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Location of heat shock protein 70 and metallothionein immunoreactivity associated with copper deficiency in the CNS of lambs

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Summary

The localizations of metallothionein I and II (MT), a small molecular weight heavy metal binding proteins, and 70-kDa heat shock protein (Hsp70) were investigated by immunohistochemical techniques in brains of lambs that had been injured by congenital copper deficiency. The results were compared with those obtained from control lambs. The morphological findings of the congenital copper deficiency in the central nervous system (CNS) were recorded. The amount of copper in the brain and liver of the lambs and feed of breeding ewes and soil was also assayed by atomic absorption spectrophotometry. The amount of copper in the brain, liver, soil and feed were low. Immunohistochemically, MT and Hsp70 expressions were found to be markedly increased in the CNS of congenital copper deficient lambs compared with control lambs. MT immunoreactivity was prominently found in the astrocytes while strong Hsp70 labelling was in both astrocytes and neurons in the cerebrum, cerebellum, thalamus/hypothalamus and medulla oblongata. Immunohistochemical labelling for both MT and Hsp70 was also seen in the pia mater, ependymal cells and choroid plexi. Present results suggest that the elevated expressions of MT and Hsp70 in astrocytes and neurons are possibly indicating that they are less susceptible to the consequences of cell stress factors and could be exploited to increase selectively their survival in copper deficiency.

Keywords: copper deficiency, heat shock protein 70, lamb; metallothionein

Neurologic diseases associated with copper deficiency in lambs include swayback (enzootic ataxia) and cerebral oedema (1, 10). Fetal copper deficiencies result in bilaterally symmetrical lesions in cerebral white matter that range from small focal changes to extensive lesions that can result in porencephaly (1, 18).

Functionally, the 70-kDa heat shock protein (Hsp70) family is a group of chaperones that assist in folding, transporting, and assembly of proteins in the cytoplasm, mitochondria, and endoplasmic reticulum (9). Hsp70 induction has been shown to take place in the central nervous system (CNS) in response to a number of stressors, such as hyperglycemic cerebral ischemia (13), and hypoxic brain injury (6). This is the first report depicting changes in Hsp70 expression in the CNS due to congenital copper deficiency in lambs.

Metallothioneins (MT) are a group of cysteine-rich, inducible, metal-containing (copper, zinc), low molecular weight proteins, expressed in most tissues and organs. MT occurs in different isomeric forms, mainly MT I and II (2). The main functions of MT are copper (Cu) and zinc (Zn) homeostasis, detoxification of heavy metals, metal transport and scavenging free radicals (5).

It is important to improve the knowledge about the regional and cellular location of MT in the mammalian brain since this information may help to interpret the neural function of MT. MT immunoreactivity also occurs after ischemic and traumatic brain injury (19). MT induction has been reported in copper-enhanced sheep brains (4). However, there are no reports concerning MT location in the brains of lambs with congenital copper deficiency.

The aim of this study was to investigate the morphological findings of copper deficiency in lambs and correlate these findings with MT and Hsp70 expressions.

Material and methods

Animals and farms. The animals were obtained from 12 farms with about 1600 pregnant ewes at different locations in the Kars region of Turkey between 2005-2006. The signs generally appeared at birth (congenital type). The lambs were born dead, died immediately after birth or were underweight. The most obvious clinical signs of the disease in the new born lambs were an inability to raise unaided, posterior un-coordination seen as hind limb weakness, posterior wobbling, unsteady gait, and/or muscular tremors and blindness. The clinical incidence of disease was about 20% n in the affected 12 farms. A total of 20 lambs were collected from the farms for necropsy and histopathology investigations. They consisted of still born (6 cases), or dead immediately following delivery (4 cases), or dead within 7 days of delivery (10 cases), and 7 lambs that had died from 0-7 days of age from various other causes and did not have brain lesions were used as controls.

Virology. Blood and tissue samples were collected from the ewes and lambs for a panel of tests including ELISA and PCR analysis for bluetongue virus, border disease virus, and Akabane virus.

