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CHRONIC COPPER POISONING IN SHEEP

STRUCTURAL CHANGES IN ERYTHROCYTES AND ORGANS *

By

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SØLI, N. E. and I. NAFSTAD: *Chronic copper poisoning in sheep. Structural changes in erythrocytes and organs.* Acta vet. scand. 1976, *17*, 316—327. — Four cases of experimental copper poisoning in sheep were examined. Light microscopical and ultrastructural alterations of erythrocytes were observed a few hours before a significant haemolysis was evident. Heinz body formation in otherwise unchanged red cells was the first morphological alteration observed. The Heinz bodies were predominantly membrane-attached. During the haemolytic crisis severe erythrocytic distortion, structural membrane alteration and Heinz body-containing ghost cells were observed. Erythrophagocytosis was mainly located to the RE cells of the spleen. Intrafollicular necroses were found in all histological sections from the spleen. Pathological changes in liver and kidney were comparable to those of earlier reports, comprising hepatocellular and renal tubular necrosis.

copper poisoning; Heinz bodies; erythrocyte ultrastructure; sheep.

The post-mortem findings associated with spontaneous chronic copper poisoning in sheep have been described by *Nordstoga* (1962). Changes in liver specific serum enzyme levels have been recorded as early as several weeks prior to the onset of the haemolytic crisis (*Todd & Thompson* 1963, *Ross* 1966, *Ishmael et al.* 1972). *Ishmael et al.* (1971) reported histological and histochemical changes occurring in liver before the onset of haemolysis. *Gopinath et al.* (1974) studied the histological, histochemical and functional changes occurring in the kidney of copper poisoned sheep prior and during the development of the haemolytic crisis. *Ishmael et al.* (1972) examined blood films

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during the haemolytic crisis, and numerous fragmented red cells, anisocytosis, poikilocytosis and polychromasia were present. Howell Jolly bodies and occasional normoblasts were also present. Heinz bodies were seen in supravitally stained films and could be present in up to 15 % of the red cells. Previously it has been reported (*Søli & Frøslie* in press) that 50—90 % of the erythrocytes contained Heinz bodies. The main purpose of the present study was to investigate by light and electron microscopy the structural properties of erythrocytes during the development of the haemolytic crisis. Results from histologic examination of other organs are mentioned briefly.

MATERIALS AND METHODS

The experimental animals were identical to those described by *Søli & Frøslie* (in press).

Four one-year-old sheep were dosed with 0.2 % aqueous copper sulphate solution ($CuSO_4 \cdot 5H_2O$ p.a. Merck Darmstadt), as a drench six days a week. During the first 48 days the animals were given 10 mg copper sulphate/kg body weight once a day, later on they received 20 mg/kg twice a day. They developed clinical symptoms after 7—13 weeks of dosing. Two conventionally fed sheep without copper supplement served as control animals. Blood samples were drawn at weekly intervals during the accumulation period and with a few hours' interval during the haemolytic crisis.

For determination of bilirubin in plasma the method described by *Hansen* (1970) was used. The procedure described by *Reitman & Frankel* (1957) was followed for determination of glutamic oxaloacetic transaminase (GOT).

Haemoglobin, red blood cell and white cell counts were performed by conventional methods. Smears of peripheral blood obtained from the anterior jugular vein were stained according to Wright's method for routine examination by light microscopy. The New Methylene Blue intravital staining method (*Schalm* 1965) was used for Heinz body counting. Vital staining with Brilliant cresyl blue (*Benjamin* 1961) was used for reticulocyte counting. Blood samples for electron microscopy were treated according to the method described by *Anderson* (1966) using acidic citrate dextrose(ACD) as an anticoagulant. The buffy coat was discarded, and the erythrocytes were prefixed in 2.5 %

glutaraldehyde and postfixed about 2 hrs. in cold 1 % phosphate buffered OsO_4 (pH 7.4) according to *Millonig* (1962). Dehydration was performed with acetone, and Araldite® was used as an embedding material. Sections were cut with a LKB ultramicrotome. They were double-stained with uranyl acetate and lead citrate and examined in a Siemens Elmiscope IA. Tissue samples from liver, kidney and spleen were fixed in buffered 10 % formalin and were processed by conventional paraffin embedding. Haematoxyline and eosin staining was used as routine staining. For particular purposes PAS-reaction and Perl's Prussian blue (*Culling* 1963) were used.

