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Research Article

Chronic Copper Poisoning in Sheep: Liver Injury

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Abstract

Pathogenesis of liver injury in chronic copper poisoning of sheep was investigated using two experiments. Test sheep were dosed orally with a 0.2 % solution of $CuSO_4.5H_2O$ at the rate of 10 ml/Kg body weight on five days of the week, until first day of the haemolytic crisis (HC). Liver samples were taken during the pre-haemolytic period and on the first or second day of the HC, liver changes were evaluated using trace element, histochemical, subcellular fractionation, ultrastructural and morphometric methods.

Copper concentrations in liver samples increased at a steady linear rate of 10.35 μg/g liver wet weight per day; uptake of copper among hepatocytes, among Kupffer cells and between zones of liver lobules was unequal. Individual hepatocytes became packed with copper loaded lysosomes and underwent degeneration and necrosis. Excess copper in liver cells was sequestered by the lysosomes leading to linear increases in volume density, numerical density and mean volume during the pre-haemolytic period. On the first day of HC, numerical density reduced, volume density remained unchanged and the mean volume increased.

Linear increase in liver copper concentration indicates that copper absorption from the gut; excretion into bile and release into sinusoids by hepatocytes occur at linear steady rates. Thus, copper homeostasis in sheep is different to that of the humans and rats.

In hepatocytes packed with copper loaded lysosomes, synthesis of new lysosomes reduced, causing decreased uptake of excess copper leading to accumulation of ionic copper in the cytoplasm, resulting in the degeneration and necrosis of the hepatocytes.

Key words: Hepatocyte; Lysosomes; Pre-haemolytic period; Haemolytic crisis; Necrosis

1. Introduction:

Copper (Cu) is a trace nutrient and exists in the animal body in stable oxidized Cu II or unstable reduced Cu I states. It has the ability to cycle between the two forms, thus is used by enzymes involved in redox reactions that are essential in biological processes, such as mitochondrial oxidative phosphorylation, iron metabolism, pigmentation, neurotransmitter synthesis, cross-linking of collagen and elastin, and free radical detoxification (Camakaris et al., 1999; Puig et al., 2002; Petris et al., 2003; Andreini et al., 2008; Nose et al., 2010). Imbalances in Cu content of the body could lead to either Cu toxicity (Eg. Wilson's disease in humans and chronic Cu poisoning in

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sheep) (Mendel et al., 2007; Wang et al., 2011) or deficiency (Eg. Menkes disease in humans) (Wang et al., 2011; Vonk et al., 2012).

Chronic Cu poisoning has been reported in sheep grazing on pastures contaminated with Cu containing compounds (Roubies et al., 2008; Oruc et al., 2009), high in Cu and/or low in molybdenum (Mo) contents (Suttle, 1991) and plants containing hepato-toxins (McLean, 1970; Howell et al., 1991). Similarly, sheep fed on concentrate feed containing high levels of Cu and/or low concentrations of Mo have developed chronic Cu poisoning (Adamson et al., 1969; Pope, 1971).

Copper accumulates primarily in the liver of chronic Cu poisoned sheep (Kumaratilake and Howell, 1987; Kumaratilake and Howell, 1989a). The distribution of Cu among the zones of a "functional liver lobule" is unequal and the pattern of distribution among these zones of a lobule appears to vary among different breeds of sheep. In Merino, Clun Forest and Cambridge breeds of sheep, the initial deposition of Cu was in centrilobular zones and extended through midlobular to periportal zones with increasing Cu loading (Ishmael et al., 1972; Kumaratilake and Howell, 1987; Haywood et al., 2005). In contrast, the deposition of Cu in Finn-Dorset and Suffolk crossed lambs and North Ronaldsay ewes extended from the periportal zones, through the midlobular zones to the centrilobular zones (King and Bremner, 1979; Haywood et al., 2005). In liver lobules, Cu accumulates in hepatocytes and Kupffer cells and the deposition of Cu in these cells is primarily within lysosomes (Kumaratilake and Howell, 1987, 1989a).

The first predominant clinical sign that develops in chronic Cu poisoned sheep is passage of dark-red urine (i.e. haemoglobin urine) that results from intravascular haemolysis and the sheep could die from this HC (Mendel et al., 2007).

The aims of this study were to induce chronic Cu poisoning in sheep with oral Cu dosing, and to investigate the changes that occur in the liver with increasing Cu loading leading to the HC. The aspects that will be investigated are:

i) Histopathological changes occurring in functional liver lobules including the portal triads using histochemical and quantitative (i.e. subjective and morphometric) methods.

ii) Distribution of Cu among the subcellular compartments of hepatocytes and the changes that occur in the organelles of the compartments, particularly the lysosomes using sub-cellular fractionation, ultra-structural and morphometric methods.

2. Experiments

2.1 Animals and their treatments

Two experiments were carried out. In experiment 1 and 2, 10 and 8 Merino sheep respectively of age 12 months were housed in individual pens with free access to water and fed 0.5 Kg per head of a proprietary feed containing Cu, iron and zinc concentrations respectively of 8.0, 37.1 and 99.7 mg per Kg dry matter. Each sheep was given 2 injections of 1.0 ml of Selvite E (Ilium, Troy Laboratories Pty, Limited, New South Wales, Australia) a week apart to prevent Selenium and Vitamin E deficiencies. Test sheep of both experiments were dosed orally with a 0.2% solution of $CuSO_4.5H_2O$ on 5 days of the week at the rate of 10 ml per Kg body weight until the first day of the HC.

2.2 Experiment 1

The aim is to investigate changes that occur in the liver with increasing copper loading in sheep during the prehaemolytic and haemolytic periods, particularly during the period immediately prior to the HC.

Ten sheep, consisted of 4 control and 6 test animals. In test sheep, blood samples were collected twice weekly until the second elevation in plasma sorbitol dehydrogenase (SD) activity occurred and thereafter daily. Second rise in plasma SD activity was taken as an indicator that liver injury has been progressing (Ford, 1967; Shaw, 1974). Acid phosphatase (AP) is an enzyme specific to lysosomes and its activity could be detected in serum when released from these organelles (Clearmont et al., 1974; Braun et al., 1989). Kumaratilake et al. (1981) have observed the highest elevation of AP activity in the serum of chronic Cu poisoned sheep occurred 1-3 days prior to the HC. Therefore, plasma SD and serum AP activities were determined in the blood samples, with the aim of identifying the period immediately prior to the HC. Furthermore, liver biopsies were obtained from the test sheep either at elevations of plasma SD or serum AP activities with the aim of obtaining a biopsy immediately prior to the HC. The test sheep were killed on the second day of the HC. Blood samples were collected from the control sheep twice weekly throughout the experiment. Control sheep were biopsied once at the end of the experiment and killed two days later. At the postmortem, liver samples were obtained from all sheep.

Liver biopsies (approximately of 0.2 – 0.8 g) were obtained by aspiration using a trocar and cannula with an internal diameter of 0.8 cm as described by Dick (1944). Details of the sheep are given in Table 1. From each liver (i.e. biopsies and postmortem samples) sample, a piece was fixed in 10% buffered formalin and processed for light microscopic investigations and another piece was processed for determination of Cu concentrations by atomic absorption spectrophotometry.

