phys Lipids* 2009; 157: 1-11.
72. Mileva M, Bakalova R, Tancheva L, et al. Effect of vitamin E supplementation on lipid peroxidation in blood and lung of influenza virus infected mice. *Comp Immunol Microbiol Infect Dis* 2002; 25: 1-11.
73. He RR, Wang M, Wang CZ, et al. Protective effect of apple polyphenols against stress-provoked influenza viral infection in restraint mice. *J Agric Food Chem* 2011; 59: 3730-3737.