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Seasonal variations of tissues and serum copper concentrations in chronic copper poisoned goats

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Abstract

Primary chronic forms of copper poisoning have been reported to occur in goats around the copper industries in Kerman province. The survey was carried out from autumn 2015 to the end of summer 2016, in Kerman province, Iran. Based on clinical signs, confirmed by necropsy and copper concentration of serum and different tissues, ten live chronic copper poisoned (CCP) goats were used in the study in each season. Blood samples were then taken. Samples from hair, liver, lung, kidney, heart and spleen were collected and copper was measured by Atomic Absorption Spectrophotometery. The highest levels of copper in lung, heart, liver, kidney and hair were observed in winter. Spleen had the highest concentration of copper in the summer. The lowest concentration of copper in serum, kidney, liver, heart, lung and hair was detected in spring. The lowest level of copper in the spleen was seen in winter. In spring, copper contents of liver had significant and positive relationships with copper level of lung, heart and spleen ($p<0.05$). Significant correlations were found between various tissues in different seasons. The highest and lowest serum concentration of molybdenum was observed in spring and winter, respectively ($p<0.05$). Serum ceruluplasmin significantly decreased in all seasons ($p<0.05$). In conclusion, the results of the current research showed that the level of copper in CCP goats is season-dependent. Significant correlations were also observed between different tissues in certain seasons in CCP goats. Ceruloplasmin decreased in CCP goats as a result of liver damage.

Keywords: Chronic copper poisoning, Goats, Season

Introduction

The presence of toxic heavy metals as a result of industrial operations is one of the serious threats to the environment (Sharma *et al*. 2009). Copper is considered as the third most important metal for industry (Northey *et al.* 2014). Copper is released into the environment by mining, farming, manufacturing operations and through waste water releases into rivers and lakes (ATSDR, 2004). Copper is also an essential trace element for all biological organisms, including bacterial cells and human (Falah *et al.* 2017). Although it is an essential nutrient, copper can also be poisonous if ingested in amounts that exceed the animal's requirement (Rosa1 *et al.* 2016; Spears, 2011; Tressman *et al.* 2000). There is a marked difference in susceptibility to copper poisoning between sheep and goats. The acute and primary chronic forms of copper poisoning have also been reported to occur naturally in goats (Merwe *et al.* 2012; Shlosberg *et al.* 1978; Solaiman *et al.* 2001; Søli *et al.* 1978). Acute copper poisoning in goat is sporadic in occurrence and result from accidental or unintentional ingestion of abnormally high doses of inorganic copper over a short period (Smith, 2015). Copper poisoning can be induced in sheep and young calves with a single oral dose of copper in the range of 20 to 110 mg/kg BW. The dose is similar in goats, with acute copper toxicity observed after an oral dose of approximately 60 mg/kg of copper sulfate (Shlosberg *et al*. 1978). In primary chronic copper poisoning, copper is ingested continuously as part of the regular ration. The level of copper in ration may be, but need not be, abnormally high, because the uptake of copper from the rumen and subsequent accumulation in the liver are conditioned by the concentrations of other minerals presenting the ration (Elshkaki *et al.* 2016). Low dietary levels of molybdenum, zinc, calcium, and sulfates can permit excessive uptake and accumulation of otherwise normal dietary levels of copper (Smith, 2015). In secondary phytogenous chronic copper poisoning, grazing of specific pasture plants, notably subterranean clover promotes the accumulation of copper in liver (Vincent, 1972). Chronic copper poisoning can be affected by various factors in farm animals, for instance environment, host, breed, season, the level and the duration of Cu intake and nutritional status. We hypothesized that degree of copper intoxication in goats may be influenced by the season (with high temperature range and varied plant species composition, heterogeneity and availability, kidding time, subsequent lactation, etc.) we also investigated the copper levels of hair, serum and different organs such as liver, lung, spleen, heart, kidney in chronic copper poisoned (CCP) goats at different seasons during a year to determine the effect of season.

Materials and methods

Animals and sampling

The study was carried out seasonally from autumn 2015 to the end of summer 2016, in Kerman province (between the latitude 30.01 degrees north and longitude 55.41 degrees east), Iran. Ten female live (3-5 years of age) mixed Shahrebabaki breed goats with clinical signs of chronic copper poisoning (anorexia, recumbency, hemolysis and jaundice in some of goats, high activity of serum aspartate aminotransferase, and gamma-glutamyltransferase) and eventual death were used in the study. Water and food were provided locally for animals. A diagnosis of copper toxicosis was made on the basis of high liver and kidney copper concentrations (compared with control) and histological evidence of hepatic necrosis.