Table 1 Details of sheep in experiments 1

Ex. No.	Group	Sheep No.	No. of days in experiment from start of Cu dosing	No. days in haemolysis	Comments
1	Control	293	111	-	Biopsied on day 109 and killed two days later
		352	111	-	
		587	111	-	
		591	111	-	
	Test	283	103	2	Obtained 5 biopsies. The last one, a day before the haemolytic crisis and killed on second day of the haemolytic crisis
		292	46	-	Obtained 2 biopsies. Died a day after the 2nd biopsy.
		294	195	2	Obtained 2 biopsies, the 2nd, a day before the haemolytic crisis and the sheep died on second day of the haemolytic crisis.
		299	98	2	Obtained 8 biopsies. The last one, a day

					before the haemolytic crisis and killed on second day of the haemolytic crisis.
1	Test	333	18	-	Obtained 2 biopsies and the sheep died immediately after the 2nd biopsy.
		354	120	2	Obtained 2 biopsies, the 2nd one, 1.5 days before the haemolytic crisis. Killed on second day of the haemolytic crisis.

In experiment 1, sheep 292 and 333 were not included in the study.

2.3 Experiment 2
The aims of this experiment were to study changes occurring in the liver and in hepatocyte lysosomes with increasing Cu loading using sub-cellular fractionation, ultrastructural and morphometric methods.

Liver samples were obtained by performing a small laparotomy incision on the right abdominal flank. Biopsies were taken from 4 sheep 2 days before the start of Cu dosing, weekly during the first 5 weeks of Cu dosing and thereafter every other week until the week before they were killed on 9, 12, 14 and 15 weeks after the commencement of Cu dosing. A biopsy was taken from each of the other 4 sheep on the first day of HC, i.e. 12, 12, 13 and 14 weeks after the start of Cu dosing.
A small piece from each biopsy was immediately immersed in 5% chilled glutaraldehyde (in phosphate buffered saline, pH 7.4, at 4^0 C), cut into approximately 1 mm cubes and processed for electron microscopy (EM) as described by Kumaratilake and Howell (1989a). Rest of the liver from each biopsy was used for subcellular fractionation.

Using the liver Cu concentrations, 24 liver biopsies (i.e. including the pre -dosing and first day of HC) were selected and divided into 6 groups, pre-dosing, 4 pre-haemolytic and haemolytic to cover a wide range of liver Cu concentrations, and the liver samples processed for electron microscopy from these biopsies were used for the morphometric analysis. Each group contained biopsies from 4 sheep.

2.4 Enzyme assays

2.4.1 Sorbitol dehydrogenase:
Activity was determined in plasma using the method described by Ford (1967) and the levels are expressed in IU/L.

2.3 ml of 0.05 M Tris buffer, pH 7.4; 0.2 ml of reduced form of Diphosphopyridine nucleotide (DPNH) (1 mg/ml) and 0.2 ml of test plasma were placed in a 3.0 ml spectrophotometer cuvette, pre-incubated for 10 minutes at 25^0 C; added 0.3 ml of 3.33 M D-fructose and the enzyme activity was determined (i.e. from the rate of change of optical density) at 340 nm using a Varian UV recording spectrophotometer. The temperature of the cuvette housing was maintained at a constant level of 25^0 C. Plasma with high activity was diluted with the buffer to obtain a linear rate of reaction.

The colour of the plasma samples obtained during HC interfered with the spectrophotometric reading, thus such samples were not used in the assay.

2.4.2 Acid phosphatase:
Activity was determined in serum and liver fractions by the method described by Walter and Schutt (1974) after adjusting the pH of the buffered substrate to 4.5 (Shannon et al., 1977). Levels are expressed in IU/L for serum and moles per min per Kg protein for liver fractions.

0.1 ml of test serum was added to 1.0 ml of the substrate, 4.5 mM 4-nitrophenyl phosphate in 0.1 M acetic acid-sodium acetate buffer, pH 4.5; incubated at room temperature (20^0) for 10 minutes and the reaction was stopped by adding 1.0 ml of 1 N NaOH. Absorbance was measured at 405 nm using a Varian UV spectrophotometer. The amount of 4-nitrophenol liberated per unit time is taken as the measure of phosphatase activity. Standard assay contained 0.04 μM of 4-nitrophenol.

The colour of the plasma samples obtained during HC interfered with the spectrophotometric reading, thus such samples were not used in the assay.

2.5 Copper concentrations
Copper concentrations in liver or sub-cellular fractions of liver were determined using a Varian Techtron atomic absorption spectrophotometer (Model 1200).

Liver or subcellular fractions (about 4.0 ml) were digested in a mixture of 10.0 ml concentrated HNO_3, 2.0 ml of concentrated H_2SO_4 and 2.0 ml of 70 % $HClO_4$ for 2 hours at 140^0 C, and for ½ an hour at 410^0 C. The digests were diluted in glass-distilled water to appropriate dilutions and the Cu concentrations were determined using a Varian Techtron atomic absorption spectrophotometer (Model 1200). It consisted of an air-acetylene burner and a specific Cu lamp, and the Cu concentrations were measured at 324.8 nm with the slit width setting at 0.7 nm.

Copper concentrations of the test samples were calculated from the absorbance measured by the spectrophotometer using a standard curve. Each time, the Cu concentrations were determined in test samples, a standard curve was constructed using Cu standard solutions that ranged from0.1 – 10.0 ppm. With this instrument, the standard curve constructed was linear and had r and R^2 values around 0.999 and 0.998 respectively. The test samples were appropriately diluted with glass distilled water to obtain the absorbance within the range of the above standards.

Some samples occasionally charred and they interfered with the absorbance reading, thus such samples were re-digested using additional acid (i.e. 5 – 10 ml concentrated HNO_3) to obtain a clear liquid.

2.6 Histochemistry
Liver samples were fixed in 10% buffered formaldehyde, dehydrated through a graded series of ethanol and embedded in paraffin wax (Pearse, 1972).

2.6.1: Staining for liver histopathology: Five to seven μm thick wax sections of liver were stained by routine Haematoxylin and Eosin method and Periodic Acid-Schiff technique with and without prior incubation with diastase (Pearse, 1972).

2.6.2: Staining for copper: Five to seven µm thick sections were stained for Cu with Rubeanic acid (RA) method as described by Lindquist (1969).

2.6.3: Evaluation of hepatocyte and Kupffer cell histopathology: In liver sections, degenerating hepatocytes, necrotic hepatocytes, focal areas of necrosis and enlarged Kupffer cells containing diastase resistant Periodic Acid-Schiff stained (DPAS) granules were given subjective scores in the following way.
No cells or focal areas – 0
Occasional cell or a focal area in a zone in a few lobules – 1
Scores of 2-10 for increasing number of cells or focal areas in lobules
Each section was scored by two people (including the author) independently.

2.7 Ultrastructure and morphometry

2.7.1 Ultrastructure: One µm thick sections were cut from six EM blocks selected from each liver biopsy, desirable areas identified, ultrathin sections taken, mounted on 200 mesh Cu grids and examined in an electron microscope.

