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The Greater Susceptibility of North Ronaldsay Sheep Compared with Cambridge Sheep to Copper-induced Oxidative Stress, Mitochondrial Damage and Hepatic Stellate Cell Activation

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Summary

Sheep of the semi-feral North Ronaldsay (copper-sensitive) and domesticated Cambridge (copper-tolerant) breeds were compared in respect of pathological changes and protein expression in the liver as a result of excessive dietary copper. Acute mitochondrial damage and hepatic stellate cell (HSC) activation with collagen synthesis occurred in response to moderate copper overload in North Ronaldsay but not in Cambridge sheep. Mitochondrial degradative changes occurred either as ballooning degeneration and rupture with subsequent autophagic degradation or as mitochondrial matrical condensation (pyknosis). In North Ronaldsay sheep prolonged exposure to copper produced mitochondrial hyperplasia and hypertrophy, and nuclear damage with necrosis. Cytosolic isocitrate dehydrogenase (IDH), an enzyme responsive to oxidative stress, was induced in the liver of Cambridge sheep receiving a Cu-supplemented diet but was undetectable in the non-supplemented control sheep. Conversely, IDH was detected at similar levels in both control and copper-supplemented North Ronaldsay sheep, indicating a lower threshold response, and an enhanced susceptibility, to oxidative stress. "Upregulation" of mitochondrial thioredoxin-dependent peroxidase reductase (antioxidant protein-1) in the hepatic cytosol of the North Ronaldsay (but not Cambridge) sheep affirmed the increased susceptibility of the mitochondria to Cu-induced oxidative stress in this breed. Likewise the upregulation of cathepsin-D indicated increased lysosomal activity and HSC activation. The findings may be relevant to copper toxicosis in human infants.

Keywords: copper; hepatic stellate cells; isocitrate dehydrogenase; mitochondria; North Ronaldsay sheep; oxidative stress

Introduction

Sheep are intolerant of dietary copper excess due to an impaired ability to excrete copper in bile, which in turn leads to liver copper accumulation and toxicity (Soli, 1980). The pathogenesis of the disease is well documented in domesticated sheep, in which copper accumulates in the liver in concentrations of 500–1000 μg/g in the cumulative (prehaemolytic) phase without clinical consequences, although sub-lethal pathological changes occur (Ishmael *et al.*, 1971; Dincer, 1994). At a liver copper concentration of >1000 μg/g, hepatic necrosis may culminate in a haemolytic crisis with profound jaundice which is generally fatal (Ishmael *et al.*, 1971). Fibrosis, limited to a mild expansion of portal connective tissue, has been reported in some copper-poisoned domesticated sheep that recovered naturally (Ishmael *et al.*, 1971) or following cessation of copper-dosing (Gopinath and Howell, 1975).

Sheep breeds vary in their susceptibility to copper. The Merino sheep is probably the most tolerant, and the North Ronaldsay the most susceptible (Wiener *et al.*, 1978). The North Ronaldsay is a primitive sheep which occupies an

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ecological niche on the copper-impoverished foreshore of North Ronaldsay island, to which it is well adapted by its powers of copper-storage (Maclachan and Johnston, 1982). If removed to the mainland, however, the North Ronaldsay sheep is in danger of succumbing to copper toxicity (Wiener *et al.*, 1977).

Distinctive pathological changes have been reported in North Ronaldsay sheep exposed to the copper-replete conditions of the mainland. These changes are characterized by a florid pericellular fibrosis similar to that occurring in non-Wilsonian hepatic copper toxicosis of infancy and childhood (Haywood *et al.*, 2001). The similarities were confirmed in a trial in which North Ronaldsay lambs received different concentrations of dietary copper, similar to those ingested by susceptible bottle-fed infants (Haywood *et al.*, 2004).

Genetic factors undoubtedly influence the response to copper, in which hepatocytes, hepatic macrophages, Kupffer cells and hepatic stellate cells (HSCs) all play a role. However, at the cellular level it is the gene-products or proteins that determine the nature of the pathological response.

To clarify the pathogenesis of copper toxicosis in North Ronaldsay sheep (and by extension childhood copper toxicosis) the present study was designed to assess protein expression and histopathological and ultrastructural changes in copper-overloaded sheep of the North Ronaldsay (Cu-sensitive) and Cambridge (copper-tolerant) breeds. It was hoped, by these means, to identify the sites responsible for the distinctive response of the North Ronaldsay sheep to copper exposure.

The pathological changes at the ultrastructural level are reported in detail here, substantiated by some major changes in protein expression.

Materials and Methods

Animals and Experimental Procedure

Fourteen North Ronaldsay ewes aged 10 months were used, of which six received a diet of hay (Cu 5 mg/kg dry weight) and 250 g/head/day of beet (Cu 5.4 mg/kg dry weight) over a 6-month period. The remaining eight animals were given the same diet except that the beet contained Cu 15 mg/kg, a supplement of $CuSO_4$ having been added. The copper-supplemented animals were killed in pairs for examination at intervals of 1, 2, 4 and 6 months after the commencement of the experiment, to achieve liver copper concentrations similar to those produced in Cambridge sheep by Simpson *et al.* (2004). The non-supplemented animals were killed in pairs at 1, 2 and 6 months. Tissues were collected and treated as described by Simpson *et al.* (2004).

Nine Cambridge ewes were housed and fed for a period as above, except that six animals received copper-supplemented pellets containing Cu 155 mg/kg copper. Full details are given in an earlier paper in this series (Simpson *et al.*, 2004).

Metal Analysis

This was carried out as before (Simpson *et al.*, 2004).

Histology

Formalin-fixed tissue was paraffin wax-embedded and sections (5 μm) were cut and stained with haematoxylin and eosin, rhodanine for copper and reticulin van Gieson for collagen.

Electron Microscopy (EM)

Samples of liver (1 mm^3) were fixed in sodium cacodylate-buffered glutaraldehyde 2.5% (v/v) for 16–24 h, transferred to Zetterquist's veronal acetate-buffered osmium tetroxide fixative for 1 h, dehydrated with acetone and embedded in epoxy resin (Spurr, 1969). Thin sections (5 μm) from several blocks obtained from two hepatic lobes (dorsal and ventral) were stained with toluidine blue and scanned under the light microscope. Subsequently, ultrathin sections from selected blocks were stained with uranyl acetate and lead citrate, placed on metal grids, examined under a transmission electron microscope (Hitatchi H 600). Whole sections were scanned and cellular changes recorded with respect to their microanatomical location.

Proteomics

The soluble protein fractions obtained from centrifugation of homogenized liver samples were separated on 2D gels on the basis of charge (pH 5–8) and size. The gels were subsequently analysed by gel comparison software and proteins whose expression pattern had been modified as a result of copper challenge excised and subjected to in-gel tryptic digestion followed by MALDI-ToF MS (matrix-assisted laser desorption ionization–time of flight mass spectrometry). The peptide mass fingerprint of the protein obtained was searched against data bases for sufficient sequence homology to identify the protein. If this proved unsuccessful, partial sequence tags were obtained by tandem mass spectrometry, which greatly increased the

chances of identification. Full details of these techniques were given by Simpson *et al.* (2004).

Results

The appearance and behaviour of all the sheep remained normal throughout.

Cambridge Sheep

Liver copper concentrations. The results of copper analysis have already been given (Simpson *et al.*, 2004).

Liver pathology. The results are given in Table 1.

