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RELATIONSHIP BETWEEN HEPATIC COPPER CONCENTRATION AND LIVER ENZYMES ACTIVITY LEVELS IN BUFFALOES

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ABSTRACT

This study was conducted on 35 buffalo calves, which were randomly collected from a buffalo farm. All calves were classified according to the determination of hepatic and serum Cu concentration. (10) of them considered as a control group, while the rest (25) considered as cu accumulated group. All calves were slaughtered in Bani Adi slaughter house (Assiut, Egypt). Blood and liver samples were collected during ordinary slaughter of the animals. Serum samples used for determination of serum copper concentration, ceruloplasmin (CP) activity, and hepatic enzyme activities (AST and GGT).Cu concentration was determined in the liver samples and feed stuffs. Results showed that the mean value of both AST and GGT enzymes activity were significant ($p<0.01$) and ($p< 0.05$) increased in the cu accumulated group than the control one. The mean values of serum cu and hepatic cu were significant ($p<0.01$) increase in the cu accumulated group than the control group and also for molybdenum while for cereloplasmin was non-significant. The mean value of cu concentration in barseem, wheat straw and concentrates were evaluated. Significant correlation between hepatic Cu accumulation, serum AST activity and serum GGT activity were recorded in this study. Analysis of Cu content in the liver with determination of both AST and GGT enzymes activity is probably the best diagnostic tool currently available for assessing the risk of increase cu accumulation.

Key words: *Copper, AST, GGT, hepatic cu accumulation, cereloplasmin*

INTRODUCTION

Copper (Cu) is one of the essential and important trace elements for the normal health and growth of animals (Rucker *et al.,* 2008). Serum copper and other biochemical variables have limited diagnostic value in diagnosis of early hepatic copper accumulation. Some researchers concluded that the most reliable factor in diagnosing early accumulation of copper by determination of the serum activates of aspartate amino transferase (AST) and gamma glutamyle transferase (GGT) (Lo´pez-Alonso *et al.*, 2006).

The most common tissue analyzed for mineral content is liver, as it is the primary storage organ for many of the essential minerals (Mc Dowell, and Arthington, 2005). Liver values are more informative and consistent as blood levels may remain normal for longer periods after liver trace elements levels commence to fall or increase indicating an early sign of trace elements deficiency or accumulation (Radostits *et al.*, 2004). Liver copper concentration is the best indicator of copper status and is the standard to compare the performance of any test used for detect copper overload. (El-salam *et al.*, 2013).

Liver Cu concentration that seemingly could be associated with subclinical chronic Cu toxicities in cattle has been observed in many countries where Cu supplements are given at concentration that is well above requirements, (Adei and Forson-Adaboh, 2008 and Leontopoulos *et al.,* 2015) or where there is contamination of pastures as a result of mining, industrial emissions, or use of organic wastes as fertilizer, also hepatic Cu concentration could be just above the normally accepted ‘‘safe’’ values (Lo´pez-Alonso *et al.,* 2000 and Tokarnia *et al.,* 2000).

There is a clear need to identify markers of early changes, with a capacity to predict risk of Cu accumulation in the liver before actual tissue or functional damage develops. Acquisition of these markers should be noninvasive, or at least less invasive than liver biopsy, and the markers should function as a sensitive index of Cu accumulation even in the absence of substantial functional damage. (Speisky *et al.*, 2003 and El-salam *et al.*, 2013).

Hepatic enzyme activities, together with Cu concentration in blood, liver, and kidney, are among

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the most widely used clinical tools for diagnosis of chronic Cu toxicities, (Auza *et al.,* 1999) because hepatic cell damage leads to a suddenrelease of hepatic enzymes into the blood during the stages of increase hepatic copper.

Ceruloplasmin (Cp) is a metalloenzyme with oxidase activity that is associated with iron and copper homeostasis (Szczubiał *et al.,* 2008). Each molecule of ceruloplasmin contains six to eight atoms of copper which influence its biological activity. Cereloplasmin has been used diagnostically to confirm copper deficiency (Laven and Livesey, 2007). Ceruloplasmin (Cp) functions include transportation of copper in the blood to various tissues, oxidizing minerals most notably iron and manganese, and scavenging oxygen radicals to protect cells. Copper is involved in the antioxidant system through its presence in several significant proteins. Copper is present most commonly in the proteins ceruloplasmin and superoxide dismutase (SOD). Ceruloplasmin activity is diminished or absent without sufficient copper (Gropper *et al.,* 2005).