RESULTS

Plasma analyses

In the first part of the accumulation period the GOT activities in plasma were within normal ranges for sheep nos. 2, 3, 4, (60 to 100 Sigma-Frankel units) and slightly increased for sheep no. 1 (150 to 225 Sigma-Frankel units). Three to four weeks prior to the haemolytic crisis, however, the plasma GOT activities for all four experimental sheep were increased and ranged from 500 to 700 Sigma-Frankel units.

Bilirubin in plasma was found to be 0.18—0.25 mg/100 ml plasma in the first part of the accumulation period. Bilirubin values started increasing at the same time as plasma GOT activities, and at the onset of the crisis plasma bilirubin was found to be 0.6—1.3 mg/100 ml plasma.

Peripheral blood smears

All the experimental animals were found to have normal haematological values with respect to haemoglobin, haematocrit, red blood cell and leucocyte count during the accumulation period. Erythrocyte morphology and differential white cell count were without remarks. At the time of the haemolytic crisis the differential cell count shifted from lymphocyte dominant to neutrophilic with about 70 % neutrophils. The monocytes counted for about 20 % of the white cells in the last days prior to the haemolytic crisis. In the accumulation period Heinz bodies were only occasionally seen in up to 1 % of the erythrocytes. However, 2—4 hrs. before measurable haemoglobin could be observed in plasma, Heinz bodies were found in 50—60 % of the red cells. At

that time GSH in the erythrocytes had fallen below normal values and an increase in methaemoglobin content in the red cells was measured (*Søli & Frøslie* in press). The number of erythrocytes containing Heinz bodies increased during the crisis up to 90 % of the red cells. Heinz bodies were commonly seen free in the plasma and were assumed to originate from lysed cells.

Marked variations in red cell size and shape along with contracted and crenated cells were seen as soon as the crisis was significant. Red cell ghosts empty of haemoglobin, but retaining their Heinz bodies, were frequently seen (Fig. 1). The reticulocyte count which was low during the accumulation period was found to increase to 5—10 % after about 20 hrs. of significant haemolysis, with the occurrence of a few normoblasts in peripheral blood smears. Thrombocyte counts were not performed, but from the visual examination of blood smears the thrombocyte number was apparently increased.

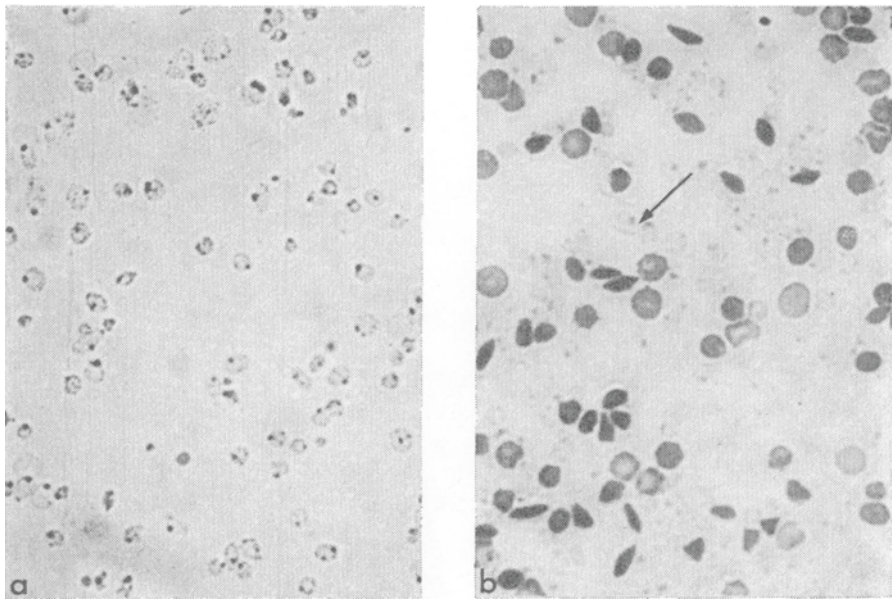

Figure 1a. Blood smear showing Heinz bodies in most erythrocytes. New Methylene Blue. × 425.