Light microscopy. After 3 months of copper supplementation (liver copper concentration 400–500 μg/g) a fine deposit of rhodanine-positive granules (at the limit of light microscope resolution) was identified in perivenular hepatocytes (zone 3), which by 4 months (liver copper concentration >1000 μg/g) had extended to all lobular zones. Histopathological changes were absent at 3 months, but at 3.5–4 months nuclear anisoploidy, apoptotic bodies, hepatocellular mitosis and a few inflammatory microfoci dispersed throughout the lobular parenchyma were present. The Kupffer cells occasionally contained brown pigment and were stained positively for copper by rhodanine.

Electron microscopy. With a moderate liver copper concentration of 400–500 μg/g hepatocyte changes varied. Dilated smooth endoplasmic reticulum (SER) cisternae were present in some hepatocytes; mitochondria were occasionally mildly swollen with increased matrical granularity, which tended to obscure the cristae. Rough endoplasmic reticulum (RER) was normal and organized into parallel arrays. Electron-dense lysosomes were present, but not in great numbers. Kupffer cells occasionally contained electron-dense particles but for the most part appeared inactive. HSCs, which were sparsely dispersed throughout the lobules and quiescent, consisted of ovoid cells containing one or two lipid (retinol) droplets at the nuclear pole.

At a liver copper concentration of 1000 μg/g and over, abundant apoptosis and apoptotic bodies were identified, both free within the sinusoids (Fig. 1) and phagocytized within hepatic parenchymal cells, some still viable and others undergoing dissolution (Fig. 2). The lesions were not confined to any one zone. Hepatocytes undergoing necrosis were identified by the breakdown of the plasma membrane and degeneration of cytoplasmic

Table 1
The pathological response of the livers of Cambridge and North Ronaldsay sheep to excesss dietary copper

Liver copper concentration (?g/g)	*Liver changes in Cambridge sheeps*	*Liver changes in North Ronaldsay sheep*
<300 (Controls)	Hepatocytes: normal Kupffer cells: activated (+) HSCs: quiescent	Hepatocytes: normal Kupffer cells: activated (+) HSCs: quiescent
400–550	Hepatocytes: mitochondria, matrix granularity; lysosomes, +; SER, proliferation and dilatation +; RER, parallel arrays Kupffer cells: activated+ HSCs: quiescent	Hepatocytes (periportal zone): mitochondria, swelling and balloon degeneration with rupture ++; SER, proliferation and dilatation +; RER, disaggregation (+); lysosmes, + Kupffer cells: activated ++ HSCs: activated + to ++, collagen synthesis
800–900	Not examined	Hepatocytes (panlobular): mitochondria, (i) balloon degeneration with rupture ++ to +++, (ii) matrical condensation (pyknosis) ++; peroxizomes, ++; lysosomes, +; SER and RER as above Kupffer cells: activated ++ HSCs: activated ++, collagen synthesis
1000–1400	Hepatocytes: mitochondria, matrix granularity and swelling +; lysosomes, +; SER, swelling + to ++; RER, disaggregation +; apoptosis, ++; necrosis, + Kupffer cells: activated + HSCs: quiescent	Hepatocytes (panlobular): mitochondria, (i) balloon degeneration with rupture +, (ii) pyknosis +, (iii) hypertrophy and pleomorphism ++ to +++; SER, ++; RER, disaggregation and degranulation + to +++; apoptosis, (+); necrosis (periportal), + to ++/+++ Kupffer cells: activated ++ to +++ HSCs: activated ++, collagen synthesis

SER, smooth endoplasmic reticulum; RER, rough endoplasmic reticulum; HSCs, hepatic stellate cells. (+) to +, negligible to minimal; ++, moderate; +++, marked.

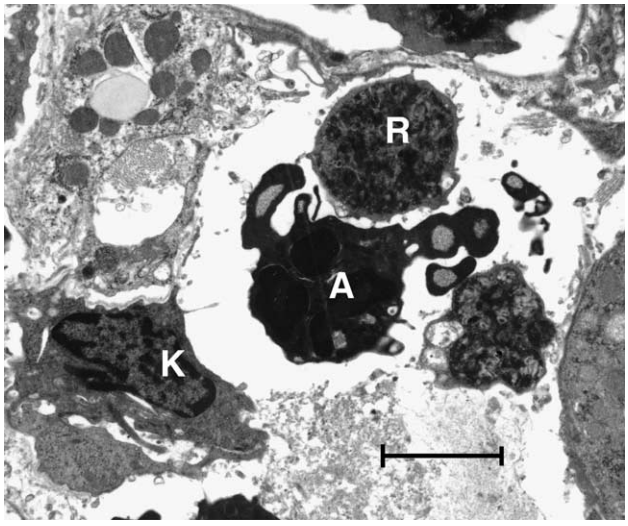


Fig. 1. Cambridge sheep liver (Cu 1017 μg/g). Apoptotic hepatocyte (A) in sinusoid showing surface protuberances (blebbing), marked condensation of cytoplasmic contents with organelle and nuclear remnants. Residual body (R) and inactive Kupffer cell (K). EM. Bar, 3 μm.

organelles within autophagic vacuoles (cytolysosomes; Fig. 2). Residual bodies containing both floccular and electron-dense undegraded material were identified (Fig. 1 and 2). Nuclear degenerative changes consisted of peripheral chromatin condensation and pyknosis.

Some Kupffer cells contained electron-dense material; they appeared unreactive and were not engaged in phagocytosis of apoptotic bodies (Fig. 2). HSCs were uniformly quiescent.

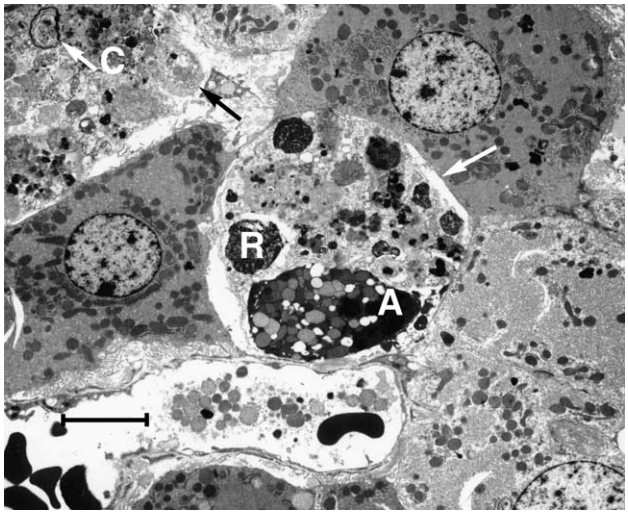


Fig. 2. Cambridge sheep liver (Cu 1017 μg/g). Viable hepatocyes juxtaposed to necrotic hepatocytes (arrows) showing dissolution of plasma membrane and cytoplasmic organelles. Apoptotic body (A), residual body (R), concentric whorled cytolysosome (C). EM. Bar, 5 μm.

North Ronaldsay Sheep

Liver copper concentrations. Two sheep that received a Cu-supplemented diet and were killed 1 month after the commencement of the experiment had liver copper concentrations (μg/g) of 528.0 and 364.8; at 2 months there was little change (491.5 and 543.9). Subsequently, however, liver copper increased strikingly (879.9 and 899.5 at 4 months and 993.9 and 1489.9 at 6 months). The results in sheep receiving the non-supplemented diet were highly variable (295.8 and 337.3 at 1 month; 187.8 and 421.8 at 2 months; 539.6 and 426.3 at 6 months), in contrast to equivalent values in Cambridge sheep (Fig. 3).

Liver pathology. The results are given in Table 1.