Blood samples were taken via jugular vein in plain tubes to obtain sera after centrifugation. Sera were then kept at -20°C until assayed. Hair samples were taken from the dorsal aspect of the neck of these animals and then washed and dried. Samples from dorsal part of liver, lung, kidney, heart and spleen were collected immediately after death. These tissues were stored in formalin 10% until analyzed for copper content. Samples (serum and tissues) from control cases were obtained from 10 otherwise healthy cases far from copper industries in different seasons.

Measurement of copper, molybdenum and ceruloplasmin contents of serum, tissues and hairs

In order to measure the copper contents of tissues, hair and serum, 150 mg of tissues, 500 µl of serum and 250 mg of hair samples were processed and digested. Copper was measured by Atomic absorption Spectrophotometery technique (Atomic Absorption/Flame Emission Spectrophotometer AA-670-Shimadzu, Japan). Molybdenum was also determined by atomic-absorption spectrophotometry. Moreover, Ceruloplasmin was measured by nephelometric assay.

Statistical analysis

Data, presented as mean ± SE statistical differences of copper content in different samples, were analyzed among various seasons by One-Way ANOVA and LSD post-hoc test. The serum concentration of molybdenum among different seasons was also compared by One-Way ANOVA and LSD post-hoc test. The correlation between the copper content of serum and different tissues and hair in various seasons of copper poisoned goats was analyzed by Pearson's correlation test and regression formulas were obtained. The correlations among copper contents of all samples were also assayed by this test. Statistical analysis of data was performed by using SPSS software (SPSS for Windows, version 11.5, SPSS Inc., Chicago, Illinois). The level of significance was set at $p<0.05$.

Results

Cases were necropsied and the poisoning was confirmed by pathognomic pathological findings (slightly yellow liver with rounded margins, icteric mucous membranes and adipose tissues, gunmetal kidney, dark red–brown urine, necrosis of hepatocytes) and serum copper concentration. Copper contents of serum, various tissues and hair (ppm; mean ± SD, wet weight) of poisoned goats at different seasons during a year are presented in Table 1. The highest levels of copper in lung, heart, liver, kidney and hair were observed in the winter. Spleen had the highest amounts of copper in the summer. The lowest amounts of copper in serum, kidney, liver, heart, lung and hair were detected in the spring. However, the lowest level of copper in spleen was seen in winter. It is notable that the severity of poisoning was evaluated on the basis of the concentration of copper, mainly in liver as compared to the liver copper concentration in control cases. The relationships among copper contents of all samples of copper poisoned goats in each season are presented in Table 2. The concentration of copper in the serum was not significantly correlated with copper content of other tissues in all seasons. In spring, copper contents of liver had significant and positive relationships with copper level of lung, heart and spleen ($p<0.05$). Copper contents of lung and heart had significant and positive relationships in spring. In summer, kidney had positive correlation with liver. In the summer, lung had positive correlation with heart. In fall, kidney had positive correlation with lung and heart. Also, in fall, lung had positive correlation with heart.

Table 1. Copper contents of serum, tissues and hair (ppm; mean±SD) of copper poisoned goats at different seasons (n=10).

Seasons	Serum	Kidney	Liver	Lung	Heart	Spleen	Hair
Spring	$7.90±0.35^{a}$	$29.28±7.00^{a}$	$88.10±16.56^{a}$	$23.19±3.46^{a}$	$29.13±5.21^{a}$	$63.34±8.75^{a}$	$14.49±4.66^{a}$
Summer	$8.25±0.68^{a}$	$30.60±5.32^{a}$	$134.85±56.77^{a,b}$	$26.41±4.19^{a}$	$29.24±8.44^{a}$	$64.82±20.11^{a}$	$15.15±11.61^{a}$
Autumn	$8.21±0.20^{a}$	$30.08±5.48^{a}$	$137.19±36.53^{a,b}$	$28.17±2.77^{a}$	$29.96±2.06^{a}$	$32.83±8.55^{b}$	$21.96±8.88^{a}$
Winter	$8.11±0.26^{a}$	$33.20±3.09^{a}$	$172.31±116.43^{b}$	$35.57±5.55^{b}$	$34.67±0.86^{b}$	$20.14±4.73^{c}$	$46.41±37.07^{b}$
Control	$4.04±1.16^{b}$	$18.22±4.10^{b}$	$21.68±7.32^{c}$	$16.06±3.77^{c}$	$18.96±6.38^{c}$	$17.19±6.08^{d}$	$3.87±0.80^{c}$

In each row, different letters indicate significant differences ($P<0.05$).