2.7.2: Light microscopic morphometric analysis: One µm thick sections stained with Toluidine blue were used for light microscopic morphometric analysis. Each section was point counted at 5 different sites using a 100 point eyepiece graticule (Graticules Ltd, Kent, UK.) at a magnification of approximately 400. Points overlying hepatocytes (normal, degenerating and necrotic), central veins, portal triad spaces and fibrous tissue were counted and the volume density (Vv) of each component was calculated as described by Kumaratilake (1984). Cells showing pyknosis, karyolysis or loss of structural details of the nucleus were counted as necrotic and cells that are otherwise abnormal (i.e. swollen and vacuolated) were counted as degenerating cells. The remainder of the cells was counted as normal. Liver mass was used as the reference volume.

2.7.3 Electron microscopic morphometric analysis: Morphometric analysis was carried out as described by Kumaratilake and Howell (1989a). Volume density, numerical density (Nv) and mean volume (V) of hepatocyte lysosomes were determined. Hepatocyte cytoplasm was used as the reference volume for Vv and Nv (Loud, 1968; Weibel et al., 1969).

2.8 Subcellular fractionation
Liver samples were homogenized and separated into nuclear (N), mitochondrial (MH), lysosomal (ML), microsomal (MI) and Cytosol (CY) fractions as described by Kumaratilake and Howell (1989b). In each fraction, the concentration of Cu and specific activity of AP were determined. The results of the latter presented in Kumaratilake and Howell (1989b).

2.9 Statistical analysis:
T-test, two way analysis of variance, correlation and regression analyses were used for the analysis of liver Cu concentrations and morphometric data of the liver and lysosomes (Kirkwood and Sterner, 2003).

3. Results

3.1 Results of Experiment 1

Liver biopsies and samples obtained at the time of killing or after death:

The details of the liver biopsies obtained during the pre-haemolytic periods, liver samples obtained after killing or death including the Cu concentrations are presented in Table 2.

Table 2 Details of liver biopsies and samples (at time of death or killing) obtained from sheep of Experiment 1

Group	SheepNo.	Biopsy No. or sampled at	Days from start of HC	Cu concentration µg/g wet weight
Control	293	1	-	212.34
		K	-	164.50
	352	1	-	258.46
		K	-	262.53
	587	*	-	*
		K	-	252.47
	591	1	-	434.46
		K	-	389.38
	Mean ±SE	-		267.22±46.29 **
Test	283	1	-37	451.30
		2	-35	463.50
		3	-31	474.97
		4	-27	548.63
		5	-2	*
		6	-1	778.15
		K	+2	803.32
	294	1	-28	*
		2	-2	*
		3	-1	1343.33
		D	+2	1135.06
	299	1	-63	294.28
		2	-61	301.65
		3	-56	364.48
		4	-23	725.34
		5	-21	747.78
		6	-18	794.63
		7	14	*
		8	-3	*
		9	-1	988.32
		K	+2	1010.46

	354	1	-6	632.95
		2	-1.5	654.73
		3	-1	*
		K	+2	688.74
	Mean±SE	-	-	909.40±100.45 **P<0.001

Cu = copper, K = killing, D = death, HC = haemolytic crisis, SE = standard error of the mean, * = the biopsy was not successful or did not have enough tissue, ** = Mean and P value for liver samples obtained after death or killing, P = probability

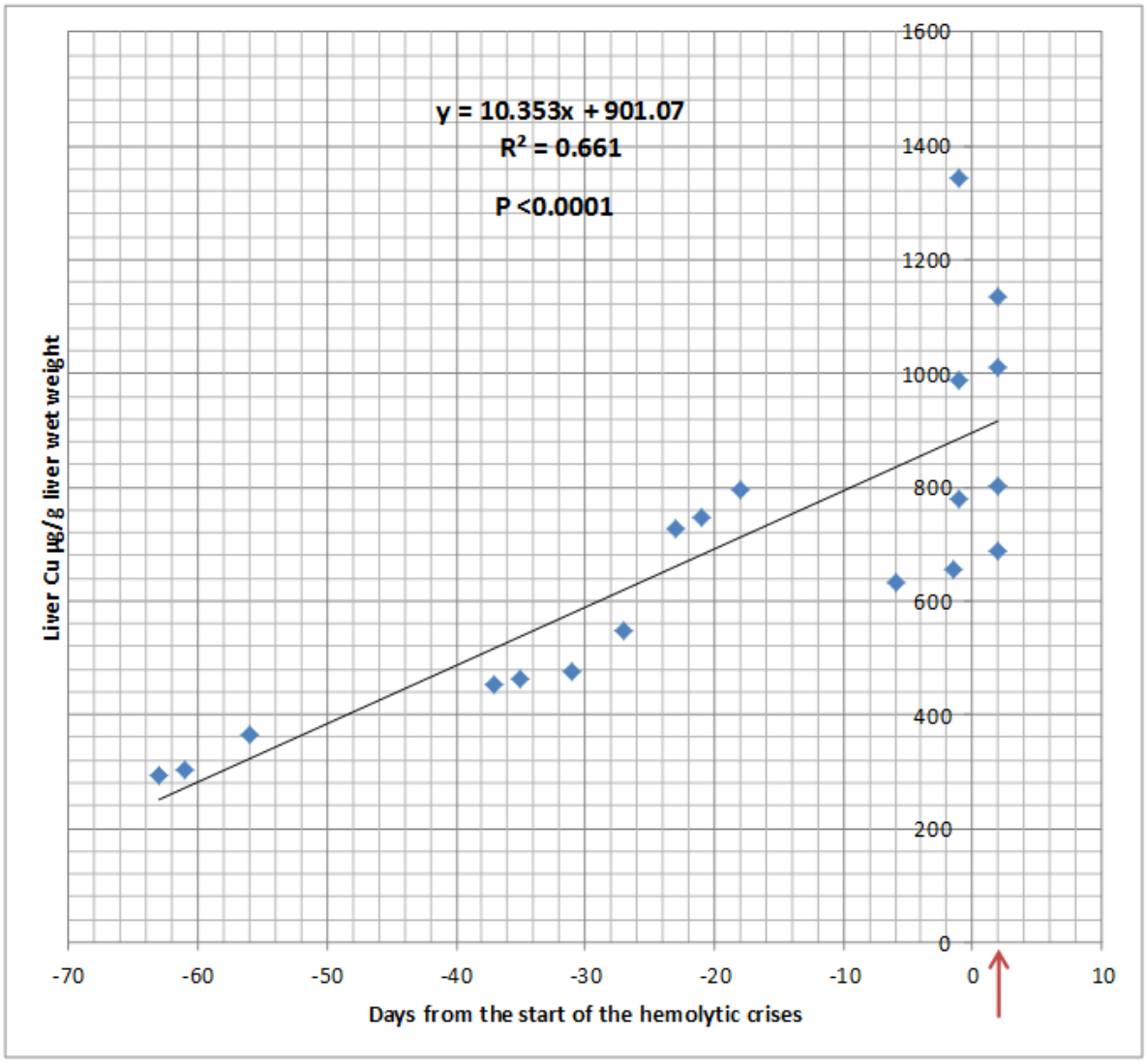

Fig. 1. Linear regression analysis of copper concentrations of liver biopsies or liver samples against the number of days from the start of the hemolytic crises

X axis based from the start of haemolytic crises
Y axis liver copper concentration of biopsies or liver samples
Arrow = Second day of hemolytic crises, P = Probability.

