# An evaluation of some oxidative and enzymatic biomarkers in different stages of naturally occurring copper poisoning in sheep

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## Abstract:

**BACKGROUND:** The early stage of Copper poisoning is difficult to be clinically diagnosed in sheep and has not been documented clearly yet. **OBJECTIVES:** To assess biomarkers in predicting early Copper poisoning in sheep, blood samples were collected from several groups of animals from a naturally Copper poisoning occurrence in an industrial region. **METHODS:** Animals were divided into four experimental groups; Group A: far from polluted region (Control group), Group B: inside polluted region, apparently healthy and without any clinically jaundice manifestation, Group C: slightly with jaundice signs and hemolytic crisis phase, and Group D: with clear jaundice signs. After collecting blood samples from each group, the serum was analyzed for evaluation of liver enzymes and oxidative stress parameters in different stages of Copper poisoning. In each blood sample, CPK, GGT, AST, ALT, total thiol (T-SH) group, and total proteins were determined. The Copper concentration in the serum, liver, and kidney of the dead animals in group D were also determined. **RESULTS:** There were significant differences in the blood parameters in group C illustrated by elevated level of serum AST, CPK, and GGT activities and total thiol (as biomarker of oxidative stress) when compared to control groups. In group D, these enzymes, in addition to T-SH, and the total protein were significantly ($p \leq 0.05$) different from those of the control and the other groups. Measurement of Copper in serum, liver, and kidney of group D (at the end stage of hemolytic phase) confirmed Copper poisoning in these groups. **CONCLUSIONS:** Based on the findings of the present study, the measurement of the liver enzyme activities and total thiol just closed to critical hemolytic phase could be reliable biomarkers for predicting Copper poisoning in sheep.

## Introduction

Copper is an essential trace element for feeding animals (Carcia-Vaquero et al., 2011) but can be toxic if over-supplemented. Copper toxicity is a chronic, highly fetal hemolytic crisis primarily affecting sheep and goat (Castro et al., 2007) due to their low capacity

to eliminate Copper from the body (Bremner, 1998; Spiesky et al., 2003). These animals can accumulate Copper in the liver even at concentrations of 500 to 1000 mg/kg without showing the clinical signs of toxicity. At a liver concentration of 1000 mg/kg, the clinical signs of Copper toxicity appear and may culminate in a hemolytic crisis with profound jaundice, which is generally fetal (Ishmael et al., 1971; Mendel et al., 2007). Due to a defect of control hemolytic phase in affected animals, it is necessary to have some biomarkers to predict this accumulation in the early phase of poisoning before the incidence of hemolytic phase and death. Excessive exposure to Copper results in an increase in oxidative stress and thus causes liver cell damage (Maxie, 2007; Reis et al., 2010; Reis et al., 2005; Isani et al., 2003; Luza and Speisky, 1998). Therefore, measurement of some liver enzyme activities and proteins oxidation, as a marker of oxidative stress, might be trustable biomarkers to detect the early phase of Copper toxicity in sheep.

## Materials and Methods

**Geographical area:** The study was performed in herds inhabiting areas where the presence of Copper has been confirmed in soil and plants because of neighboring to a Copper melting factory. There were also some reports of Copper poisoning (Mozaffari et al., 2009; Taghipour bazargani et al., 2009).

**Animal experiment:** A total of 82 Shaal sheep, aging from 3 to 4 years, were randomly selected. The animals were examined clinically, and based on clinical findings and the region were assigned into one of the four groups of the study as follows: Group A (n= 23): apparently healthy, in distance as far as 100 kilometers away from the contaminated zones; Group B (n=21): apparently healthy, inside the polluted zone; Group C (n=21): slightly affected sheep with subclinical signs of toxicity excluding Jaundice; Group D (n=17): clearly affected sheep with acute signs of toxicity including jaundice. In group D, sheep died few days after blood sampling. Blood were collected from jugular vein through a catheter into propylene tubes containing heparin and centrifuged at 2000 rpm for 10 min until serum were harvested. All serum samples were frozen at -20 degrees of Celsius and shipped to the Toxicology and Animal Poisoning Research Center at the Faculty of Veterinary Medicine, University of Tehran.

**Total SH (Thiol) groups assay:** For the measurement of systemic oxidative stress, the concentration of total SH groups in plasma from sheep in each group was measured by using a spectrophotometer (Beckman, DU 650) at 412 nm, using dithionitrobenzoic acid (DTNB; Sigma, Germany) as the reagent. In this assay, Colorless DTNB is converted to yellow 5-Mercapto-2- nitrobenzoic acid in the presence of thiol compounds that has an absorption maximum at 412 nm (Hu and Dillard, 1994).