Table 2. The relationships among copper contents of all samples in copper poisoned goats at each season (n=10)

Enzymes	Spring mean±SE	Summer mean±SE	Autnmn mean±SE	Winter mean±SE	Control mean±SE
Ceruloplasmin	0.117±0.03b	0.115±0.03b	0.115±0.02b	0.112±0.02b	0.241±0.08a
Molybdenum	0.95±0.23 a	0.81±0.15	0.79±0.09	0.58±0.17 b	0.57±0.16 b

In winter, kidney had positive correlation with heart. But lung had significant and negative correlation with spleen. Serum ceruloplasmin and molybdenum concentration of CCP goats at different seasons during a year are presented in Table 3. The highest and lowest serum concentration of molybdenum was observed respectively in spring and winter ($p<0.05$). A significant negative correlation was found between the molybdenum of serum and hair copper content of copper in winter ($r=-0.899$, $p<0.05$). No significant difference was found between ceruloplasmin in different seasons. Significant low concentration of ceruloplasmin in CCP goats was observed in different seasons when compared with control values. The minimum level of ceruloplasmin was observed in winter. Although, the data were analyzed for presenting the regression lines, none of them were significant.

Table 3. Serum ceruloplasmin (g/L) and molybdenum (ppm) concentration of copper poisoned goats at different seasons (n=10)

Seasons		Serum	Kidney	Liver	Lung	Heart	Spleen
Spring	Kidney	-0.550					
	Liver	-0.159	0.202				
	Lung	-0.035	-0.232	0.674*			
	Heart	0.120	-0.449	0.664*	0.959*		
	Spleen	0.256	-0.355	0.704*	0.349	0.546	
	Hair	-0.005	0.214	0.305	-0.701	-0.045	0.306
Summer	Kidney	-0.431					
	Liver	-0.265	0.651*				
	Lung	0.310	-0.278	0.447			
	Heart	0.164	0.309	0.585	0.725*		
	Spleen	0.452	-0.277	-0.306	0.401	0.594	
	Hair	0.169	0.369	0.440	0.218	0.417	0.054
Autumn	Kidney	0.013					
	Liver	0.532	-0.128				
	Lung	-0.182	0.934*	-0.470			
	Heart	0.105	0.981*	0.052	0.857*		
	Spleen	0.085	-0.056	0.066	-0.127	-0.123	
	Hair	0.121	0.191	0.049	-0.140	0.182	0.226
Winter	Kidney	0.336					
	Liver	-0.131	0.332				
	Lung	-0.415	-0.339	-0.376			
	Heart	0.204	0.800*	-0.139	0.226		
	Spleen	0.350	0.233	0.441	-0.989*	-0.354	
	Hair	-0.323	-0.015	-0.152	0.134	0.095	-0.148

Stars indicate significant relationships between copper contents of samples ($P<0.05$).
In each row, different letters indicate significant differences ($P<0.05$).