Figure 1b. Blood smear showing anisocytosis and poikilocytosis. Heinz bodies are visible in ghosts (arrow). Wright stain. × 800.

At an advanced phase of the crisis, multiple aggregates of thrombocytes, and degenerated white cells were observed. Intracellular erythrocyte fragments could be seen in some monocytes.

Electron microscopical examination

The ultrastructure of erythrocytes during the accumulation period was unremarkable. From electron microscopical examination of blood samples drawn at the haemolytic crisis, the erythrocyte population could roughly be divided into different cell types according to morphology: 1) Normal erythrocytes, indistinguishable from the erythrocytes of control animals; 2) red cells containing Heinz bodies which were distorted in shape, they were, however, found to have normal electron density of the cytoplasm suggesting no leakage of haemoglobin (Fig. 2); 3) Red cells revealing partial or complete loss of haemoglobin but retaining their Heinz bodies. Fragmentation of the cell membrane was commonly seen at this phase of haemolysis (Fig. 3). In the two first mentioned cell types, discontinuities of membranes

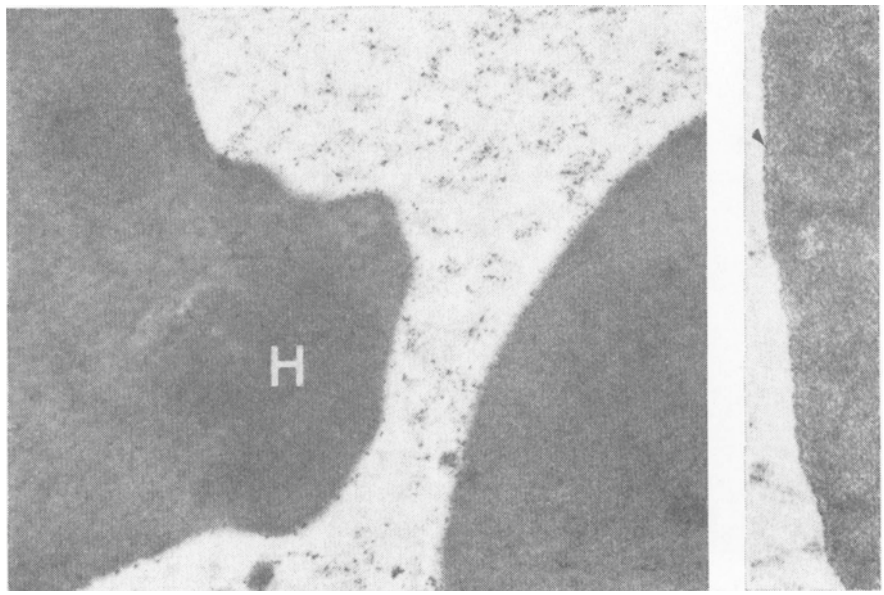

Figure 2. Electron micrograph of Heinz body located in a protrusion in an erythrocyte (H). × 40000.

Inset: Part of erythrocyte from the same animal four weeks before haemolytic crisis. Note three-laminar cell membrane (arrow). × 60000.

were not observed. The three-laminar structure normally present in red cells which was also demonstrable in the red cells of the experimental animals during the accumulation period, could not however be seen in the red cells of blood drawn at the haemolytic crisis (Fig. 2).

Several Heinz bodies were usually present in one cell. The Heinz bodies were of varying size and were almost invariable attached to the cell membrane. Fragments of erythrocytes and free Heinz bodies in the cytoplasm were frequent findings.

Post-mortem changes

Sheep no. 1 died on day 3 of the crisis, the three other experimental sheep (nos. 2, 3, 4) were killed in extremis on the second day of the crisis.