Light microscopy. At a moderate copper overload of 400–550 μg/g, changes were restricted to nuclear anisoploidy. At higher Cu concentrations of 800–900 μg/g, hepatic parenchymal swelling was

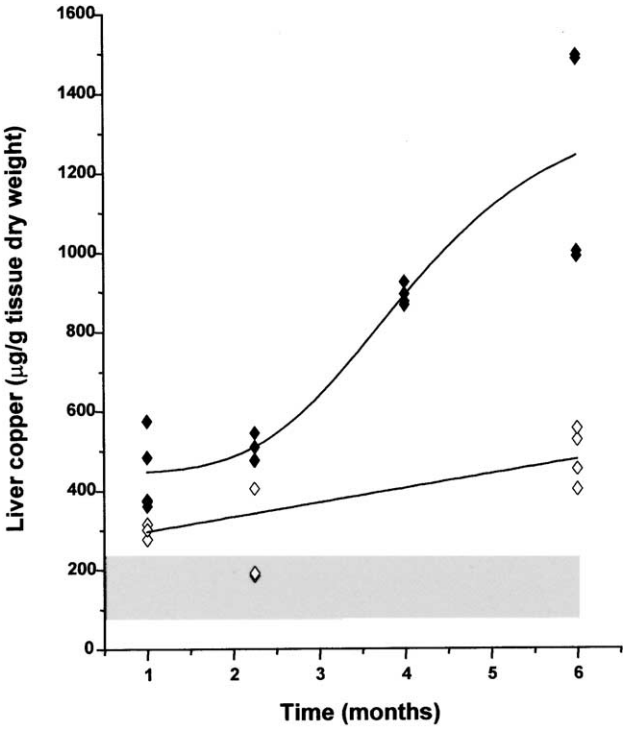


Fig. 3. Copper content in liver of normal and copper-loaded North Ronaldsay sheep. Samples from the perfused dorsal and ventral lobes of control (open symbols) or copper-loaded (closed symbols) sheep. The results are superimposed on previously published values (Simpson *et al.*, 2004) for normal liver copper values in Cambridge sheep (shaded).

noticeable in periportal zones (zone 1) of liver lobules, in which hepatocytes had a rarified and often vacuolated appearance. Kupffer cells were plump and prominent and often contained rhodanine-positive granules. At Cu concentrations of approximately 1000 μg/g and over, there was evidence of irreversible damage in many periportal hepatocytes (20%), with increased cytoplasmic eosinophilia and karyolysis. Mild inflammatory changes were represented by polymorphonuclear or mononuclear microfoci and a low grade general increase in polymorphs.

Histochemically, Cu was seen to be present in a fine granular form (at the limit of the light microscope resolution). Excess Cu, unlike that in copper-supplemented Cambridge sheep, accumulated particularly in periportal lobular zones.

Electron microscopy. The livers of control sheep with hepatic Cu concentrations of <300 μg/g did not differ from their Cambridge counterparts, consisting of unremarkable hepatocytes and Kupffer cells, and quiescent HSCs.

At moderate copper loading (400–550 μg/g), however, marked changes were identified in Ronaldsay livers, in contrast to those of the equivalent Cambridge sheep. These changes affected hepatocytes and mesenchymal cells (Kupffer cells and HSCs).

The hepatocytes occupying the periportal lobular zone (zone 1) were mildly distended with dilated SER. Their mitochondria were swollen with increased matrical granularity or radial clumping and blunting of cristae, and ballooning of mitochondria with rupture of mitochondrial membranes was common (Fig. 4). Ruptured mitochondria often contained concentric membrane whorls within their ballooned portions (Fig. 5), the apparent prelude to cytolysosome formation (Fig. 6). SER was abundant and RER was mainly arranged in parallel arrays. By contrast, hepatocytes from the intermediate and periacinar lobular zones (zones 2 and 3) contained

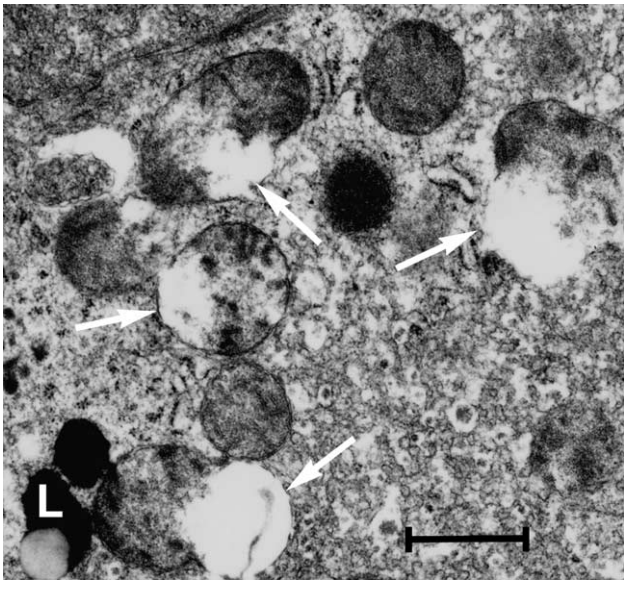


Fig. 4. North Ronaldsay sheep liver (Cu 544 μg/g). Distressed mitochondria within periportal hepatocyte showing ballooning and rupture of limiting membranes (arrows). Electron-dense lysosome (L). EM. Bar, 1 μm.

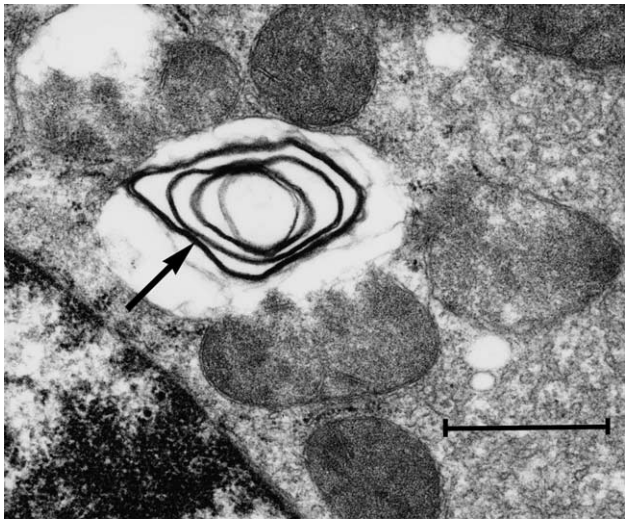


Fig. 5. North Ronaldsay sheep liver (Cu 492 μg/g). Ballooned and ruptured mitochondrion within periportal hepatocyte, including concentric whorled mitochondrial membrane (arrow). EM. Bar, 1 μm.

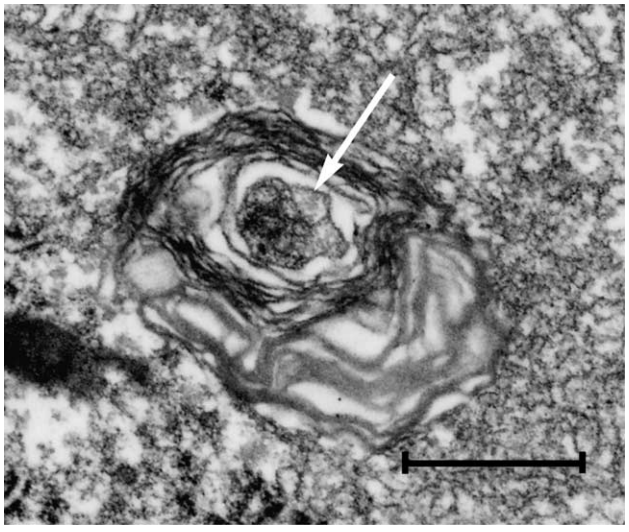


Fig. 6. North Ronaldsay sheep liver (Cu 1400 μg/g). Cytolysosme within hepatocyte; whorled membranes enclosing degraded organelle (arrow). EM. Bar, 1 μm.

mitochondria with mild swelling and increased matrical granularity.