Metal analysis. Liver and brain samples from the affected lambs and ration and soil samples were taken from all farms and pastures where the ewes had grazed. The samples were dried to a constant weight and homogenized. They were then acid digested with a mixture of nitric, perchloric and sulphuric acids and the copper content was measured by atomic absorption spectrometry.

Necropsy and histopathology. A complete necropsy was performed on all animals. Following the macroscopical evaluation tissue samples were taken from the cerebrum, cerebellum, thalamus/hypothalamus, medulla oblongata and spinal cord and fixed in 10% buffered formalin. Selected blocks were processed and embedded in paraffin wax. Sections (8-10 µm) were then cut from each block for histological examination and immunohistochemical labeling.

Immunohistochemistry. Sections from all the tissue samples were cut 5-7 µm and processed for immunohistochemical examination by a standard streptavidin-biotin--peroxidase method. Mouse monoclonal antibodies that react with human and rabbit MT-I and MT-II. (Clone: E9, Dako Corporation, CA, Carpinteria, USA, code M0639) and Hsp70 (Clone: BRM-22, Sigma) were used at dilutions of 1 : 600 and 1 : 1000 for 60 min, respectively. Negative control tissue sections were incubated with normal rabbit serum.

Analysis of immunostaining results. The percentage of the total area of the MT and Hsp70 positive cells was assessed semi-quantitatively under a light microscope with a 10 × ocular with grids and a 40 × objective. The labeling intensity in a given cellular compartment was assessed on a semi-quantitative basis. The findings were categorized as follows: (0) no positively staining cells; (1; weak) 1-5%; (2; moderate) 6-10%; (3; marked) > 11% of cells positive.

Statistical analysis. Statistical analysis of the results was performed using Mann Whitney and Kruskal-Wallis tests. $P < 0.01$ was considered the limit of statistical significance. All analyses were performed by Minitab 12 statistical package (Minitab Inc, Pennsylvania, USA).

Results and discussion

PCR and ELISA analysis of blood and tissue samples collected from the ewes and lambs revealed that all the samples were negative for the bluetongue virus, border disease virus, and Akabane virus.

The copper content in liver and brain of the lambs was very low (5.66 ± 0.72; 4.34 ± 0.41 ppm), respectively. The copper content in the soil samples and diet of breeding ewes was also low (1.18 ± 0.32; 0.3 ± 0.07 ppm), respectively. The concentrations of copper in the liver, brain, soil and feed are shown in tab. 1.

Grossly evident cerebral changes were present in all cases. There were marked fluid-filled cavitations in the cerebral hemispheres (fig. 1 A). Transverse sections of the cerebral hemispheres revealed clear fluid resembling normal cerebrospinal fluid. The lesions affected the cerebral white matter bilaterally and varied from small focal lesions to extensive cavitations (fig. 1 B). These cavitations were, in most cases, connected to lateral ventricles. Septum pellicidum was not present. A mild reduction in size of the cerebellum was present in 3 cases (case Nos. 17-19) and a marked reduction in size was present in 1 case (case No. 20). The cerebellum was of normal appearance in the remaining 16 cases.

Tab. 1. Brain and liver copper concentrations (ppm, dry matter) of studied lambs ($\bar{x} \pm Sd$)

Samples	Control lambs	Copper deficient lambs
Liver	28.55 ± 7.37	5.66 ± 0.72
Brain	13.12 ± 1.07	4.34 ± 0.41
Ration	–	0.30 ± 0.07
Soil	–	1.18 ± 0.32