Liver Cu concentrations: The Cu concentrations of the liver samples obtained from the test sheep after killing or death were significantly higher than those of the control animals ($P<0.001$) (Table 2). Linear regression analysis of the Cu concentrations of the liver samples (i.e. of test sheep) against the number of days from the start of HC to the time of sampling of the liver samples indicated that the liver Cu concentration increased linearly ($R^2 = 0.66$ and $P< 0.0001$). This linear increase occurred at a steady rate of 10.35 μg/g liver wet weight per day during the oral Cu dosing (Fig. 1).

Histopathology and histochemistry: Copper concentrations of the liver samples of control sheep 591 were similar to early liver biopsies of test sheep 283 and 299, thus samples from sheep 591 were excluded from this part of the experiment.

In control sheep 293, in some liver lobules, occasional necrotic hepatocytes were seen in midlobular or periportal zones and areas of neutrophil infiltration were observed in midlobular zones. In addition, liver biopsies of the 4 control sheep had small number of Kupffer cells containing small amounts of DPAS positive granules, in the centrilobular and midlobular zones of some lobules. In these lobules, the majority of these cells were seen immediately around the central veins.

In test sheep, the number of individual hepatocytes that were degenerating or necrotic and Kupffer cells that were enlarged with DPAS positive granules increased with increasing liver Cu concentrations. The liver biopsy 1 of sheep 299, which had a Cu concentration of 294.3 μg/g wet weigh, such cells were seen in the centrilobular and midlobular zones of some lobules. In biopsies with higher Cu concentrations, the number of degenerating and necrotic hepatocytes, and enlarged Kupffer cells with DPAS positive granules increased, and they were seen in all 3 zones of a lobule. The number of lobules containing such cells also increased. In biopsies taken 1 – 1.5 days prior to the HC from sheep 283, 294 and 354, a marked increase in the number of individual degenerating and necrotic hepatocytes and enlarged Kupffer cells (i.e. containing DPAS positive granules) was seen in most lobules. However, such an increase in degenerating and necrotic hepatocytes was not observed in the liver samples taken one day prior to or on the 1st day of HC from sheep 299. Liver samples taken on the 2nd day of the HC from sheep 294, 299 and 354, and 1.5 days prior to HC from sheep 354 had focal areas of necrosis in centrilobular zones (i.e. adjacent to the central vein). In sheep 299, the focal areas extended into the midlobular zones of some lobules. In sheep 283 focal areas of necrosis were not seen.

The degenerating and necrotic changes of the hepatocytes and the enlargement of kupffer cell with DPAS positive granules were given subjective scores independently by the two people including the author and results are presented in Table 3 (see materials and methods for details of the scoring).

Table 3 Subjective scores of liver samples of sheep of Experiment 1: For changes that occurred in hepatocytes and Kupffer cells.

Group	Sheep No.	Days From HC	Sample No.	Degenerating or necrotic hepatocytes						DPAS positive Kupffer cells		
				Individual cells			Focal areas					
				CL	ML	PP	CL	ML	PP	CL	ML	PP
Control	293	-	1	0	1	1	0	0	0	1	0	0
		-	K	0	0	0	0	0	0	1	0	0
	352	-	1	0	0	0	0	0	0	1-2	1	0
		-	K	0	0	0	0	0	0	0	1	0
	587	-	K	0	0	0	0	0	0	1-2	1	0
	Total			**0**	**1**	**1**	**0**	**0**	**0**	**5**	**3**	**0**
Test	283	-37	1	3	3	3	0	0	0	2-3	2-3	1-2
		-35	2	1	2	1	0	0	0	2-3	2-3	1-2
		-31	3	1	0	1	0	0	0	3	3	2
		-27	4	3	2	3	0	0	0	3	3	2-3
		-1	5	5	10	1	0	0	0	*	*	*
		+1	K	1	1	1	0	0	0	5-6	4	2-3
	294	-28	1	2	2	2	0	0	0	3-4	4-5	2
		-1	2	10	10	10	0	0	0	7	6	3-4
		+1	K	6	6	2	4-5	0	0	6	6	3-4
	299	-63	1	2	1	0	0	0	0	1	1	0
		-61	2	1	0	1	0	0	0	2	1	0
		-56	3	1	1	0	0	0	0	1-2	1	0
		-23	4	1	1	1	0	0	0	2	2	1
		-21	5	1	2	1	0	0	0	2	2-3	1
		-18	6	1	2	2	0	0	0	2-3	4	2-3
		-14	7	2	2	2	0	0	0	3	4	2-3
		-1	8	1	3	1	0	0	0	4-5	5-6	3
		+1	K	1	2	1	6	1-2	0	4	3-4	2
	354	-6	1	6	4	2	0	0	0	4-5	3-4	2
		-1.5	2	8	8	4	1	0	0	5-6	4	2-3
		+1	K	9	4	4	2	0	0	6	2-3	2
	Total			**66**	**66**	**43**	**13.5**	**1.5**	**0**	**71.5**	**66**	**37.5**

HC = haemolytic crisis, DPAS = diastase resistant Periodic Acid-Schiff stained granules, CL = centrilobular zone, ML = midlobular zone, PP = periportal zone, K = killed, D = death, * = sections poorly stained, thus details other than necrotic cells were not clear

In addition to the above changes, large hepatocytes, and hepatocytes with large nuclei, vacuolated nuclei, and mitotic figures were seen. Also, there were focal areas infiltrated with neutrophils or mononuclear cells. The former areas were more commonly seen in the mid lobular zones.

All hepatocytes contained DPAS positive granules in the cytoplasm, the size and the number of granules among hepatocytes within a lobule and between lobules varied. Isolated hepatocytes

packed with the granules were seen; the numbers of such hepatocytes increased in liver biopsies with increasing Cu concentrations and their distribution pattern within a zone and among zones within a lobule were very similar to the distribution pattern of isolated degenerating and necrotic hepatocytes. Some of the hepatocytes packed with DPAS positive granules had enlarged nuclei or nuclei with necrotic changes. In portal triads, macrophages containing DPAS positive granules were also observed. The number and the size of the macrophages in portal triads varied, and their number and size increased with increasing Cu concentrations of the liver biopsies.

The distribution pattern of granules stained positively for Cu with the RA method in hepatocytes, Kupffer cells and macrophages in portal triads were similar to those of DPAS positive granules in respective liver samples. Isolated hepatocytes and Kupper cells packed with RA positive granules were seen with increasing Cu concentrations of the liver samples. The distribution pattern of these cells within the liver lobules was similar to those cells packed with DPAS positive granules in respective liver samples (Fig. 2). In addition, macrophages containing RA positive granules were seen in the portal triads with increasing liver Cu concentrations (Fig 2). The distribution pattern of the macrophages containing RA positive granules in the portal triads was similar to those cells containing DPAS positive granules in the respective liver samples.