Discussion

When the soils of farmland are polluted with copper, animals will absorb concentrations that are damaging their health (Bhavani *et al.* 2014). Copper is a well-documented cause of liver toxicity in many domestic species, including sheep, dogs, cats, horses, cattle, goats, pigs, and camelids (Carmalt *et al.* 2001; Morgan *et al.* 2014). Sheep are the most sensitive domestic animals to Cu toxicity because their Cu excretory mechanism is less efficient (Hefnawy *et al.* 2015; Kurek *et al*. 2017). Although there is an abundance of clinical data for sheep, there are relatively few published reports of copper toxicosis in goats (Adam *et al.* 1977; Solaiman *et al*. 2001). Goats are reportedly less susceptible to copper intoxication than sheep (Soli *et al.* 1978). Angora goats may be more sensitive to copper toxicity than meat and dairy goats (Hart, 2008). It is important to consider that the toxic level of copper for sheep may not be the same for goats, as the copper requirements of goats may actually be higher (Solaiman *et al*. 2001). Increased absorption of copper is not easily achieved but abnormally high excretion is still more difficult, so that there is the general tendency for copper to accumulate (Sarath, 2014). It has been hypothesized that individual hepatocytes become packed with copper loaded lysosomes and synthesis of new lysosomes reduce, causing decreased uptake of excess copper leading to accumulation of ionic copper in the cytoplasm, resulting in the degeneration and necrosis of the hepatocytes (Sarath, 2014). When the liver's capacity to accumulate copper is overloaded, usually after stressful events such as nutrition, traveling, pregnancy, lactation, strenuous exercise, disease and malnutrition, breakdown of copper-containing lysosomes occurs and results in severe hepatocellular disease and a release of copper from liver into the bloodstream that leads to intravascular hemolysis (Hefnawy *et al*. 2015; Howard *et al*, 2014; Soli, 1980). It has been reported that the toxicity of Cu can occur anytime and peak incidence usually is in the fall and winter (Chariman *et al*. 1975 Church *et al*. 1988; Kimberling, 1988). Copper concentration can vary among plant species and varieties. Different plant species contain different levels of Cu (Hopkins *et al*. 1994; Minson, 1990).

Villares *et al.* (2002) stated that the concentrations of different elements in an organism can vary with the season, independent of environmental concentrations. Some researchers have reported the highest metal contents (Cd, Cu, Ni, Pb, Sn, Zn) in plants during autumn and relatively low levels during spring (Brekken *et al*. 2004; Kim *et al*. 1994; Villares *et al*. 2002), whereas others have indicated the highest foliar levels during spring and the lowest during winter (Martin *et al*. 1982; Wilkins, 1978). In this study, the concentration of copper in specific plants grown on the area and different copper levels emitted in the region during factory periodic activity may be either higher or lower in different seasons which subsequently affect the level of copper in liver and other body organs of the goats. Comar *et al*. (1948) stated that in ruminants, the tissues showing a high concentration of copper, in decreasing order, are the liver, kidney, gastrointestinal tract, adrenals, thymus, gallbladder and bile. Those of medium concentration include the pancreas, red bone marrow, intestinal lymph, blood, spleen, heart, lung, and reproductive organs; very low concentrations of copper are found in the white bone marrow, muscle, bladder, ligament, cartilage, bone, eye and nerve tissues. It is obvious that the liver, which shows the highest copper concentration, serves as the chief storage organ (Comar *et al.* 1948).