Changes were also apparent in mesenchymal cells. Kupffer cells were prominent, with large plump nuclei, and frequently contained electron-dense spheroidal bodies, identified as copper-containing lysosomes and consistent with the rhodanine-positive granules seen under the light microscope. About 20% of HSCs appeared activated, i.e., while still possessing their identifying lipid retinoid vacuole within the cytoplasm, they also displayed an intimate relation to extracellular collagen fibrils, which appeared to be emanating from their cytoplasm. The collagen fibrils either lay in close apposition to HSCs within the space of Disse bounding the sinusoids (Fig. 7) or had penetrated between the intercellular junctions of hepatocytes (Fig. 8).

At higher copper loading (800–900 μg/g), ballooning and rupture of the mitochondria were sufficiently marked in periportal lobular hepatocytes to cause considerable rarefaction of the cytoplasm (Fig. 9). Changes were now found in the inner lobular zones (zones 2 and 3), mitochondrial swelling, balloon degeneration and rupture being common; pyknotic mitochondria could also be identified, distinguished by intense matrical condensation with electron-lucent cristae (Fig. 10a,b). Not all mitochondria, however, were degenerate and some showed evidence of replication in the form of a transecting membrane (Fig. 10b). Aggregates of spheroidal, homogeneous, electron-dense microbodies or peroxizomes were seen in the vicinity of damaged mitochondria (Fig. 10a). Small numbers of electron-dense bodies, unequivocally identified as lysosomes by the presence of single membrane, absence of cristae and inclusion of occasional fat droplets (see Fig. 4), were present within hepatocytes. Activation of Kupffer cells was characterized by their increased size, prominent nuclei and

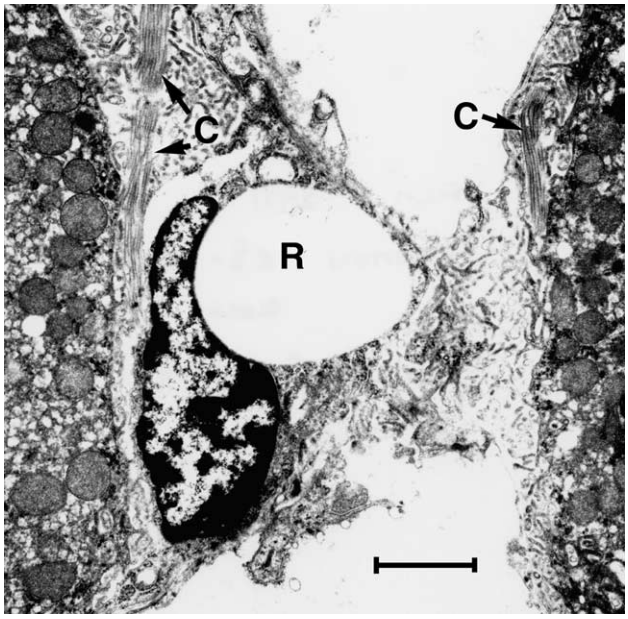


Fig. 7. North Ronaldsay sheep liver (Cu 544 μg/g). Activated hepatic stellate cell (HSC) in perisinusoidal space, juxtaposed between hepatocytes. Collagen fibrils (C) in space of Disse. Retinol vacuole (R) within HSC. EM. Bar, 2 μm.

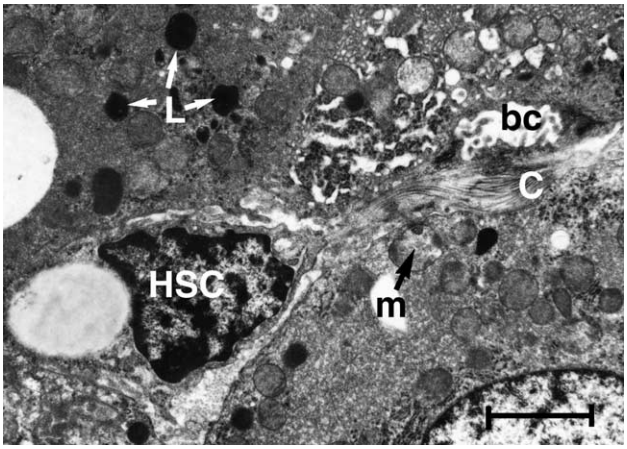


Fig. 8. North Ronaldsay sheep liver (Cu 544 μg/g). Activated HSC with collagen fibrils (C) penetrating between intercellular junctions of adjoining hepatocytes. Bile canaliculus (bc). Lysosomes (L). Mitochondria with peripheral flocculent aggregates (m). EM. Bar, 2 μm.

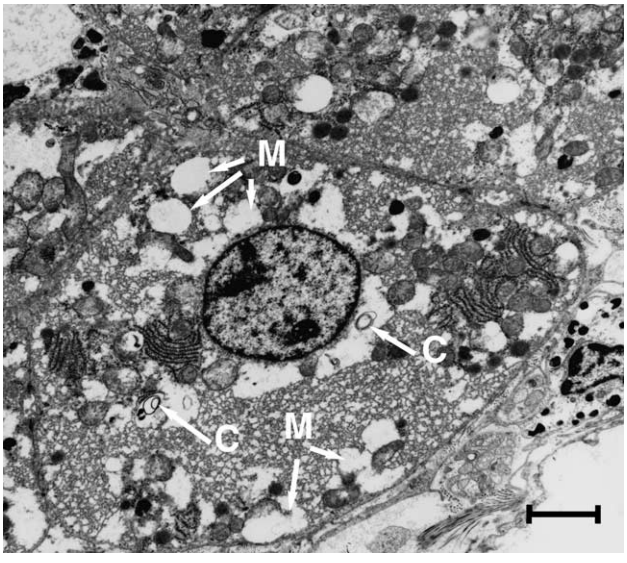


Fig. 9. North Ronaldsay sheep liver (Cu 880 μg/g). Periportal hepatocyte distended by vacuoles created by rupture of grossly ballooned mitochondria (M), including concentric whorled membranes (C). EM. Bar, 2 μm.