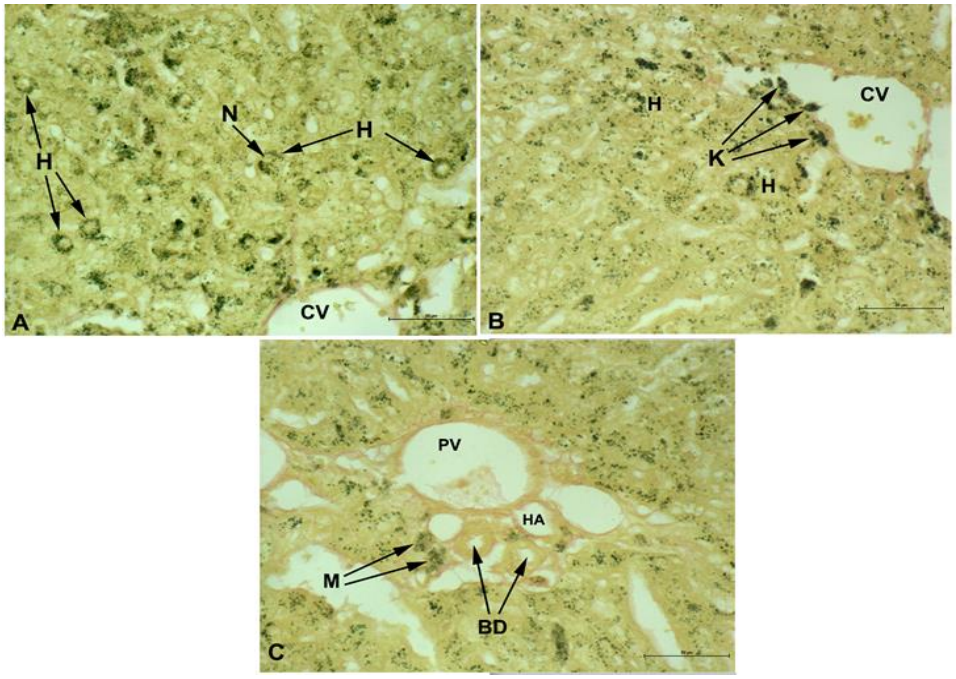

Fig. 2. A liver sample from first day of hemolytic crises stained with rubeanic acid method for copper (copper content 1239.21 µg/wet weight).

A – centrilobular zone - hepatocytes packed with copper containing granules. B - centrilobular zone – Kupffer cells loaded with copper containing granules. C – A portal tract – macrophages loaded with copper containing granules. H= Hepatocytes packed with copper containing granules. N = nucleus of hepatocytes. CV = central vein. K = Kupffer cells loaded with copper containing granules. M = Macrophages loaded with copper containing granules. PV = Portal vein. HA = hepatic artery. BD = Bile ducts. Scale = 50 µm.

3.2 Results of Experiment 2

Morphometry:

Volume density measurements of the liver components of five pre-haemolytic groups are presented in Table 4. Volume densities of intact hepatocytes decreased, while those of degenerating and necrotic hepatocytes, and fibrous tissue increased with increasing Cu loading. The Vv of total hepatocytes and cavities of central veins and portal triads did not change.

Table 4 Changes in volume density of liver components in pre-dosing and pre-haemolytic liver biopsies obtained from sheep of Experiment 2

Components of liver		Volume density ($\mu m^3/100\mu m^3$ of liver) - Mean± SE					Two-way analysis of variance	
							Between Cu ranges	Between Sheep
Hepatocytes	Intact normal	96.06 ±0.23	94.18 ±0.69	94.47 ±0.49	92.45 ±0.71	90.63 ±0.59	<0.005	NS
	Degenerating	0.15 ±0.07	0.50 ±0.18	0.41 ±0.13	0.59 ±0.11	0.90 ±0.22	<0.025	NS
	Necrotic	0.0	0.02 ±0.01	0.37 ±0.11	0.87 ±0.11	2.02 ±0.11	<0.005	NS
	Total	96.21 ±0.18	94.70 ±0.79	95.26 ±0.46	93.90 ±0.79	93.55 ±0.84	NS	NS
Space of cavities in central vein and portal triad		2.59 ±0.16	3.55 ±0.49	3.14 ±0.51	3.00 ±0.61	3.50 ±0.77	NS	NS
Fibrous tissue		1.20 ±0.06	1.75 ±0.56	1.61 ±0.26	3.10 ±0.46	2.96 ±0.17	<0.01	NS
Liver Cu (µg/g liver ww) - Mean± SE		88.4 ±7.3	305.5 ±14.38	500.4 ±13.66	616.5 ±0.86	732.5 ±38.67		
		Pre-dosing	Pre-haemolytic					

ww = wet weight; SE = standard error of mean, NS = not significant

Volume density, Nv and mean volume V of hepatocyte lysosomes in the 6 liver groups have been published (Kumaratilake and Howell, 1989a). In this (i.e. current) study, mean Vv, Nv and V of hepatocyte lysosomes of the pre-dosing and the 4 pre-haemolytic groups were pooled and regression analyses against the liver Cu concentrations were performed for each morphometric parameter. Linear regression analyses indicated that Vv (R^2 = 0.972, P<0.002), Nv (R^2 = 0.986, P<0.0007) and V (R^2 = 0.935, P<0.007) increased linearly with increasing liver Cu concentrations. These increases in Vv, Nv and V of lysosomes occurred at the rates of 0.01 $\mu m^3/100\ \mu m^3$ cytoplasm, 0.01 lysosomes/100 μm^3 cytoplasm and 0.0009 μm^3 respectively for every 1.0 µg/g wet weight rise in liver Cu (Fig. 3). However, in samples obtained on the 1st day of haemolysis, Vv did not change significantly, Nv reduced and V increased compared to those values of the pre-haemolytic group four (Table 5).