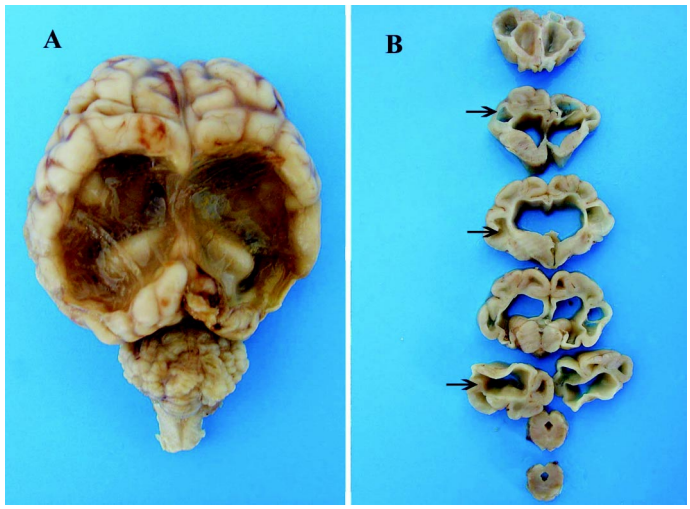

Fig. 1. (A) Brain of a still born lamb affected with copper deficiency. Note marked fluid-filled cavitations of the cerebral hemispheres. (B) Transverse section of the brain of a four days old lamb affected with copper deficiency. Note bilateral cyst formation (arrow) of the subcortical white matter and dilated lateral ventricles

Severe cerebral lesions were present in all copper deficient lambs. The lesions included degeneration and cyst formation and porencephaly within the cerebral white matter. These were the most prominent features. The remaining white matter showed extensive losses of myelinated nerve fibers which were replaced by a loose glial meshwork of reactive astrocytes and their processes (fig. 2 A and B). These cavities had sharply demarcated edges with thin glial walls, and showed very little, if any, evidence of microglial activity or phagocytosis (fig. 2 A). In severe cases, regular cellular layers of the cerebral cortex were not visible. Laminar or patchy neuronal necrosis was present in the cortex with rarefaction and vacuolation of the neuropil. The cytoplasm of small pyramidal cells present in the middle layer of the cerebral cortex showed extensive eosinophilia, pyknosis or loss of nucleus. Gitter cell proliferation was quite limited and was mainly observed in the white matter and particularly around the cavitations. Widening of Virchow-Robin spaces was present in all the brains. In the brainstem, lesions were most frequently seen in the neurons of the red and vestibular nuclei. The main feature was the presence of neuronal degeneration characterized by vacuolisation, central chromatolysis, swelling of the cell body, and hyalinization (fig. 2 C). Laminar neuronal necrosis with mineralization was also observed (fig. 2 D). Similar lesions were less frequently seen in the pontine, facial and olivary nuclei. The spinal cord was affected in all cases. Large multipolar nerve cells were present in the cervical and lumbosacral regions indicating chromatolysis, vacuolisation, and necrosis. Lesions in the spinal cord white matter included tract degeneration which involved both axons and myelin sheaths and was confined to the ventral columns.