Large numbers of livestock in many parts of the world consume diets that do not meet the dietary requirements (Ogundiran *et al.,* 2012 and Wang *et al.,* 2013). Continued ingestion of diets that are deficient, imbalanced or excessively high in a mineral induces changes in the form of concentration of the mineral in the body tissues and fluids, so that it falls below or rises above the tolerable limits. In such circumstances biochemical lesions develop, physiological functions are affected adversely and structural disorders may arise (Suttle and Jones, 2000 and Moral *et al.,* 2008). Many factors that alter copper metabolism influence copper accumulation by enhancing the absorption or retention of copper. Low levels of molybdenum or sulfate in the diet are important examples to decrease the absorption of Cu. (Akhtar *et al.,* 2007).

Identification of animals of Cu accumulation is important to avoid not only economic losses due to subsequent severe disease or death, but also to avoid subclinical disease, and to adapt Cu supplementation to physiologic needs. It also has been reported that dietary supplements leading to Cu accumulation in the liver at concentration only slightly above normal (around 125 mg/kg of wet weight) induce negative effects on animal performance, in terms of reduced feed intake and average daily gain (Engle and Spears, 2000).

The aim of the study reported here was to evaluate the suitability of serum AST and GGT activities as potential markers of hepatic Cu accumulation in buffalo calves.

MATERIALS AND METHODS

Animals:
A total number of 35 buffalo calves were included in this study. Which were randomly collected from Alhawatka buffalo farm during the period from August to December 2015.All animals were dewormed according to the farm records. All calves were classified according to the determination of hepatic and serum levels of copper (10) of them considered as a control and 25 consideredascu accumulated group. All calves slaughtered in a slaughter house, Assiut, Egypt.

Blood Sampling
Blood and liver samples were collected during ordinary slaughter of the animals.

For separation of serum samples, blood sample (20 ml) was collected from each calfe from the jugular vein into plane tube without anticoagulants. The blood samples were left to clot, then centrifuged to provide non haemolysed serum and frozen at −20°C until analysis. Serum samples used for determination of serum copper levels, ceruloplasmin (CP) activity, and hepatic enzyme activities (AST and GGT). Liver sample (about 100g) was taken from the caudal lobe of each calfe immediately after slaughter. Samples were placed on ice immediately after collection, and were transported to the laboratory for further preparation.

Biochemical Assays:
Serum Cu, AST and GGT were measured spectrophotometrically by using biodiagnostic test kits. Serum molybdenum was determined by using an atomic absorption spectrophotometer.

The activity of ceruloplasmin was measured by Assay Max Ceruloplasmin ELISA Kit (Catalog No. EC4001-1 Assaypro) A polyclonal antibody specific for ceruloplasmin had been pre-coated into a specific microplate with removable strips. Ceruloplasmin in standards and samples were tested for copper using spectrophotometer (UV/ VIS spectrophotometer Optizem 3220 uv MECASYS Co., LTD, Korea).Competed with a biotinylated ceruloplasmin sandwiched by the immobilized antibody and streptavidin- peroxidase conjugate. All unbound material was then washed away and a peroxidase enzyme substrate was added. The color development was stopped and the intensity of the color was measured.

Liver samples: (one gram) were digested in a mixture of 2:1: 0.5 nitric acid (HNO, 65%, Perchloric acid ($HCLO_4$, 60%) and sulphuric acid (H_2SO_4, 97%) respectively. The samples were further diluted and aspirated into an atomic absorption spectrophotometer.

Feed samples:
The animals were fed according to the farm strategy depended mainlyon green berseem (Trifolium Alexandrinum) as it is the commonly available during winterseason plus some concentrates mixture and wheat straw. The commercial concentrate mixturein this district was supplied by certain company and consisted of; undecrticated cotton seedcake; course wheat bran; crushed corn; rice kernel and polish and salt. Samples were collected from the farm before animals go to the slaughter house. Feed samples were dried, ashed and the HCl-insoluble ash was determined according to Marshal (1993).

Copper and molybdenumwere determined by atomic absorption spectrophotometer (AA-640, Shmadzu Co., Ltd, Japan). The preparation of ration for copper determination wasdone according to Hady (1986).

Statistical analysis:
Recorded data were analyzed statistically using analysis of variance (ANOVA). The statistical differences between means were estimated by Duncons Multiple Range test. The computation was facilitated by statistical package SPSS (2000).Association between hepatic Cu accumulation and each of the blood parameters analyzed was assessed using the Pearson correlation coefficient.