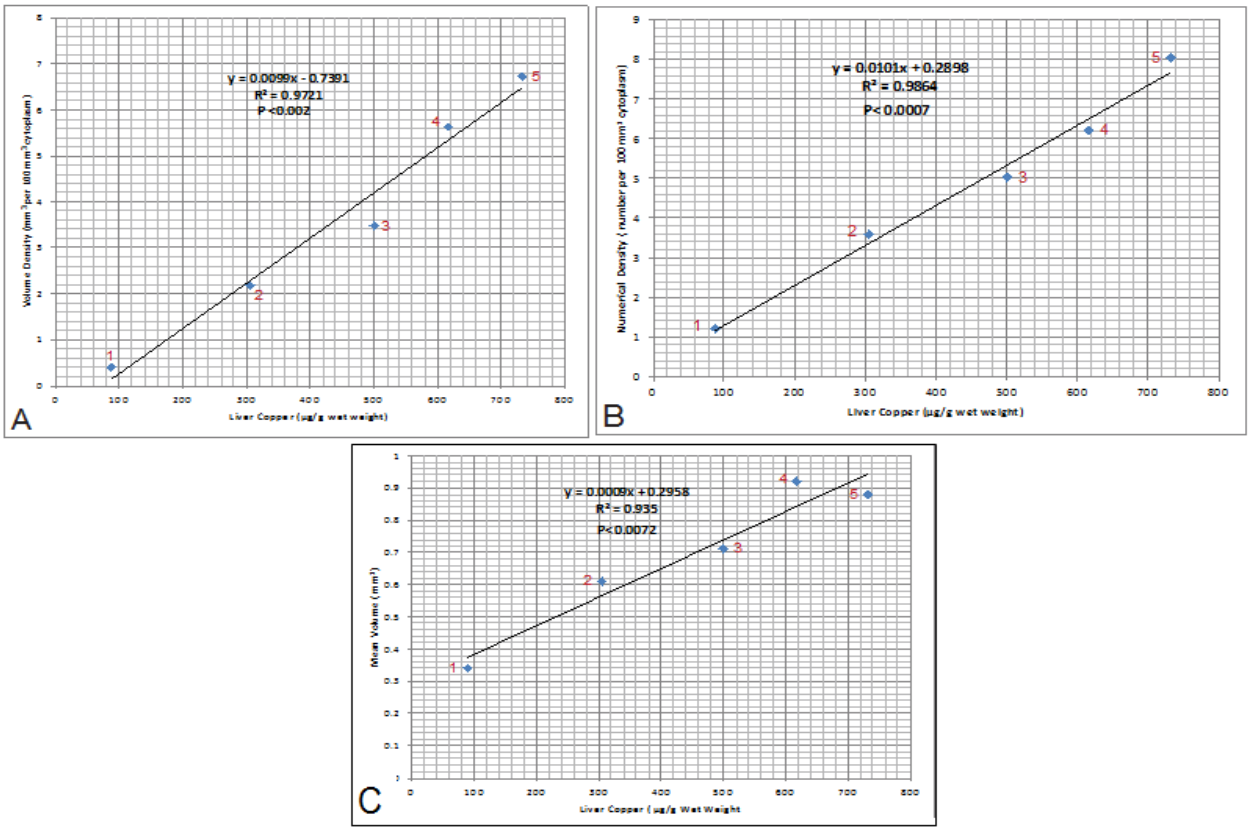

Fig. 3. Linear regression analyses of hepatocyte lysosomal mean volume density, numerical density and mean volume against mean live copper concentrations.

A - Volume density, B – Numerical density, C – Mean volume, P = Probability, 1 = pre-dosing group, 2 – 5 = prehaemolytic groups 1-4 respectively.

Table 5 Volume density, numerical density and mean volume of electron-dense hepatocyte lysosomes in liver samples obtained from sheep of pre-haemolytic group four and on first day of haemolytic crises in Experiment 2

Group	**Liver Cu range and Mean±SE of concentration (µg/g wet weight)**	**Volume desity (mm^3per $100mm^3$ cytoplasm) Mean ±SE**	**Numerical density (number per $100mm^3$ cytoplasm) Mean ±SE**	**Mean volume (mm^3) Mean±SE**
Pre-haemolytic group four (n=4)	662.6 – 827.1 732.48±38.67	6.72±0.63	8.05±0.87	0.88±0.15
First day of haemolytic crises (n = 4)	791.0 – 1199.1 (1073.49±95.55)	6.47±0.81 NS	4.43±0.56 P<0.025	1.51±0.20 P<0.05

SE = standard error of mean; n = number of sheep; P = probability; NS = not significant

Ultrastructure:
Hepatocytes contained electron dense, membrane bound lysosomes, the size of these organelles within hepatocytes varied, and the number and the size of these lysosomes varied among hepatocytes within a zone and between zones of a liver lobule. In liver samples with high Cu concentrations, individual hepatocytes packed with electron dense lysosomes were seen; some of these cells were either degenerating or necrotic. The large electron dense lysosomes in hepatocytes were nodular and appeared to have formed by the fusion of several small lysosomes. Kupffer cells also contained electron dense lysosomes and some of these cells were packed with lysosomes. In addition, macrophages of the portal triads also contained electron dense lysosomes.

Subcellular fractionation:
Copper concentrations of the fractions and the specific activity of AP in the fractions have been published (Kumaratilake and Howell, 1989b). In this (i.e. current) study, the Cu concentrations of each liver fraction of the individual sheep (i.e. in predosing, prehaemolytic and first day of haemolysis groups) were pooled and regression analyses were carried out against the liver Cu concentrations (Fig. 4). Regression analyses indicated that Cu concentrations of N, MH, ML, MI and CY fractions increased linearly at the rate of 0.3486 µg (R^2 = 0.946, P<0.0001), 0.2928 µg (R^2 = 0.951, P<0.0001), 0.1325 µg (R^2 = 0.914, P<0.0001), 0.0373 µg (R^2 = 0.79, P<0.0001) and 0.1875 µg (R^2 = 0.891, P<0.0001) respectively for every 1.0 µg rise in Cu concentration of 1 g of liver wet weight (Fig. 4).

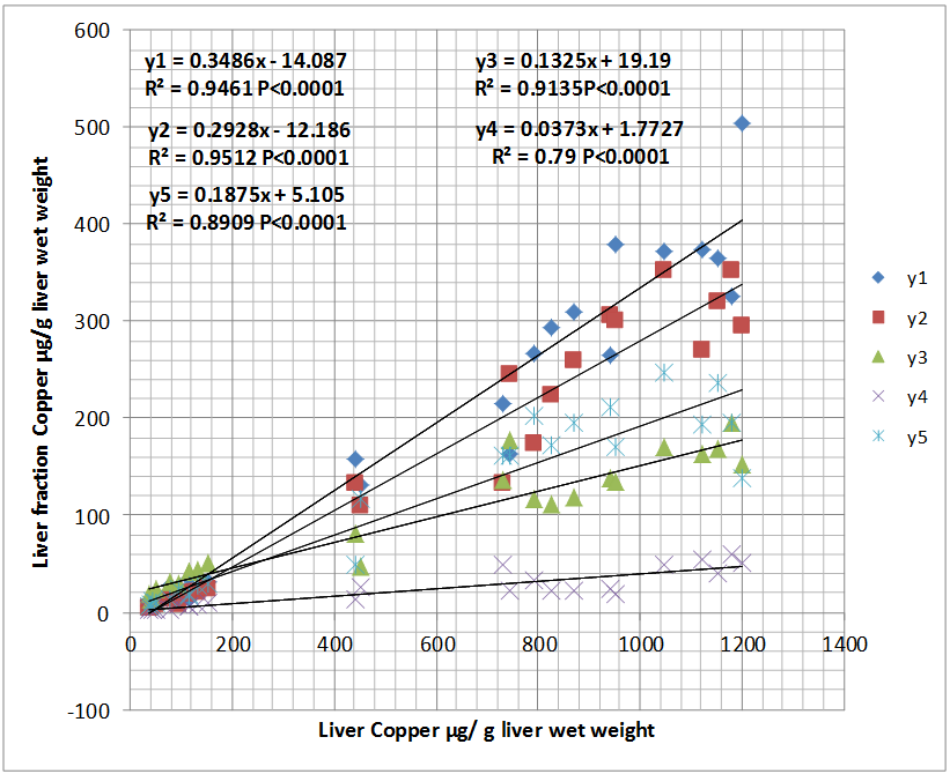

Fig. 4. Linear regression analysis of liver fraction copper concentrations against the liver copper concentrations.

Y_1 – Nuclear fraction copper
Y_2 – Mitochondrial fraction copper
Y_3 – Lysosomal fraction copper
Y_4 – Microsomal fraction copper
Y_5 – Cytosol fraction copper
Y axis = Liver fraction copper concentration
X Axis = Liver copper concentration

4. Discussion

The DPAS and RA positive granules seen in hepatocytes, Kupffer cells and macrophages of portal triads in liver samples taken from the sheep of Experiment 1 (Table 3 and Fig. 1) represent the lysosomes that are loaded with Cu (Goldfischer and Sternlieb, 1968; Ishmael et al., 1971; Kumaratilake, 1984; Gross et al., 1989). In agreement, electron microscopic investigation of the liver samples of sheep of Experiment 2 revealed that lysosomes of hepatocytes, Kupffer cells and macrophages of the portal triads were loaded with electron dense material. X-ray microanalysis investigations of the livers of Cu loaded animals have revealed that the electron dens material packed in the lysosomes is Cu (Nakanuma et al., 1979; Gross etal., 1989; Yagi et al., 1992; Bremner, 1998; Klein et al., 1998).