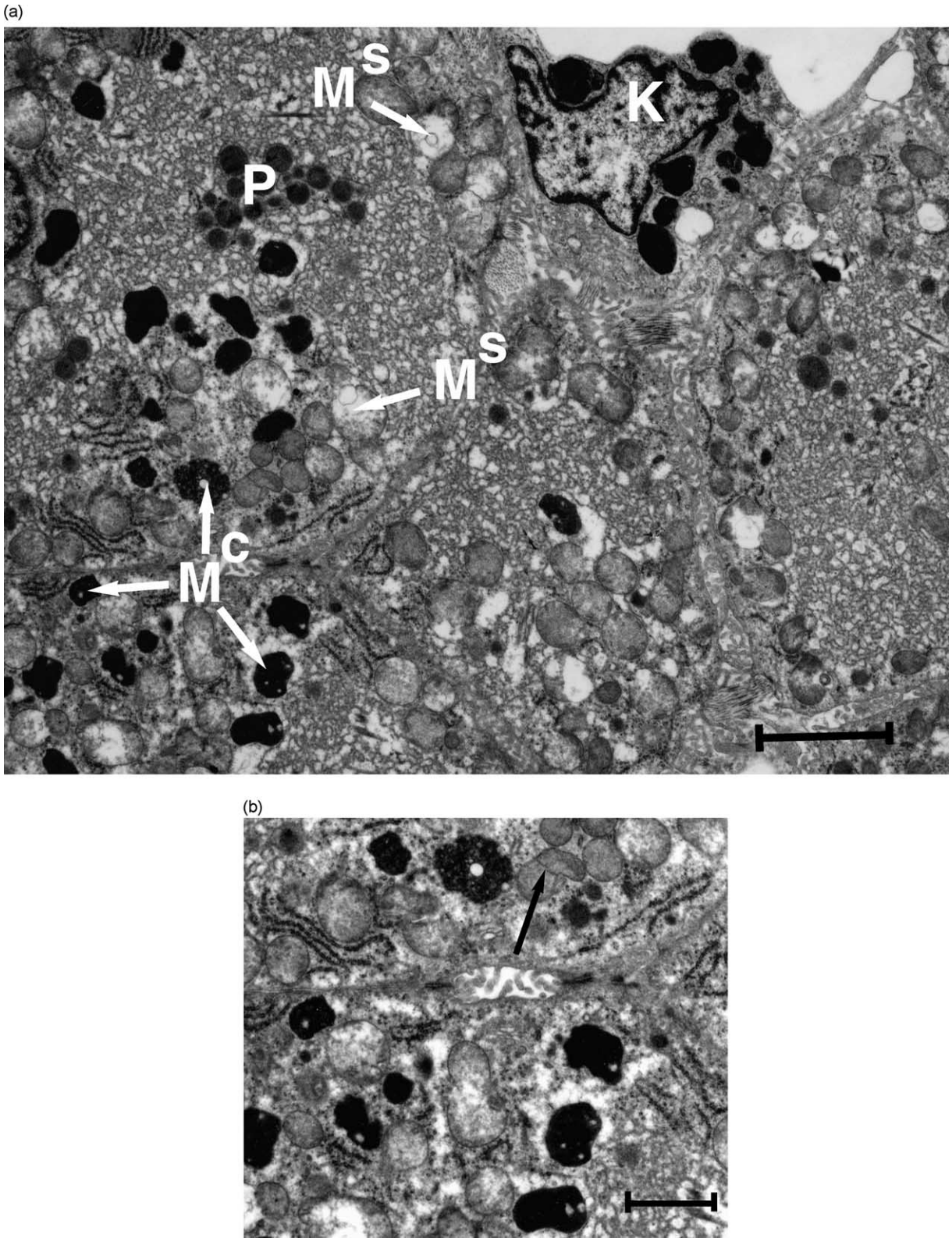


Fig. 10a,b. North Ronaldsay sheep liver (Cu 880 μg/g). (a) Hepatocytes (intermediate zone) with distressed swollen mitochondria exhibiting ballooning and rupture (M^s) and mitochondria with matrical condensation and dilated electron-lucent cristae (M^c), peroxizomes (P) and Kupffer cell (K) with electron-dense lysosomes. Hypertrophied and dilated SER. EM. Bar, 2 μm. (b) Pyknotic mitochondria with dilated electron-lucent cristae, contrasting with swollen mitochondria with granular contents; also, a dividing mitochondrion with transecting membrane (arrow). EM. Bar, 1 μm.

electron-dense lysosomes (Fig. 10a). Hepatocyte SER was hypertrophied, and RER, which showed modest degranulation, was generally arranged in parallel arrays. Occasional lipid vacuoles were identified. Increased numbers of HSCs were activated, with associated collagen synthesis, but these mesenchymal changes showed no regional predilection.

At a high liver Cu concentration (1000 μg/g) the swollen and ruptured hepatocyte mitochondria had become less evident and at a concentration of 1400 μg/g could no longer be identified. By contrast, hepatocellular mitochondria showed increased numbers (hyperplasia), hypertrophy (megamitochondria) and marked pleomorphism, often with bizarre forms. These altered mitochondria exhibited either a uniform matrix with recognizable cristae or a matrix with flocculent densities; alternatively, some mitochondria were pyknotic with an electron-dense matrix containing electron-lucent structures identified as dilated cristae (Fig. 11 and 12a,b). The SER was still dilated, but compaction with increased homogeneity was present in some hepatocytes and the RER showed some disaggregation and degranulation (Fig. 12a). Once again electron-dense bodies, unequivocally lysosomes, were relatively few in number. The nuclei of many periportal hepatocytes were often noticeably enlarged (polyploidy) and contained more than one nucleolus; the chromatin showed a tendency to dispersal.

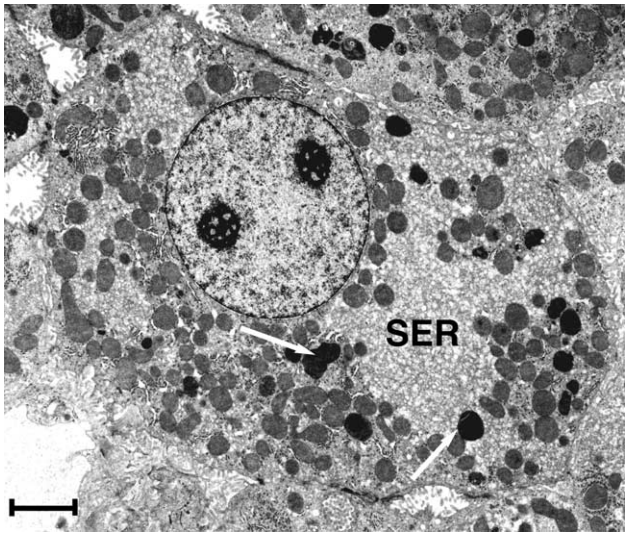


Fig. 11. North Ronaldsay sheep liver (Cu 1400 μg/g). Hepatocyte (periportal zone), enlarged (polyploid) nucleus with two nucleoli; also, numerous enlarged, frequently pleomorphic mitochondria, some of which exhibit matrical condensation (pyknosis) (arrows). SER abundant and dilated. EM. Bar, 2 μm.