RESULTS

Table 1: Mean and standard error values of serum AST and GGT activities in buffalo calves.

Parameter	Control group	cu accumulation group	P value
AST (U/L)	117±18	131±28.2	<0.001
GGT (U/L)	11	24	<0.050

Table 2: Mean value of serum and hepatic cu and serum cereloplasmin (CP).

Analyte	Control group	cu accumulation group	P value
Serum Cu (mg/L)	0.384±0.11	0.923±0.175	<0.01
Hepatic cu mg/kg wet weight	69.2±5.4	192.4±7	<0.01
Cereloplasmin (mg/L)	134±1.9	165±3.4	n.s
Molybdenum (ug/dl)	43.80±2.4	36.71±1.9	<0.05

n.s=non significance

Table 3: Mean value of copper in different feed stuffs on dry matter basis.

Feed stuff	copper	Molybdenum
Barseem (mg/kg)	17.4±1.32	3.31±0.15
Wheat straw (mg/kg)	5.6±0.41	1.96±0.13
Concentrates (mg/kg)	11.8±0.98	2.11±0.12

Table 4: Pearson's correlation between liver copper concentration, serum Cu, Cp, AST, and GGT.

Analyte	Hepatic Cu	
	Pearson's correlation	P
Serum Cu (mg/L)	0.141	<0.240
Cp (mg/L)	-0.085	<0.427
AST (U/L)	0.261	<0.035
GGT (U/L)	0.269	<0.027

Table 5: Mean values of Serum Cu, Hepatic Cu, AST, and GGT in both groups.

	Serum Cu(mg/L)	Hepatic Cu mg/kg wet weight	AST(U/L)	GGT(U/L)
Control group	**0.384±0.11**	**69.2±5.4**	**117±18**	**11**
cu accumulation group	**0.923±0.175****	**192.4±7****	**131±28.2****	**24***

** significance at P<0.01 and * significance at P<0.05.

DISCUSSION

The results in table (1) revealed that the mean value of AST and GGTenzymes activity in control buffalo calves were 117±18 and11 u/l while calves with hepatic cu accumulation showed a mean value 131±28.2 and 24u/l. The mean value of both AST and GGTenzymes activity were significant (p<0.01) and (p <0.05) increase in the cu accumulated group than the control one. These results are in agreement with those reported by (Lo´pez-Alonso *et al.,* 2006 and Antonio *et al.,* 2008).

Several authors have postulated that hepatic enzymes may also be useful asearly markers during the long-term, subclinical phase of hepatic Cu accumulation (Humann–Ziehank *et al.,* 2001 and Laven *et al.,* 2004). On the basis of the fact that, during this silent phase, some cells undergo necrosis, leading to increases in enzyme activities in the blood.

Table (2) showed the serum and hepatic Cu concentrations and serum Cpconcentration in control group were 0.384±0.11 mg/L, 69.2±5.4 mg/kg wet weight and 134±1.9 mg/L while these values in the cu accumulated group were 0.923±0.175mg/L., 192.4±7 mg/kg wet weight and 165±3.4 mg /L respectively.

The mean value of serum cu and hepatic cu were significant (p<0.01) increase in the cu accumulated group than the control group. The mean value of serum molybdenum was significant (p<0.05) increase in the cu accumulated group than the control group while cereloplasmin was non-significant. These results are in agreement with those reported by (Bidewelland Livesey, 2002 and Lo´pez-Alonso *et al.,* 2006).

Results of this study indicate that, in buffalo with moderate hepatic Cu accumulation, neither the serum cu nor CP concentration, (the 2 main parameters typically used in the diagnosis of Cu deficiency), (Tessman *et al.,* 2001), are significantly associated with hepatic Cu concentration.

Table (3) the mean values of cu concentration in barseem, wheat straw and concentrates were 17.4±1.32, 5.6±0.41 and 11.8±0.98 respectively. the mean values of molybdenum concentration in barseem, wheat straw and concentrates were 3.31±0.15, 1.96±0.13 and 2.11±0.12 respectively.

Ingestion of quantities of Cu slightly higher than required may cause accumulation in the hepatic cells. Generally, the copper content of the different Egyptian feedstuffs was ranged between 4.5 and 16.7 mg/kg confirming the adequacy of copper to satisfy the animal's requirement which on average is10 mg/kg established by Anonymous. (1996 and 2005) and Ogundiran *et al.* (2012), and more than the critical level reported by NRC (1984). Molybdenum contents in all fodders examined were adequate, where dietary requirement of molybdenum for cattle and buffaloes was 0.2-7 mg/kg (Church, 1988).