In test sheep of experiment 1, with oral Cu dosing, the liver Cu concentrations increased linearly at a steady rate of 10.35 µg/g liver wet weight per day (Fig. 1). The absorption of Cu from the gut should also have to occur at a linear steady rate to maintain the increase in the liver Cu concentration at a linear steady rate. Absorption of Cu in mammals is mainly from the duodenum and stomach (Lutsenko et al., 2007; van den Berghe and Klomp, 2009), thus in sheep, it should be mainly through the duodenum and the abomasum. In mice, rats, pigs and humans absorption of copper has been identified to occur via the high affinity Cu importer, CTR1 protein located on the apical membrane of the enterocytes (Petris et al., 2003; Guo et al., 2004; Molloy and Kaplan, 2009; Nose et al., 2010) and in sheep too, the CTR1 protein may be involved in the absorption of Cu. Copper in the diet (i.e. in Cu II state) has to be reduced into Cu 1 (i.e. cuprous) state before entering into enterocytes via CTR1 proteins (Georgatsou et al., 1997; Lee et al., 2002). Therefore, Cu that is in Cu II (cupric) state in Copper sulfate ($CuSO_4.5H_2O$) given to the test sheep orally, have to reduce to Cu1 before entering into enterocytes via CTR1 proteins. In humans, it has been found that at high extracellular Cu levels, CTR1 proteins are endocytosed and degraded to reduce/limit the entry of Cu into enterocytes (Petris et al., 2003; Guo et al., 2004; Molloy and Kaplan, 2009; Nose et al., 2010), thus this may be one of the mechanisms that regulates Cu homeostasis. In sheep, if the regulation of the CTR1 proteins is dependent on the Cu concentrations in the lumen of the gastrointestinal tract as in humans, the liver Cu concentrations of the test sheep would not have increased linearly at a steady rate. This indicates that either the regulation of uptake of Cu by CTR1 proteins in sheep is different to that of the human or it is similar, but absorption of Cu also occurs through an alternate pathway and this pathway could maintain the rate of absorption of Cu linearly at a steady rate, particularly when the dietary Cu concentrations are high. Findings of Zimnicka et al. (2011) indicated that anion exchangers in the apical membrane of the enterocytes play an important role in the absorption of Cu in humans. A similar mechanism may be operating in sheep, when the Cu concentrations of the diet are high.

Copper that has entered into the enterocytes leaves the cells through the basolateral membrane into the peri-cellular space in lamina propria and then passes into the rich capillary network that drains it into the hepatic portal system. Copper exporter (Cu transporting P-type ATPases), ATP7A is involved in the transport of Cu across the basolateral membrane and the genetic abnormality of this exporter leads to Cu deficiency in humans causing Menkes disease (van den Berghe and Klomp, 2009; Wang et al., 2011). In sheep too, ATP7A could be the Cu transporter in the basolateral membrane. In the test sheep, the linear increase in liver Cu concentrations at a steady rate (Fig 1) may also indicate that there is no rate limiting factor in the transport of Cu via the basolateral membrane into the portal circulation.

In test sheep, to maintain a linear increase in liver Cu concentrations at a steady rate, the uptake of Cu from the perisinusoidal spaces (i.e. Cu absorbed from the digestive tract) into the liver cells, excretion of Cu into bile by the hepatocytes and the release of Cu back into the perisinusoidal space from hepatocytes (i.e. for distribution into body) have to occur at steady rates. In agreement with this, Gooneratne (2011) did not observe a significant increase in biliary Cu excretion in sheep with increasing Cu content of the diet. However, in humans and rats, the amount of Cu excreted in the bile increased with increasing size of the hepatocyte Cu pool (Gross et al., 1989; Tao and Gitlin, 2003). These indicate that the Cu homeostatic mechanisms in hepatocytes of sheep are different to those of humans and rats.

The linear increases in Vv, Nv and V of hepatocyte lysosomes at steady rates with increasing liver Cu concentrations, indicate that as the uptake of Cu into the liver cells increased, new lysosomes were formed and they and existing lysosomes sequestered the excess Cu in hepatocytes. Thus, the volume (i.e. mean volume) of these lysosomes increased and these changes are reflected by the increase in Vv of lysosomes. All these happened at a linear steady rate (Figs. 1 and 3). Similarly, the concentrations of Cu in liver fractions increased linearly at steady rates with increasing liver Cu concentrations (Fig. 4). The increases in Cu concentrations of the N and MH fractions were associated with elevations in the specific activities of AP (i.e. a lysosomal enzyme), while the elevation of the Cu concentration of the ML fraction was not associated with an elevation of the specific activity of AP (not shown here) (Kumaratilake, 1984; Kumaratilake and Howell, 1989b). These lysosomal changes indicate that as the lysosomes sequestered Cu, they became heavy and sedimented with the MH and N fractions in order. Therefore, most of the increase in the concentrations of Cu in the N and MH fractions resulted from the sedimentation of Cu loaded heavy lysosomes with these fractions. According to the Linear regression analysis, the Cu concentration of N, MH and ML fractions increased linearly at a steady rate of 0.3486 μg, 0.2928 μg and 0.1325 μg respectively for every 1.0 μg rise in the Cu concentration of a gram of liver wet weight (Fig 4). These indicate that approximately 77% of the Cu that entered into the liver cells was taken up by the lysosomal system. Thus, the lysosomal system prevents the built up of Cu in the cytosol (i.e. in cytoplasm) into toxic levels. In sheep liver, Cu was taken up by the lysosomes of hepatocytes, Kupffer cells and macrophages of the portal triads (Fig 2). The uptake of Cu by hepatocytes and Kupffer cells is from the blood in perisinusoidal spaces. Lymph from the perisinusoidal spaces drains into the portal triads (Ohtani and Ohtani, 2008), thus the uptake of Cu by the macrophages in portal triads is likely to occur from the lymph. In test sheep of this group, the hepatic lymph nodes had large number of macrophages containing granules stained positively for Cu (not shown here) (Kumaratilake, 1984). These indicate that small part of the Cu taken up by the liver was drained

away in lymph. It should be mentioned that the major part of the Cu that was sequestered in lysosomes was in hepatocytes (Fig. 2).

The trans-Golgi network, which is a distinct part of the secretory pathway (Lutsenko et al., 2007) may have sedimented with the MI fraction, which took up about 3 - 4% of the Cu that entered into the liver cells (Fig. 4). Part of this Cu may represent the Cu that will be secreted into blood (.ie. by incorporation into ceruloplasmin). 18 – 19% of the Cu that entered into in liver cells was taken up by the cytosol fraction (Fig. 4). Copper is transported in hepatic portal vein in Cu II form bound to albumin, transcuprein or histidine; at the hepatocytes (i.e. at basolateral membrane), the Cu is reduced to Cu I and taken into the liver cell via CTR1 copper transporters. Ionic Cu I is toxic, thus in cytosol, it is bound to metallothionine and reduced glutathione, and is transported to target structures like mitochondria and trans-golgi network bound to Cu chaperones (Robert and Sarkar, 2008; de Romana et al., 2011). Copper in the MI and CY fractions represent the mobile pool of Cu. In normal hepatocytes, CTR2 Cu transporters located in lysosomal membranes may move stored Cu from the lysosomes into the cytosol to maintain the proportion of Cu in this compartment (Wang et al., 2011) at physiological levels.