All four sheep showed similar gross lesions. Visible mucous membranes were yellow-brown in colour. The carcasses were dull yellow with yellow-brown discolouration of body fat. The abdominal cavity contained an excess of brown fluid in two animals. The livers were yellowish brown. The kidneys were swollen,

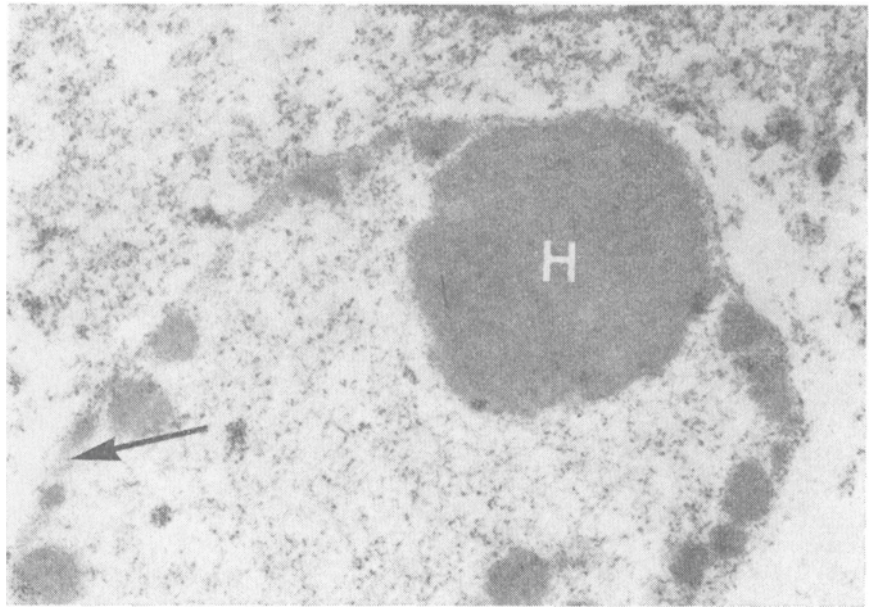

Figure 3. Electron micrograph showing ghost cell of erythrocyte containing one large (H) and several small Heinz bodies. Arrow indicates defective membrane. × 60000.

enlarged and black coloured. The bladders either contained dark coloured urine or were empty.

Histology

Livers. Livers from the sheep that died and those of the sheep that were killed showed similar lesions. Focal necroses without regular location within the lobuli were characteristic findings. The necrotic areas contained slight numbers of inflammatory cells. Bile canalicular thrombi were present, and intracellular yellow to brownish pigment granula were deposited in hepatic and Kuppfer cells. Some part of the pigment exhibited positive reaction with Perl's staining for iron. Erythrocyte remnants were located in some RE-cells.

Kidneys. Proximal tubules showed degenerative changes including cellular desquamation and vacuolation and areas with regular necrosis. The tubular lumina contained casts of granular eosinophilic material evidently containing haemoglobin. The glomeruli were moderately swollen with varying numbers of pyknotic nuclei and exudation of homogenous or granular material in the Bowman capsular space (Fig. 4). Kidney sections

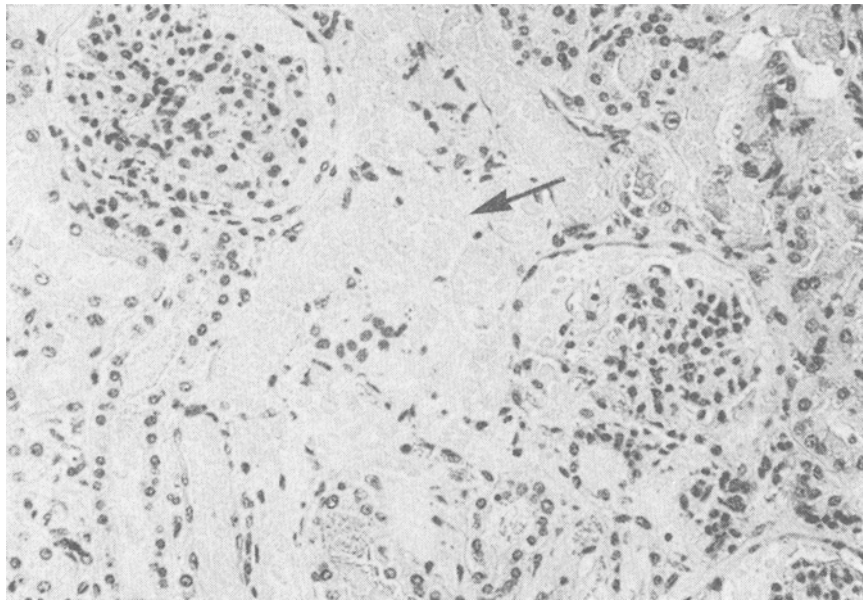

Figure 4. Kidney section. Arrow indicates tubular necrosis. Haematoxylin-eosin stain. × 200.

stained by Perl's Prussian Blue showed relatively small amounts of iron containing material in the tubular epithelial cells in experimental animals killed during the haemolytic crisis. PAS-staining of kidney sections demonstrated clearly, by visualization of the naked basal lamina of the tubular epithelium, that severe epithelial necrosis had taken place.