In fact, the trace elements concentrations for animals' in both serum and liver will therefore depend on the mineral contents of feed and forage, the level of dietary sources intake, and the availability of minerals (Kamalu *et al.,* 2006; and Khan *et al.,* 2007). Also many environmental and plant factors affect the mineral concentrations of forage plants; which include, species or strain, variety, soil type, the climatic conditions of different seasons during plant growth, stage of maturity of forage plants and other management practices (McDowell and Arthington, 2005 and Wang *et al.*,2013).

Table (4 and 5) showed significant correlation between hepatic Cu accumulation, serum AST activity and serum GGT activity, these results are in agreement with those reported by Blakley and Hamilton, 1985, Stoszed *et al.,* 1986, Lo´pez-Alonso *et al.,* 2006 and Antonio *et al.,* 2008). These results indicated that hepatic Cu and CP concentrations are not correlated at either normal or high amounts of hepatic Cu accumulation. This is because, once the animal's liver reaches adequate Cu status, CP as well as Cu-dependent enzymes such as superoxide dismutase in erythrocytes attain maximal activity that is not increased with further hepatic Cu accumulation (Baker *et al.,* 1999 and Rock *et al.,* 2000) in addition, CP values vary with factors, such as age and sex, (Fisher *et al.,*1990) and increase rapidly in response to factors other than Cu excess, such as exercise and various inflammatory and infection conditions. (Harris, 1997).

CONCLUSION

Analysis of Cu content in the liver is probably the best diagnostic tool currently available for assessing the risk of increase cu accumulation. Hepatic biopsy specimens should be regularly obtained from animals at risk for increase Cu concentration (e.g., highly Cu-supplemented diet or grazing in contaminated areas).

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العلاقه بين تركيز النحاس فى كبد الجاموس ومستوى نشاط انزيمات الكبد

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أجريت هذه الدراسة على عدد ٣٥ عجل جاموسى تم إختيارها عشوائيا من احدى المزارع بمحافظه اسيوط لإيجاد العلاقه بين تخزين النحاس فى أكباد الجاموس ومستوى انشطه انزيمات الكبد. حيث تم تجميع عينات دم وكذلك عينات كبد من هذه المجموعه علاوه على عينات من الغذاء المقدم لهم داخل المزرعه. وتم تتبع هذه الحيوانات من المزرعه حتى دخولها المجزر. وقد تم تقسيمها الى مجمعتين الأولى المجموعه الضابطه (١٠ عشره حيوانات) والثانيه المجموعه الممثله لزياده تراكم النحاس (٢٥ حيوان). وأستخدمت عينات المصل النقى لقياس نشاط انزيمى الأسبرتيت أمينوترانسفيريز والجاما جلوتاميل ترانسفيريز ونسبه تركيز النحاس ونسبه السيريلوبلازمين فى مصل الدم. وتم تعيين نسبه تركيز النحاس فى الكبد وفى الغذاء المقدم داخل المزرعه. وقد أوضحت النتائج وجود زياده معنويه يبين متوسط نشاط كلا من الانزيمين (انزيمى الأسبرتيت أمينوترانسفيريز والجاما جلوتاميل ترانسفيريز) فى المجموعه المتراكم بها النحاس عن المجموعه الضابطه وكذلك وجد أن هناك زياده معنويه فى كل من تركيز النحاس فى مصل الدم والكبد فى المجموعه المتراكم بها النحاس عن المجموعه الضابطه وهو كذلك بالنسبه لتركيز الموليبدينوم، كما اوضحت النتائج عن عدم وجود أى علاقه معنويه مع نشاط السيريلوبلازمين. وقد تم تقدير نسبه تركيز النحاس فى الغذاء المقدم للحيوانات. وقد وجد ارتباط بين المجموعه المتراكم بها النحاس ونشاط كلا من (انزيمى الأسبرتيت أمينوترانسفيريز والجاما جلوتاميل ترانسفيريز) وكذلك نسبه تركيز النحاس فى السيرم. وقد خلصت الدراسه الى ان تحليل تركيز النحاس بالكبد وتقدير نشاط كلا من انزيمى الأسبرتيت أمينوترانسفيريز والجاما جلوتاميل ترانسفيريز) تعد وسيله جيده لتقدير مدى خطوره تراكم النحاس.