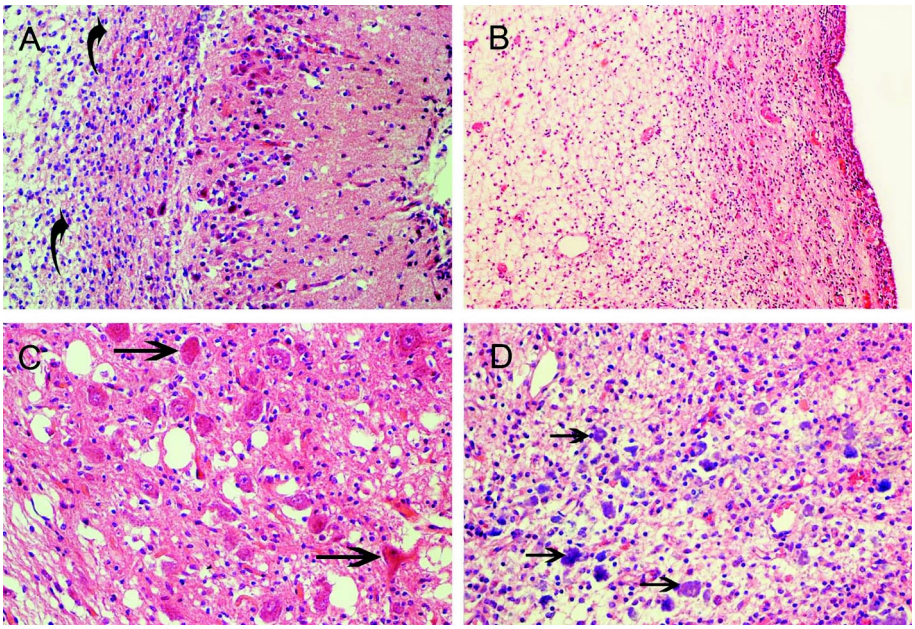

Fig. 2. Congenital copper deficiency. (A) In the cerebral cortex, degeneration and cyst formation within cerebral white matter surrounded by astrocytes (arrows). HE. × 260. (B) Status spongiosis around the lateral ventricle of the cerebral cortex. HE. × 110. (C) The neuronal vacuolisation, central chromatolysis, swelling and hyalinization of neurons (arrows) of the brain stem. HE. × 260. (D) Status spongiosis, neuronal degeneration, necrosis and mineralization (arrows) of the brain stem. HE. × 260

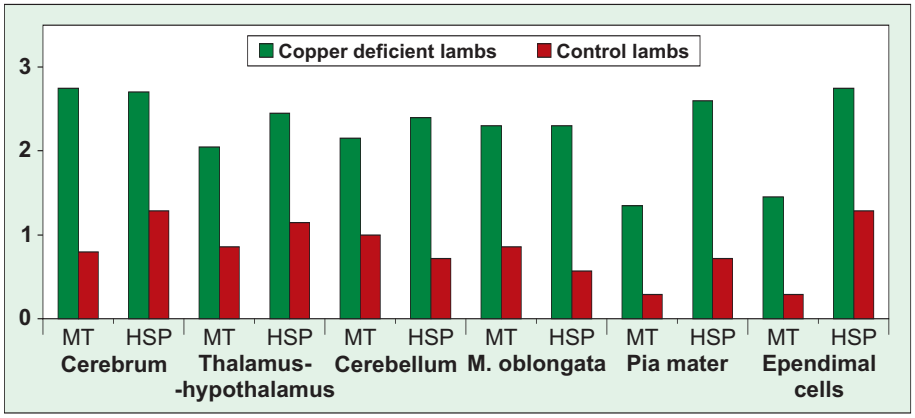

Fig. 3. Mean immunohistochemical score* of the Hsp70 and MT levels in the studied lambs

Explanation: *the labelling intensity in a given cellular compartment was categorized as follows: (–) no positively staining cells; (1; weak); (2; moderate); (3; marked) staining

In various places in the cerebellum the Purkinje cells indicated some loss, chromatolysis, necrosis or coarse vacuolisation. There was a widening of the normally thin external granular layer and a corresponding thinning and rarefaction of the internal granular layer. The external granule cells had decreased in number and some showed degenerative changes. In one case (case No. 20) with severe cerebellar hypoplasia there were folial changes which varied from presumably partial formation to complete destruction of the cortical parts. Prominent Purkinje cell loss and focal to diffuse depletion of granule cells were evident.

Markedly increased MT and Hsp70 immunoreactivity were found in all the copper deficient lamb brains examined. The mean immunohistochemical score of Hsp70 and MT compared with copper deficient and control lamb brains are shown in fig. 3.

Enhanced MT immunoreactivity occurred in the astrocytes and their processes in copper deficient lamb brains (fig. 4 A and B). Cerebral MT localization was prominent in the astrocytes surrounding the cavitations

(fig. 4 A). MT expression decreased gradually from substantia alba to substantia grisea. Intense cytoplasmic and intranuclear MT immunoreactivity was present in the astrocytes forming a narrow band beneath the pia mater. Rather weak and sparse MT immunoreactivity occurred in the cerebral neurons.

Marked Hsp70 immunoreactivity was found in the astrocytes surrounding lesions present in the substantia alba (fig. 5 A). Prominent Hsp70 labeling also occurred in the endothelial cells of the vessels present in the newly formed cavitations and astrocytes. Both neurons (chiefly in the pyramidal neurons) and astrocytes of the stratum polymorphicum adjacent to substantia alba and endothelial cells of the vessels showed Hsp70 immunolocalization. Hsp70 expression was less pronounced in the stratum moleculare.