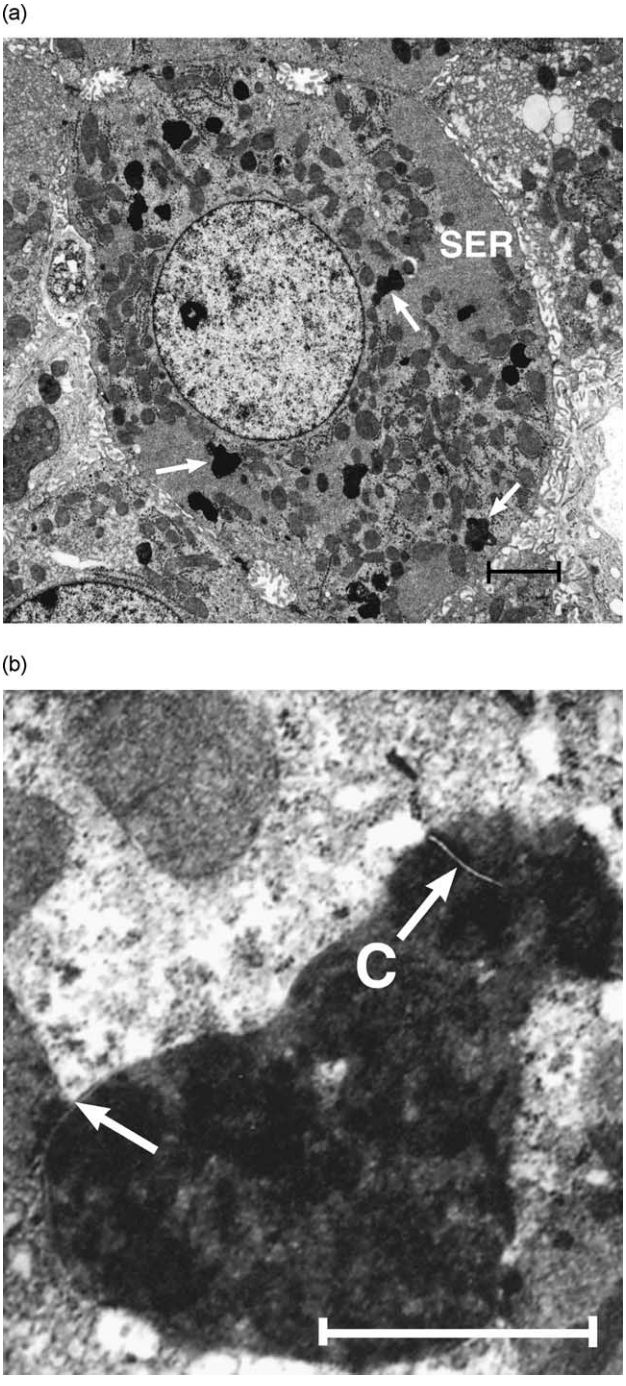
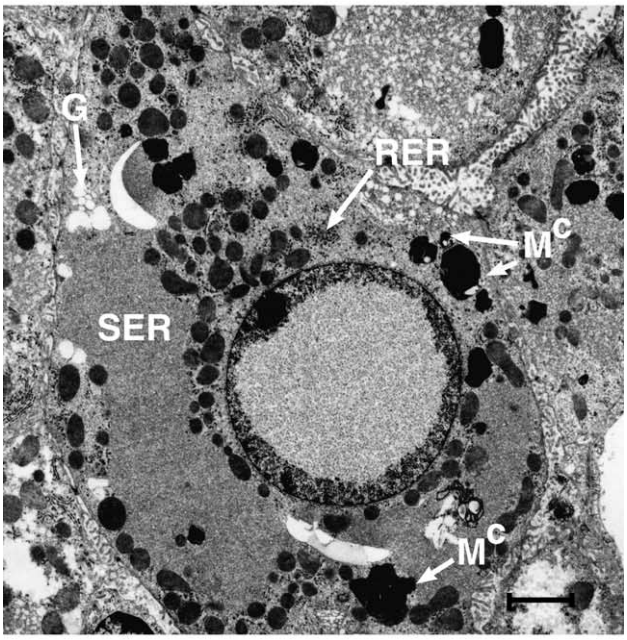


Fig. 12a,b. North Ronaldsay sheep liver (Cu 1400 μg/g). (a) Hepatocyte (periportal zone) with enlarged nucleus showing dispersal of chromatin. Cytoplasm contains large numbers of hypertrophied, pleomorphic mitochondria, some of which show intense matrical condensation (arrows), while others show flocculent densities. SER shows increased homogeneity and granularity. RER shows some disaggregation and degranulation. EM. Bar, 2 μm. (b) Pleomorphic hepatocyte mitochondrion with matrical flocculent densities, electron-lucent crista (C) and double limiting membrane (arrow). EM. Bar, 0.5 μm.

(a)



(b)

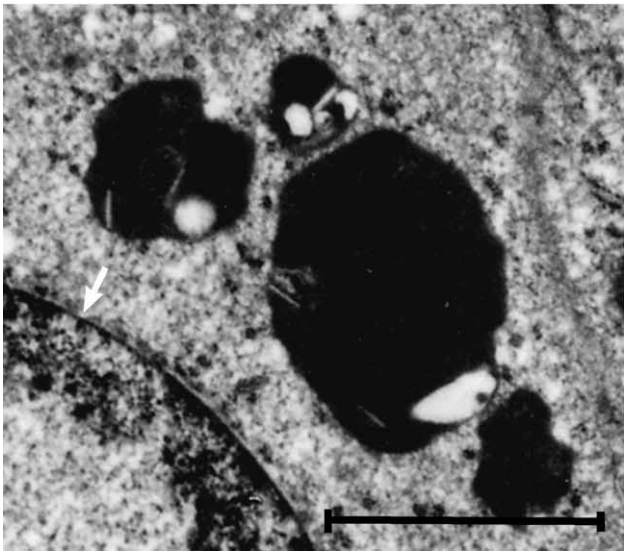


Fig. 13a,b. North Ronaldsay sheep liver (Cu 1400 μg/g). (a) Necrotic hepatocyte (periportal zone) with central nuclear chromatolysis, peripheral margination of extant chromatin and nucleolus. Mitochondria (M^c) are pleomorphic with many hypertrophied bizarre forms and show nearly uniform matrical condensation. Smooth endoplasmic reticulum (SER) uniformly homogeneous and finely granular. Rough endoplasmic reticulum (RER) shows marked disaggregation and degranulation. Golgi apparatus (G) is dilated. EM. Bar, 2 μm. (b) Pyknotic hypertrophied hepatocyte mitochondrion with intense matrical condensation and dilated electron-lucent cristae. Note discontinuity of nuclear outer membrane (arrow). EM. Bar, 0.5 μm.

Necrosis, particularly in the periportal zones of liver lobules, was now a striking feature, greatly outweighing apoptosis. This contrasted with the situation in Cambridge sheep counterparts. Hepatocytes undergoing necrosis were identified by nuclear chromatolysis (Fig. 12a), frequently culminating in a complete central depletion of chromatin, leaving a residual rim with nucleolar margination ("signet ring" appearance) (Fig. 13a). Mitochondria increasingly were of the pyknotic variety with marked dilatation of cristae (Fig. 13b). In addition, the outer nuclear membrane was incomplete or missing (Fig. 13b). The cytoplasm had become condensed, with a uniformly homogeneous and finely granular SER (Fig. 12a and 13a); the RER was disaggregated and degranulated (Fig. 13a).

Kupffer cells were much in evidence, being hypertrophied and containing electron-dense lysosomes. HSCs were frequently enlarged, with copious cytoplasm and obvious stellate prolongations that contained numerous fine filaments. The majority of the HSCs showed evidence of activation with some collagen synthesis; however, retention of the retinoid droplet, often reduced in size, suggested that full phenotypic transformation had not yet taken place.

Proteomics

Fig. 14 shows a 3dimensional representation of a protein identified by MALDI-ToF MS and peptide mass fingerprinting as cytosolic $NADP^+$-dependent isocitrate dehydrogenase (IDH). In Cambridge sheep (Simpson *et al.*, 2004), IDH was induced in response to a Cu-supplemented diet and was present in the livers of sheep with both moderate (Fig. 14) and high Cu concentrations but was not detected in the livers of control Cambridge sheep (Fig. 14). In contrast, IDH was present in the livers both control and copper-supplemented North Ronaldsay sheep (Fig. 14). Moreover Cu-loaded North Ronaldsay sheep, unlike Cambridge sheep, showed a 4–5 fold increase in mitochondrial thioredoxin-dependent peroxide reductase (antioxidant protein-1) (Fig. 15). In addition, cathepsin D was increased (Fig. 16) in 2D gels derived from cytosolic liver samples of North Ronaldsay sheep given high doses of copper.