**Hepatic enzyme activities and total serum protein measurement:** The enzymatic activities of serum Aspartate amino transferase (AST), Alanine amino transferase (ALT), Creatine Phosphokinase (CPK), Gamma-glutamyl transferase (GGT), and total serum protein were determined using an auto analyzer (Eppendorf, Germany) and commercial kits (Pars Azmoon Co., Iran).

**Measurement of Copper concentration in serum, liver, and kidney:** The concentration of Copper in serum, liver and, kidney in different groups of the study were determined via a atomic absorption instrument, according to Stahr (Stahr, 1991). Briefly, 50 g of liver and kidney were taken and dried for 48 h in an oven at 120ºC to remove the water content and to obtain a constant weight (about 68% water) then weighed and dry ashed in a muffle furnace at 450°C. The ash was dissolved in 1.0 N HCI and analyzed on a flame atomic absorption instrument (GBC, 906). The serum

Table 1. Hepatic Enzymes, total Thiol, Total proteins and Copper concentration in serum, liver and kidney of different phase of Copper poisoning. a,b,c,d Means in the same column with different superscripts differ statistically ($p<0.05$).

| | Group A (Control) | Group B | Group C | *Group D* |
|---|---|---|---|---|
| **Hepatic Enzymes (IU/l±SE)** | | | | |
| **AST** | 110.47 ± 34.6$^{a}$ | 124.00 ± 31.6$^{ab}$ | 149.13±36.3$^{c}$ | 398.23 ± 2.5$^{d}$ |
| **ALT** | 20.66 ± 5.9$^{a}$ | 22.40 ± 3.8$^{ab}$ | 22.43± 3.8$^{ab}$ | 56.57 ± 13.7$^{c}$ |
| **GGT** | 35.14 ± 13.9$^{a}$ | 38.40 ± 17.53$^{a}$ | 54.52±20$^{b}$ | 98.07 ± 69.6$^{c}$ |
| **CPK** | 14141.8± $^{a}$ | 158.45 ± 60.3$^{a}$ | 213.66 ± 6$^{b}$ | 6840 ± 8$^{c}$ |
| **Total Protein(gr/dl±SE)** | 7.09 ± 0.5$^{a}$ | 7.17 ± 0.5$^{ab}$ | 7.340.5±$^{ab}$ | 9.83 ± 2.2$^{c}$ |
| **Total Thiol(mmol/l±SE)** | 0.116 ± 0.02$^{a}$ | 0.110 ± 0.03$^{ab}$ | 0.0850.03±$^{c}$ | 0.040 ± 0.01$^{d}$ |
| **Copper(PPM±SE)** | | | | |
| **Serum** | - | - | - | 5.78 ± 2.5 |
| **Liver** | - | - | - | 1019.9 ± 139 |
| **Kidney** | - | - | - | 56.76 ± 7.1 |

samples (_0.75 ml) were diluted to 2.0 ml with distilled deionized water and mixed with 2.0 ml of 1.4 M HCl. And, after 30 min, 2.0 ml of 1.23 M trichloroacetic acid was added to deproteinate the sample. The supernatant was analyzed on a GBC atomic absorption instrument (Stahar, 1991).

**Statistic procedures:** All of the data related to different treatments were analysed according to ANOVA. Duncan's multiple range test was used for comparing the means of parameters to see if there were significant differences ($p\leq0.05$).

## Results

The data are presented in Table 1 and Figure 1. No significant difference ($p>0.05$) was found in the serum activities of ALT, AST, GGT, CPK, and total thiol and total protein content between group B and controls. In the group C, the plasma activities of AST, GGT, CPK, and total thiol were statistically different from those in controls. In addition, the serum ALT activity and the total protein in serum from group C was similar to controls. Biochemical profiles of AST, GGT, CPK, and total thiol displayed considerable diversity in group B when compared to those in group C; however, it was not statically significant ($p\geq0.05$). In addition, data analysis revealed that there is a significant difference in the plasma ALT, AST, CPK, GGT activity, total thiol, and total protein levels between group D and other groups. The concentration of Copper in serum, liver, and kidney of the sheep in group D was 5.78 ± 2.51, 1019.89 ± 139.04 and 56.76 ± 7.12 ppm, respectively.

## Discussion

This study was designed to identify reliable biomarkers indicating Copper toxicity before hemolytic crisis phase. The study was performed in the south province of Iran where several studies have reported the natural occurrence of Copper poisoning in sheep and goats. (Mozaffari et al., 2009; Taghipour bazargani et al., 2009). The concentrations of Copper reported by these studies are comparable to that we found in the kidney and liver of the sheep in our study and thus can be attributed to the presence of Copper pollution in soil and plants in the region. Mozaffari et al. (2009) showed that the concentration of Copper in isolates from dead sheep with acute hemolytic signs of Copper poisoning in the same area exceed the permitted limit (150 mg/kg), up to 7.97 folds in the liver (1196.9 ±20.6 mg/kg) and 9.14

folds in the kidney(137.2 ± 8.96 mg/kg). The authors concluded that the concentration of Copper found in water and plants was too high and would be a source for Copper poisoning (Mozaffari et al., 2009).