Spleen. Intrafollicular necroses were regularly found. The phenomenon seemed to be associated with obstruction of central follicular arteries (Fig. 5). Intracellular erythrocyte and fragmented erythrocytes were commonly seen, as were iron containing pigment granules, in phagocyting cells in the red pulp and the perifollicular area.

DISCUSSION

Changes in GOT and bilirubin were comparable to those described in earlier reports, and so were the post-mortem gross lesions as well as histopathological changes in liver and kidney (*Nordstoga* 1962, *Ishmael et al.* 1971, 1972, *Gopinath et al.* 1974).

The follicular necroses in the spleen are suggested to originate

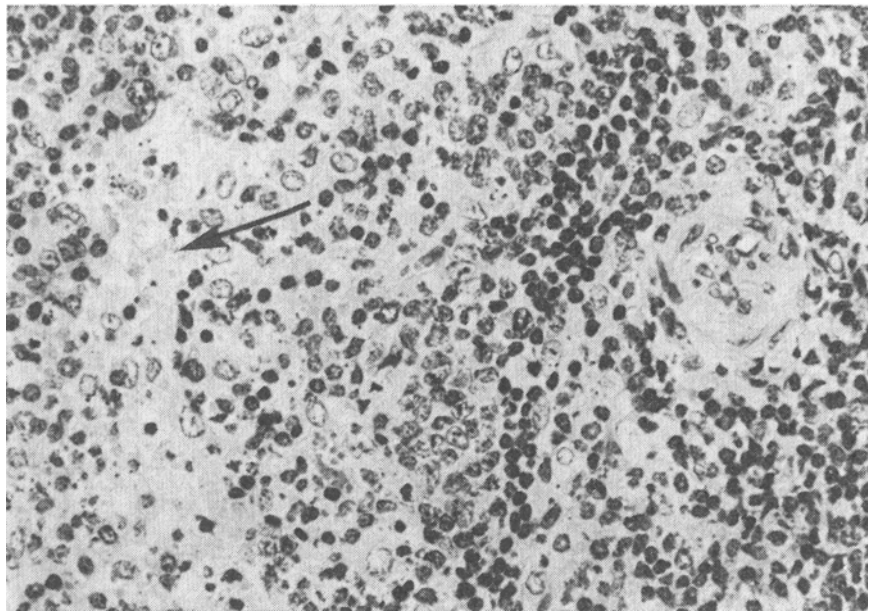

Figure 5. Spleen section showing follicular necrosis (arrow). Note obliterated artery.
Haematoxylin-eosin. × 425.

from hypoxaemia caused by obstruction of the central arteries. Whether the vascular changes are directly caused by an effect of copper on the vascular wall, or by thrombi produced by agglutination of abnormal erythrocytes cannot be explained at present. In vitro investigations on human red cells have demonstrated that Cu^{++} among other multivalent metallic cations is able to attach proteins to the cell surface and cause agglutination by Coombs serum (*Jandl & Simmons* 1957). In addition to the morphological changes reported in previous investigations, findings in the present material indicated that extravascular haemolysis must have occurred, as judged from the erythrophagocytosis in RE-cells, especially of the spleen. This finding was not unexpected, since Heinz body anaemia of different origins in other animal species is commonly associated with erythrophagocytosis in the RE-system (*Harris & Kellermeyer* 1970). For chronic copper poisoning in sheep the haemolysis, however, is of minor importance compared to the intravascular one. Most compounds that may give oxidative damage, including copper, converse reduced glutathione to its oxidized form, resulting in oxidation of haemoglobin to methaemoglobin and the denaturation and precipitation of haemoglobin in the form of intracellular Heinz bodies (*Jandl et al.* 1960, *Søli & Frøslie* in press). The cells containing Heinz bodies are destroyed at an accelerated rate in vivo (*Jandl et al.*). Three mechanisms have been proposed for the accelerated rate of destruction of red cells containing Heinz bodies: 1) decreased red cell deformability due to the mechanical presence of rigid bulky Heinz bodies resulting in either splenic sequestration (*Rifkind* 1965) or intravascular haemolysis (*Lubin & Desforges* 1972); 2) chemical and/or mechanical changes in red cell membranes caused by the attachment of Heinz bodies to the inner surfaces of cell membranes via mixed disulphide linkages (*Allen & Jandl* 1961, *Rifkind, Lessin et al.* 1972); 3) direct oxidative injury to the red cell membrane (*Kosower et al.* 1969, 1971).