Discussion

Both the Cambridge and North Ronaldsay sheep accumulated excessive liver Cu concentrations (1000 μg/g) from similar basal levels within the

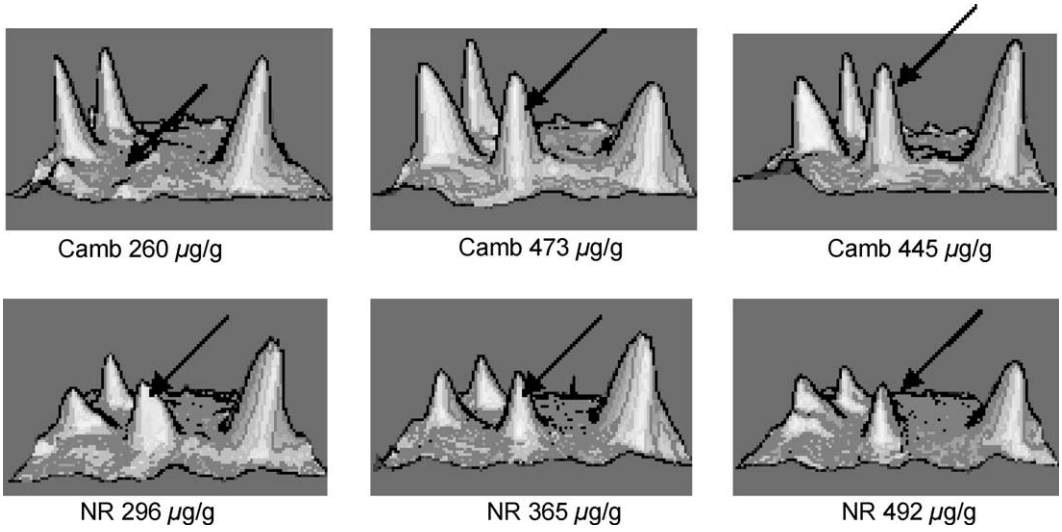


Fig. 14. The expression of cytosolic $NADP^+$-dependent isocitrate dehydrogenase in Cambridge and North Ronaldsay sheep with moderate Cu-overload. The region of the 2D gel encompassing a protein identified as cytosolic $NADP^+$-dependent isocitrate dehydrogenase by MALDI-ToF MS and peptide mass fingerprinting is shown for two Cu-loaded Cambridge (Camb) and North Ronaldsay (NR) sheep and their control counterparts (arrows). The copper concentration in each individual liver (mean of dorsal and ventral lobes), determined by ICP-MS, is indicated.

observation period of 4–6 months. However, the Cu dose level administered to the North Ronaldsays (15 mg/kg) was only one-tenth of that given to the Cambridge sheep. Moreover, the unsupplemented North Ronaldsay sheep, unlike their Cambridge counterparts, continued to accumulate copper on diets containing as little as 6 mg/kg. This reaffirmed the propensity of the North Ronaldsay sheep to accumulate potentially toxic concentrations of copper when removed from their Cu-impoverished habitat.

Early in the trial, moderate copper loading (400–550 μg/g) provoked marked changes in the livers of the North Ronaldsay but not Cambridge sheep. Severe damage was incurred by the hepatocyte mitochondria of the North Ronaldsay sheep, accompanied by reactive changes in local mesenchymal cells, including HSC activation and collagen

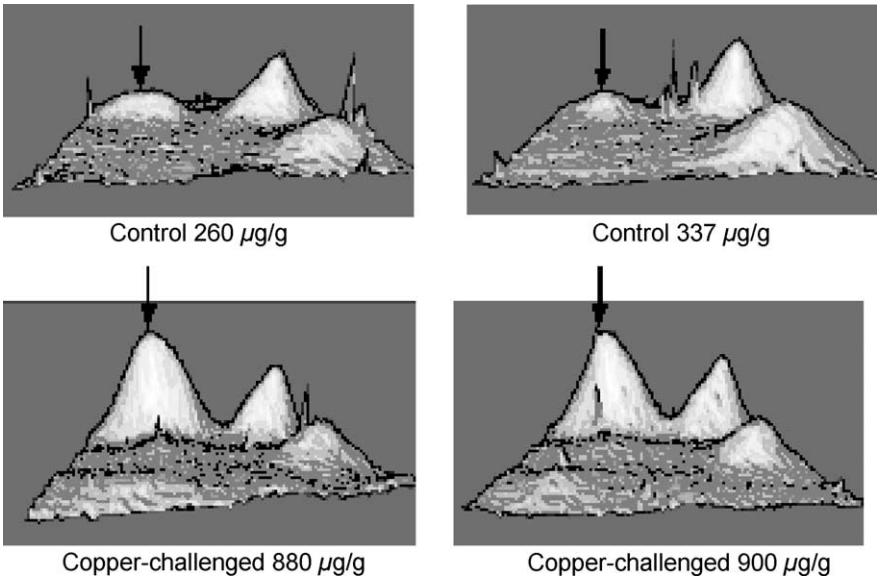


Fig. 15. The expression of thioredoxin-dependent peroxide reductase in control and copper-supplemented North Ronaldsay sheep. The regions of the 2D gels encompassing a protein identified as thioredoxin-dependent peroxide reductase by MALDI-ToF MS and peptide mass fingerprinting is shown for control and Cu-challenged North Ronaldsay sheep (arrows). The copper concentration in each individual liver (mean of dorsal and ventral lobes), determined by ICP-MS, is indicated.

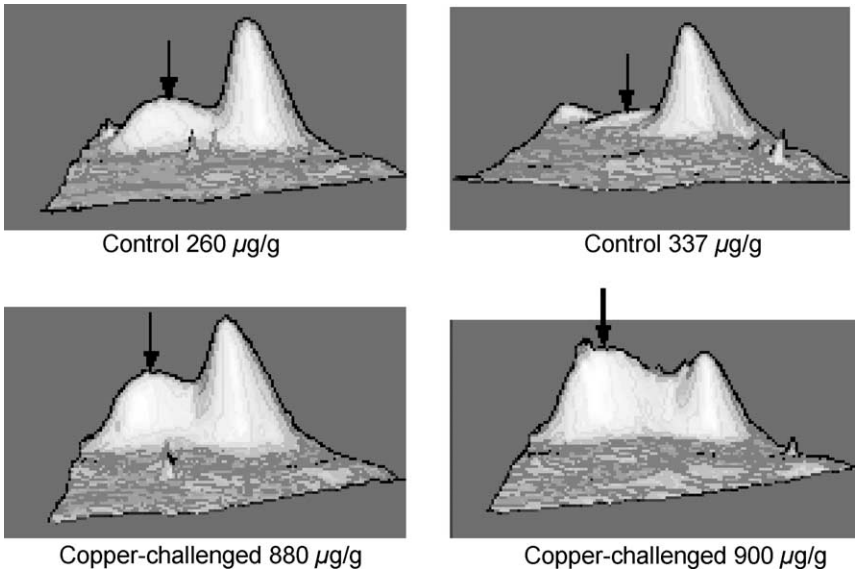


Fig. 16. The expression of cathepsin D in control and copper-supplemented North Ronaldsay sheep. The regions of the 2D gels encompassing a protein identified as cathepsin D by MALDI-ToF MS and peptide mass fingerprinting is shown for control and copper-challenged North Ronaldsay sheep (arrows). The copper concentration in each individual liver (mean of dorsal and ventral lobes), determined by ICP-MS, is indicated.

synthesis. Established differences in response were exacerbated in copper overload (>1000 μg/g) in that apoptosis preceded and overshadowed necrosis in the Cambridge livers, whereas in the North Ronaldsays necrosis was dominant. Laying down of collagen took place progressively along sinusoids and between intercellular junctions, presaging the pericellular fibrosis that characterizes the established disease in North Ronaldsay sheep, and which impairs nutrient exchanges and disrupts intercellular communications (Haywood *et al.*, 2001, 2004).

Copper poisoning has been studied in domesticated breeds of sheep, at the light microscope level (Ishmael *et al.*, 1971; Dincer, 1994) and ultrastructurally (King and Bremner, 1979). The pathological changes in Cambridge sheep livers accorded with these earlier reports. King and Bremner (1979) highlighted the incidence of apoptosis and established the importance of autophagy in contributing to cell death (necrosis). However, although limited expansion of the portal tracts by fibrous tissue was reported by these authors and by Gopinath and Howell (1975), the state of the HSCs in copper toxicosis was not examined.