The unequal distribution of Cu among the hepatocytes within a zone and between zones of liver lobules as indicated by the deposition of DPAS and RA positive granules (Table 3 and Fig. 2) is an interesting phenomenon. Kupffer cells also demonstrated the same phenomenon (Table 3 and Fig 2). The reason and the mechanism for the unequal uptake of Cu by the hepatocytes and Kupffer cells are not clear. The transport of Cu from the perisinusoidal space into hepatocytes and from hepatocytes into the perisinusoidal space are primarily via Cu transporters CTR1 and ATP7A respectively. Furthermore, the biliary excretion of Cu into the canaliculi (i.e. across the canalicular membrane) is via ATP7B (Vonk et al., 2008; van den Berghe and Klomp, 2009; Wang et al., 2011). Whether these Cu transporters or some other Cu transporting mechanism/s is/are regulating the unequal uptake of Cu among hepatocytes and Kupffer cells is not certain. The distribution of Cu between zones of a liver lobule was unequal (Table 3) and the mechanism/s that causes/cause the unequal distribution of Cu between the zones of a liver lobule is also not clear. The distribution pattern of Cu between zones of a liver lobule is also varying among sheep breeds (Ishmael et al., 1972; King and Bremner, 1979; Kumaratilake and Howell, 1987; Haywood et al., 2005). The unequal distribution of Cu among hepatocytes and among Kupffer cells within a zone , and between zones in a liver lobule may be a protective adaptations to prevent necrosis of large number of hepatocytes at the same time with increasing Cu concentrations of the liver. Furthermore, the uptake of part of the Cu that is reaching the livers of the test sheep by the Kupffer (Table 3 and Fig,2) cells may be reducing the Cu burden on hepatocytes to maintain the normal liver functions.

Individual hepatocytes that took up Cu at a higher rate than other hepatocytes were the cells that became packed with Cu loaded lysosomes and underwent degeneration and necrosis, and such cell numbers were higher in central and mid lobular zones (Tables 3 and 4; Fig. 2). Electron microscopic investigation of the Cu loaded liver samples confirmed that the hepatocytes that showed degenerative and necrotic changes were packed with electron dense lysosomes. The Vv, Nv and V changes of hepatocyte lysosomes during the pre-haemolytic period and on the first day of the HC (Fig 3 and Table 5) may represents the changes that would have occurred in the hepatocytes that became packed with Cu loaded lysosomes (Fig. 2) and underwent degeneration or necrosis. The excess Cu that entered into hepatocytes was taken up by the lysosomal system of the cell to protect

it from the harmful effects of the Cu. This Cu was taken up by the existing lysosomes as well as the new lysosomes that were being synthesized by the hepatocytes. Thus, as the Cu concentration of the hepatocytes increased, the V and Nv of hepatocyte lysosomes increased leading to an overall increase of the Vv of the organelles (Fig. 3). Liver Cu concentrations increased linearly at a steady rate during Cu dosing (Fig. 1), thus the above lysosomal measurements also increased at a linear steady rate during the prehaemolytic period (Fig. 3). Reduction in the Nv and the remaining of the Vv unchanged (Table 5) indicate that as the hepatocytes became fully packed with Cu loaded lysosomes, the ability of the cells to produce new lysosomes decreased, thus reducing the sequestration of excess Cu entering into the liver cells by the lysosomal system. This would have led to accumulation of ionic Cu I within the cell and saturation of the Cu binding metallothionine and reduced glutathione in the cytoplasm. This in turn would have caused the unbound Cu I, which is toxic to accumulate in the cytoplasm, resulting in the changes that lead to degeneration and necrosis of the hepatocytes.

The number of degenerating and necrotic hepatocytes (i.e. individual hepatocytes) increased with increasing liver Cu concentrations and the number of such cells increased sharply, in liver samples that were obtained 1-1.5 days prior to or on the second day of the HC (Tables 3 and 4). In addition, focal areas of necrotic hepatocytes were seen, particularly in the liver samples taken on the second day of the HC (Table 3). Copper from the necrotic hepatocytes (i.e. particularly from lysosomal, cytosolic and microsomal compartments) liberates into the blood in the perisinosoidal spaces. Therefore, the sharp increase in the number of necrotic hepatocytes from about 1.5 – 1 day prior to the HC (Table 3) will increase the blood Cu concentrations sharply, precipitating the HC. Elevation of plasma Cu concentrations on consecutive days immediately prior to the HC and the highest elevation of plasma Cu concentrations 1 – 2 days prior to the HC or on the first day of HC have been reported in chronic Cu poisoned sheep (Kumaratilake et al., 1981; Kumaratilake, 1984). The mechanisms causing the intravascular lysis of the red blood cells precipitating the HC is not clear.

5. Conclusion

Sheep given Cu orally as a 0.2 % solution of $CuSO_4.5H_2O$ at the rate of 10 ml/Kg body weight on 5 days of the week, the liver Cu concentrations increased linearly at a steady rate of 10.35 μg/g liver wet weight per day. This linear increase in liver Cu concentration at a steady rate indicates that in sheep, the mechanisms that regulate the absorption and excretion of Cu from the gastrointestinal tract and via the bile respectively are different to those in the humans and rats.

The uptake of Cu among hepatocytes and among Kupffer cells witin a zone and the distribution pattern of Cu in these cells between zones of a liver lobule are unequal. The mechanisms for these phenomena are not clear. Copper concentrations of N, MH, ML, MI and CY fractions increased linearly at steady rates with increasing liver Cu concentrations and 18 – 19% and 3 – 4 %, and about 77% of the Cu that entered into the liver cells were taken up by the CY and MI compartments and the lysosomal system of hepatocytes respectively. The Cu in MI and CY fractions represent the mobile pool and the lysosomal system sequestered the excess Cu that entered into the liver cells to protect the cells from the harmful effects of Cu. Increase in the concentrations of Cu in N and MH fractions resulted primarily from the sedimentation of Cu loaded heavy lysosomes with these fractions. Sequestration of Cu into the lysosomal system increased the Vv, Nv and V of lysosomes at a linear, steady rate with increasing liver Cu concentrations during the prehaemolytic period. In the

liver samples obtained on the first day of the HC, the Nv and Vv of hepatocyte lysosomes reduced and did not increase respectively. These changes in lysosomes may represent the changes that would have occurred in hepatocytes that became packed with lysosomes and underwent degeneration and necrosis. Decrease in Nv indicates, in hepatocytes packed with lysosomes, the ability to synthesize new lysosomes reduced, reducing the uptake of excess Cu into the lysosomal system. Thus, ionic Cu will accumulate in the cell after the saturation of Cu binding proteins in the cytoplasm. Free ionic Cu is toxic and may have caused the degenerative and necrotic changes seen in hepatocytes.

Copper from the necrotic hepatocytes liberates into the blood, the sharp increase in the number of necrotic hepatocytes from 1-2 days prior to the HC, may have markedly increase the blood Cu concentrations leading to the HC.

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