Up to now, our attempt to identify trustable biomarkers in an early diagnosis of Copper poisoning has been inconclusive. However, measurement of the liver content of Copper through biopsy has been shown by many studies to be useful in sheep, but not so popular due to invasive manner of this method (Lopez-Alonso et al., 2005). In the acute phase of the poisoning, Copper accumulation in the liver, more than 1000 ppm, results in hepatocytes necrosis and sudden release of Copper into the blood (Ishmael et al., 1971; Mendel et al., 2007). However, there is not a certain opinion on when liver bonding capacity to accumulate Copper would be saturated (Simpson et al., 2004). Nevertheless, the mean level to reach this stage in our study was 1019.89 ± 139.06, with min- max 817.70 and 1272.08 mg/kg, respectively. A report from Ishmael et al. (1971) has shown that Copper can accumulate in the liver in concentration of as high as 500 to1000 mg/kg in cumulative phase (per-hemolytic phase) without having clinical consequences. In contrast with the other biomarkers, Copper release from liver into plasma follows a constant manner and elevates to a critical level, just few days before critical phase, and thus serum Copper is a trustable biomarker of early Copper toxicity (Ozer et al., 2008). It is also emphasized that levels of Copper in urine increases 24 hours before hemolytic phase (Lewis et al., 1997) which just could be considered as an additional biomarker for Copper exposure as well. Meanwhile, one study reported that the Copper contents of RBC (Cyclic Citrullinated Peptide) decreased after Copper intoxication (Hu and Dillard, 1994). With respect to the mechanism of Copper intoxication, it is believed that it is involved in the Fenton reaction (Isani et al., 2003, Luza and Speisky, 1998); therefore, its accumulation in liver results in increased level of free Copper which in turn results in increased lipid peroxidation, elevation in free radicals, and membrane damage in hepatocyte's organelles such as lysosyme (Myers et al., 1993; Gooneratne and Howell, 1980). At the same time, oxidation of thiol containing proteins and bonding of Copper with thiol groups will subsequently lead to completely disruption of RBC membrane and extra venous heamolysis (Lopez-Alonso et al., 2005). Shortly after exposure to Copper, liver enzymes such as Arginase, AST, GGT, LDH, and Sorbitol dehydrogenase (SDH) has been shown to increase in blood 3 to 6 weeks before starting the hemolytic phase (Lewis et al., 1997). These studies also took advantage of elevated levels of BUN, GGT, AST, ALT, and ALP as certain indicators of liver damage at the end stage of Copper poisoning (Lewis et al., 1997). In aprevious report by Lopez-Alonso et al. (2006) the level of liver enzymes in the silent phase of Copper toxicity in cattle was within the normal limits and there was a positive relationship between liver concentration of Copper and the ALT and GGT level in blood (Lopez-Alonso et al., 2006). Many studies reported an increase in the level of liver enzymes in blood; however, they did not indicate any relation among liver enzyme activities and the level of Copper in liver and clinical findings after experimental toxicity, as well (Humann-ziehank et al., 2001; Laven et al., 2004). Meanwhile, in a research study by Ortolani et al. (2003) it was stated that the increased level of GGT followed by AST was the best way to indicate Copper-loading in sheep during the pre-hemolytic phase. It seems that decreased feed consumption and subtle losses of the body weight are associated with a hemolytic crisis in few days after feeding sheep with Copper-rich diets (Ortolani et al., 2003). In the present study, we followed up four groups of sheep in different stages of Copper intoxicity by feed or water from a Copper smelting facto-

ry (Mozaffari et al., 2009, Taghipour bazargani et al., 2009). In group B, there was not any significant difference between any measured parameters against controls. In group C, the blood level of GGT, AST, CPK, and total thiol, but not ALT and total protein, were significantly different from that of control's ($p<0.05$). According to the present data, in group D with hemolytic signs including hemoglubinoria, jaundice, polydepsia and lose of appétit, all blood parameters were changed, and statistically significant differences were found when compared to controls and other groups. On the other hand, rather a slight difference of CPK between groups B, C, and D with signs of jaundice showed liver damages or may be muscles (Gooneratne and Howell, 1980) a few days before the critical stage of hemolytic phase.

In conclusion, all blood parameters measured in this investigation (liver enzymes and total Thiol) were not successful in predicting the hemolytic phase and thus early Copper poisoning in sheep. To the best of our knowledge, measurement of Copper content of liver has been suggested to be the most convenient parameter in the diagnosis of early exposure to Copper.

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