Ishmael *et al.* (1971) reported initial deposition of histochemical copper in centrilobular zone 3 in copper-loaded Clun Forest lambs, a finding repeated in Merino sheep by Kumarilatike and Howell (1986). However, King and Bremner (1979) reported the converse in copper-loaded Finn-Dorset×Suffolk ewes. The findings from our own studies have been equally conflicting. Thus, copper deposition in the Cambridge trial followed the pattern established by Ishmael *et al.* (1971), whereas excess copper accumulation in the North Ronaldsays had more in common with the findings of King and Bremner (1979); certainly the onset and severity of lesions were particularly marked in the periportal zones.

Variations in the microanatomical distribution of lesions in copper-storage diseases were summarized by Fuentealba *et al.* (1989b), who found some relation with intralobular copper retention. Generally, there would seem to be a relationship to metabolic zonation of liver function along a vascular gradient of oxygen tension, which is highest periportally.

The histochemical demonstration of copper, however, has limited usefulness. At the cellular level, X-ray electron microanalysis is one possible alternative (Fuentealba *et al.*, 1989a; Haywood *et al.*, 1996).

Copper, which is a transition metal that can exist in more than one oxidation state, is highly reactive and participates in many enzyme-induced electron transfer reactions. However, if present in excess it readily catalyses the conversion of hydrogen peroxide to the hydroxyl radical, which causes lipid peroxidation of cell membranes, cleavage of nucleic acids and chemical modification of proteins. To counteract this, antioxidant systems have evolved, e.g., the glutathione system, specific intramitochondrial antoxidant systems, and catalase within the peroxizomes.

Induction of cytosolic IDH was indicative of an oxidant challenge ameliorated by upregulation of glutathione synthetase and glutathione S-transferase mu in response to moderate copper-dosing in Cambridge sheep. At higher copper overload only IDH was detectable and oxidative stress-induced injury was apparent (Simpson *et al.*, 2004). In North Ronaldsay sheep, IDH (controlling the delivery of glutathione precursors) was expressed in the livers of both control and copper-supplemented sheep, in contrast to the Cambridge sheep, in which IDH was induced solely in response to copper-supplementation. This implies that the susceptibility to oxidative stress greater in the North Ronaldsay breed.

Furthermore the disparities with regard to cell death between the two breeds at high copper overload suggest that oxidative stress is of sufficient magnitude in North Ronaldsay sheep to inhibit caspase-induced apoptosis; in addition, the prevalence of hepatic peroxisomes indicates heightened oxidative stress.

The severe mitochondrial distress culminating in membrane rupture recorded in the Cu-loaded North Ronaldsay sheep liver (copper 400–900 μg/g) in this study reaffirms the high susceptibility of this breed. Such severe injury is unknown in other copper-associated diseases in man and animals. The disruption of mitochondrial membrane integrity was supported by the increase in mitochondrial thioredoxin-dependent peroxide reductase in 2D gels derived from cytosolic liver samples of Cu-overloaded North Ronaldsay sheep. This reductase is a component of the mitochondrial peroxide-destroying system, responsive to oxidant challenge and not expected to be found outside the mitochondria (Rabilloud *et al.*, 2001).

A second distinct type of mitochondrial damage identified was matrical condensation, which varied from flocculent deposits of electron-dense material to complete mitochondrial matrical electron-density. These pyknotic mitochondria were often bizarre in shape.

Mitochondrial oxidant injury has been reported in Cu-overloaded rats (Sokol *et al.*, 1993), Wilson's disease and Bedlington terrier copper toxicosis (Sokol *et al.*, 1994). Accompanying morphological damage varies considerably, and in both rats (Fuentealba *et al.*, 1989a) and Bedlington terriers (Haywood *et al.*, 1996), it is at best muted, consisting of mild swelling and increased matrix granularity with a few matrical crystalline inclusions. Such damage resembles that recorded in the Cambridge sheep in this study. Mitochondrial abnormalities have been recorded in the early stages of Wilson's disease (Sternlieb, 1992) and Long-Evans cinnamon (LEC) coloured rats, the genetic homologue of Wilson's disease (Sternlieb *et al.*, 1995). Such changes bear a striking resemblance to the matrical condensation found in North Ronaldsay hepatic mitochondria in this study.

Ghadially (1977) found that mitochondrial involution or dissolution broadly took one of two forms, namely, either swelling (balloon degeneration) or condensation of the matrix with increased electron-density, termed mitochondrial pyknosis. Both forms were identified in Ronaldsay copper toxicosis (RCT) in this study, whereas in Wilson's disease and LEC rats mitochondrial condensation was the most prevalent finding. Failure to transport cytosolic glutathione into mitochondria, is the explanation for the mitochondrial oxidative injury occurring in alcoholic liver disease (Fernandez–Checa *et al.*, 1997) and may have been important in our study. Alternatively, unregulated intramitochondrial copper may provoke the generation of OH in a Haber-Weiss Fenton type reaction. Copper, an integral component of cytochrome oxidase located in the intramitochondrial space and imported by a protein COX17 (Glerum *et al.*, 1996) is tightly bound to this site and an unlikely candidate. The yeast mitochondrion, however, contains a dynamic pool of non-protein bound copper, responsive to cellular changes in copper concentration and localized within the mitochondrial matrix as a soluble, anionic, low molecular weight complex (Cobine *et al.*, 2004). If such a pool is a feature of mammalian liver mitochondria, it might explain the severe oxidant damage recorded in the North Ronaldsay sheep livers.

It is puzzling that the acute mitochondrial damage recorded in the early phases of copper accumulation in North Ronaldsay sheep subsided following persistent copper loading. In Wilson's disease, however, a parallel finding was reported by Sternlieb (1992), who stated "paradoxically these mitochondrial abnormalities tend to disappear…. as the disease progresses to cirrhosis". Possibly, there is an eventual adaptation (or selection) of surviving mitochondria to the adverse conditions, a suggestion supported by the pronounced mitochondrial hypertrophy and pleomorphism exhibited.

Haywood *et al.* (2001, 2004) reported that copper-induced fibrosis in North Ronaldsay sheep results from HSC activation, simulating the pericellular fibrosis that characterizes infantile copper toxicosis. Activated HSCs provide the source of modified extracellular matrix (ECM), leading to

collagen fibril formation following liver injury. HSCs, which normally store vitamin A (retinol), change from their quiescent state to an activated form, in which a sequence of responses occurs, leading to proliferation and fibrogenesis (Friedman and Eng, 2000). Initiation is provoked by reactive oxygen intermediates, amplified by depletion of antioxidants, causing rapid changes in gene expression (collagen 1 gene) that render the cells responsive to cytokines. These, notably TGF-β1 secreted by Kupffer cells and others, represent the dominant signal for ECM production and fibrogenesis. In this study acute mitochondrial oxidant damage, Kupffer cell activation and coincident HSC activation (affirmed by upregulation of cathepsin D precursor [Kristensen *et al.*, 2000]), conforms to this line of reasoning.

In conclusion, the present investigation confirms the idiosyncratic pathological response of North Ronaldsay sheep to excess dietary copper. Furthermore, it extends knowledge of the putative pathogenesis, in respect of oxidant injury, in which all the evidence points to mitochondrial vulnerability as a major contributory factor. This may be a consequence of failure of antioxidant delivery systems or, alternatively, of increased production of reactive oxygen species by uncontrolled mitochondrial copper influx.

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