Rifkind & Danon (1965) have described that Heinz bodies produced by phenylhydrazin and acetyl phenylhydrazin are formed near the center of the cell; they then rapidly migrate to the membrane and attach there. Membrane-attached Heinz bodies are able to affect the erythrocyte membrane ultrastructure as demonstrated by *Rifkind* and by *Rifkind & Danon,* as well as the chemical composition, as demonstrated by *Szelényi et al.*

(1972). The alterations included increased cholesterol and phospholipid levels in the membranes.

On the other hand, Heinz bodies in mono-methylhydrazine-incubated red cells do not migrate to the membrane, but nevertheless the cell deformability is altered by the compound. These results show that alterations in red cell deformability caused by oxidative injury may be unrelated to direct Heinz body-cell membrane interactions (*Weinstein et al.* 1975).

The type of Heinz body formation connected with copper poisoning in sheep as observed in our material is predominantly membrane-attached, similar to that of phenylhydrazin and acetylphenylhydrazin produced Heinz bodies in other animal species (*Rifkind, Rifkind & Danon*).

Furthermore the observed changes in membrane morphology may well be consistent with the alteration in membrane lipid composition described in phenylhydrazin-induced Heinz body formation, as the morphological visualization obtained by osmium fixation will most likely be influenced by changes in the membrane lipid compounds.

The results suggest that the extravascular haemolysis as well as the intravascular one is caused by the Heinz body formation and the alterations in the red cell membrane that can be found in situations with membrane-attached Heinz bodies. It cannot be excluded, however, that copper directly alter the composition of the erythrocyte membrane.

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REFERENCES

Allen, D. W. & J. H. Jandl: Oxidative hemolysis and precipitation of hemoglobin. II. Role of thiols in oxidant drug action. J. clin. Invest. 1961, *40,* 454—475.

Anderson, D. R.: Ultrastructure of normal and leukemic leukocytes in human peripheral blood. J. Ultrastruct. Res. 1966, Suppl. *9,* 1—42.

Benjamin, M. M.: Outline of Veterinary Clinical Pathology. 2nd Ed., The Iowa State University Press 1961, 43—55.

Culling, C. F. A.: Handbook of Histopathological Techniques. 2nd Ed., Butterworth & Co., London 1963, p. 308.

Gopinath, C., G. A. Hall & J. McC. Howell: The effect of chronic copper poisoning on the kidneys of sheep. Res. vet. Sci. 1974, *16*, 57—69.

Hansen, M. Aas: Kliniske leverprøver hos drøvtyggere. (Clinical liver tests in ruminants). Thesis, Oslo 1970, p. 27.

Harris, J. W. & R. W. Kellermeyer: The Red Cell. Production, Metabolism, Destruction: Normal and abnormal. Harvard University Press, Cambridge, Massachusetts 1970, p. 573.

Ishmael, J., C. Gopinath & J. McC. Howell: Experimental chronic copper toxicity in sheep. Histological and histochemical changes during the development of the lesions in the liver. Res. vet. Sci. 1971, *12*, 358—366.

Ishmael, J., C. Gopinath & J. McC. Howell: Experimental chronic copper toxicity in sheep. Biochemical and haematological studies during the development of lesions in the liver. Res. vet. Sci. 1972, *13*, 22—29.

Jandl, J. H. & R. L. Simmons: The agglutination and sensitization of red cells by metallic cations: Interactions between multivalent metals and the red-cell membrane. Brit. J. Haemat. 1957, *3*, 19—38.