In our survey, in contrast to the highest level of copper in liver, lung, heart, kidney and hair in winter, the lowest amounts of copper were observed in kidney, liver, heart, lung and hair during spring. Highest level of copper in winter may be due to the stress factors such as tough climate conditions, parturition of goats and effect of low molybdenum in this season which may contribute to the higher appearance of chronic copper poisoning clinical signs in winter. In healthy goats, the effect of season and reproductive status on copper concentration in blood serum of crossbred goats was evaluated and it was found that in relation to the season, serum Cu levels were lower in rainy seasons (José *et al*. 2011). Khan *et al*. (2006) reported that the plasma Cu^{2+} concentrations of all classes of sheep were significantly higher in winter than in summer showing the seasonal as well as physiological effects (Khan *et al*. 2006). Sivertsen and Løvberg (2014) stated that changes in environmental climate, pregnancy, parturition and lactogenesis may affect the animals and sensitize them to show clinical signs of copper poisoning (Sivertsen & Løvberg, 2014). In the present study, in order to lower the effect of excess copper in the rumen of the animals, the ammonium molybdate was added to the area at different times of the year especially in rainy seasons. Molybdenum and sulfur decrease intestinal absorption of Cu, thus low levels of mentioned elements result in higher intestinal absorption of Cu (Mecitoglu *et al.* 2017). It has been also stated that the effect of molybdenum plus sulfate is to increase accumulation of copper in the kidney and increase excretion of copper through urine (Dick, 1969). It is reported that dietary inorganic sulfate, in the absence of supplemental molybdenum had no effect upon the parameters of copper metabolism (Marcilese *et al*, 1969). Although Ammonium molybdate was provided for the area, no sulfur source (sulfide minerals) was added to the soil. This may be the reason why copper toxicity still occurs frequently in the region. Villar *et al*. (2002) reported that the most currently accepted method for prevention of chronic copper poisoning is the administration of molybdenum plus sulphur with the feed, or by parenteral administration if the animal is already in the hemolytic crisis (Villar *et al.* 2002). Of the different molybdenum compounds, tetrathiomolybdate (TTM) is the only agent shown to remove copper selectively from the hepatocyte storage protein, metallothionein, without removing copper from other copper-enzymes such as ceruloplasmin or affecting the metabolism of other essential metals such as zinc or iron (Ogra *et al.* 1998; Villar *et al.* 2002). Our study showed that the highest concentration of serum molybdenum in CCP goats was in the spring. Although the lowest concentration of copper was also observed in spring in Kidney**,** liver, lung, heart and hair, no significant correlation was observed between high serum Mb and low copper level of the above-mentioned tissues in this season. The lowest and highest levels of copper in spleen were seen in winter and summer respectively, the cause of which was unknown for us and probably some other causes should be investigated. In this study the concentration of copper in the serum of CCP goats was not significantly correlated with copper content of other tissues in all seasons. This may be due to unavailability of ceruloplasmin to carry excess copper in blood stream and subsequent excretion of the copper in urine, while at the same time the copper concentration of other tissues was still higher in different seasons. The phenomenon has been also reported in Wilson's disease in man (a disease in which copper deposits in the liver occurs) (Reed *et al*. 2018). Positive significant correlation between the serum molybdenum and hair copper content in winter, observation of the lowest concentration of serum molybdenum, may show that the level of molybdenum was still in such a low level that molybdenum did not affect the level of copper in the hair and changed independently. Non-significant multiple negative correlations among serum molybdenum and copper concentration of serum and tissues were observed during the year. This may also show that the level of molybdenum in CCP goats was in such a low level that negative correlations did not reach a significant level and animals still remained in a state of excess copper showing clinical signs of chronic copper poisoning. The copper content of liver had positive significant correlation with the copper of lung, heart in spring rather than in other seasons. This may show that concentration of copper in liver, in spring, was not in such a high level (as the main organ for storing copper); so, the lowest liver copper concentration of these tissues in spring let significant correlations be observed. The positive correlation between lung and heart in other seasons except winter may be due to the lower concentration of copper in these tissues (in contrast to copper concentration in liver) and probably close anatomical and functional relationship of these two organs. The significant negative correlation between the copper concentration of spleen and lung, in winter, is vague and because of the lower concentration of copper in these two organs, it may be a random finding. There was also a significant positive correlation between kidney and heart in winter, and among the kidney, lung and heart in autumn. This phenomenon may be due to a long term receiving of a high proportion of copper by the kidney which is in close functional proximity to heart and lung. Kidney copper had a positive correlation with liver copper in summer which may be due to concurrent change in copper concentration of kidney (as a route of excretion of CU) and liver (as the main site of copper storing organ in the body). Serum ceruloplasmin was lowest in winter when highest liver copper was observed. Moreover, significant low concentration of ceruloplasmin in CCP goats was observed in all seasons. This shows that the capability of liver, for ceruloplasmin synthesis and secretion, is significantly declined as a result of hepatic cellular degeneration and necrosis (Fox *et al.* 2000).

Mathenge (1978) reported that Plasma ceruloplasmin activity did not change in CCP sheep. He also stated that the liver function tests and plasma copper for early diagnosis of chronic copper poisoning may be of use in animals which would later undergo severe crises. Mathenge (1978) and Xu *et al*. (2018) reported a marked decrease in the concentration of ceruloplasmin in serum samples from patients with Wilson's disease (Mathenge, 1978; Xu *et al.* 2018).

In conclusion, although chronic copper poisoning is a much larger problem in sheep, our observations in the region of Kerman showed its occurrence in goats. The results of the current research showed that the level of copper in CCP goats is season-dependent and seasonal variations can affect the level of toxicity. Significant correlations were also observed between different tissues in certain seasons in CCP goats. Environment, climate, nutrition and stressors may be the main causes of copper contents of different tissues. Ceruloplasmin decreased in CCP goats as a result of liver damage.

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Conflict of interest

The authors certify that there is no conflict of interest.

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