Jandl, J. H., L. K. Engle & E. W. Allen: Oxidative hemolysis and precipitation of hemoglobin. I. Heinz body anemias as an acceleration of red cell aging. J. clin. Invest. 1960, *39*, 1818—1836.

Kosower, N. S., K. R. Song & E. M. Kosower: Glutathione. IV. Intracellular oxidation and membrane injury. Biochim. biophys. Acta (Amst.) 1969, *192*, 23—28.

Kosower, N. S., Y. Marikovsky, B. Werheim & D. Danon: Glutathione oxidation and bisphysical aspects of injury to human erythrocytes. J. Lab. clin. Med. 1971, *78*, 533—545.

Lessin, L. S., W. N. Jensen & P. Klug: Ultrastructure of the normal and hemoglobinopathic red blood cell membrane. Arch. intern. Med. 1972, *129*, 306—319.

Lubin, A. & J. F. Desforges: Effect of Heinz bodies on red cell deformability. Blood 1972, *39*, 658—665.

Millonig, G.: Further observation on a phosphate buffer for osmium solutions in fixation. *In* Electron Microscopy. Brese, S. S. jr., ed., 5th Int. Congr. Electron Microscopy. Vol. II, Acad. Press, New York 1962, p. 8.

Nordstoga, K.: Undersøkelse over en særlig form for kopperforgiftning hos sau. (Investigation on a special kind of copper poisoning in sheep). 9th Nord. Vet. Congr. Copenhagen 1962, Proc. 196—201.

Reitman, S. & S. Frankel: A colorimetric method for the determination of serum glutamic oxaloacetic and glutamic pyruvic transaminases. Amer. J. clin. Path. 1957, *28*, 56—63.

Rifkind, R. A.: Heinz body anemia: An ultrastructural study. II. Red cell sequestration and destruction. Blood 1965, *26*, 433—448.

Rifkind, R. A. & D. Danon: Heinz body anemia: An ultrastructural study. I. Heinz body formation. Blood 1965, *25*, 885—896.

Ross, D. B.: The diagnosis, prevention and treatment of chronic copper poisoning in housed lambs. Brit. vet. J. 1966, *122*, 279—284.

Schalm, O. W.: Veterinary Hematology. 2nd Ed., Lea & Febiger, Philadelphia 1965, 72—73.

Szelényi, J. G., J. H. Breuer, GY. Györffy, M. Hasitz, M. Horányi & S. R. Hollán: Changes in the erythrocyte membrane induced by Heinz-body formation. Haematologia 1972, *6*, 327—340.

Søli, N. E. & A. Frøslie: Chronic copper poisoning in sheep. The relationship of methaemoglobinemia to Heinz body formation and haemolysis during the terminal crisis. Acta pharmacol. (Kbh.) (In press).

Todd, J. R. & R. H. Thompson: Studies on chronic copper poisoning: II. Biochemical studies on the blood of sheep during the haemolytic crisis. Brit. vet. J. 1963, *119*, 161—173.

Weinstein, R. S., M. E. George & R. H. Steingart: Contribution of Heinz Bodies to alterations in red cell deformability. Toxicol. appl. Pharmacol. 1975, *32*, 545—558.

SAMMENDRAG

Kronisk kobber forgiftning hos sau. Strukturelle forandringer i erytrocytter og organer.

Fire tilfeller av eksperimentell kobber forgiftning hos sau er undersøkt. Lysmikroskopiske og ultrastrukturelle forandringer i erytrocytter ble observert noen timer før tydelig hemolyse var synlig. Dannelse av Heinz' legemer i ellers uforandrede erytrocytter var den første morfologiske forandring som ble observert. Heinz' legemene var hovedsakelig membranbundet.

Under den hemolyttiske krisen opptrådte stort antall av erytrocytter med abnorm fasong, „ghost"celler som inneholdt Heinz' legemer, og strukturforandring i erytrocyttmembranen. Erytrofagocytose var hovedsakelig lokalisert til RE-cellene i milten. Intrafollikulære nekroser ble funnet i alle histologiske snitt fra milten. De patologiske forandringer i lever og nyre tilsvarte de forandringer som er beskrevet i tidligere rapporter inkludert de hepatocellulære nekroser og de tubulære nekroser i nyrene.

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