In the pia mater and ependymal cells of the ventricular system, Hsp70 immunoreactivity was more pronounced than that of MT labeling (fig. 5 B). In the cerebellum, which showed consistently strong reactivity, intense MT and Hsp70 labeling occurred within the astrocytes (Bergman cells). The astrocytes of the substantia alba of the cerebellum often showed marked labeling of the whole cell, cytoplasmic processes and nuclei for both MT and Hsp70. While there was not any MT expression, a prominent Hsp70 reaction was found in the Purkinje cells – particularly in those regions where severe cerebellar lesions were evident (fig. 6 A).

In the thalamus/hypothalamus and medulla oblongata, moderate to marked MT and Hsp70 immunoreactivity occurred within the astrocytes of both white and grey regions (fig 4 C). While MT was negative, moderate Hsp70 immunoreactivity was seen in the nucleus paraventricularis, nucleus of the solitary tract, nucleus infundibularis and nuclei of cranial nerves of the thalamus/hypothalamus and medulla oblongata (fig. 5 C). Epithelial cells of the choroid plexus of the ventricular system showed moderate to marked Hsp70 expression (fig. 5 D). In the choroid plexus, MT staining distribution was similar to Hsp70 but staining intensity ranged from weak to moderate.

In the control brains, Hsp70 labeling was seen in all the brain regions; moderate staining was particularly

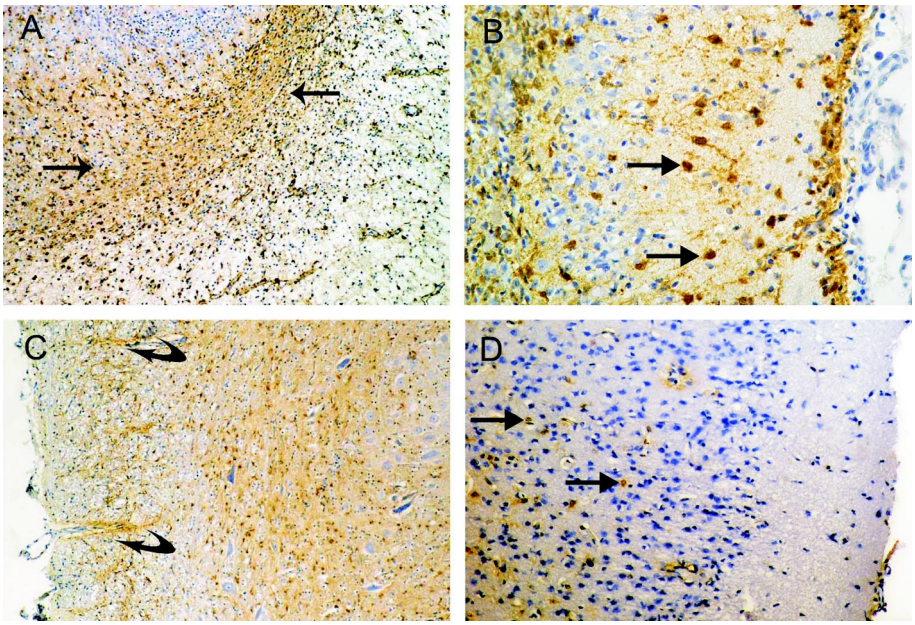

Fig. 4. MT immunoreactivity in lamb brains. Marked MT immunoreactivity in the astrocytes and their processes of the copper deficient lamb brains (A, B and C). (A) In the cerebral cortex, increased MT expression around the cystic formation (arrows). Diaminobenzidine × 110. (B) Cerebral cortex, MT immunoreactivity in the astrocytes (arrows). Diaminobenzidine × 460. (C) Medulla oblongata, MT immunoreactivity in astrocytes around the blood vessel (arrows) Diaminobenzidine × 110. (D) Cerebral cortex of the control lamb brain. Weak MT immunoreactivity in the astrocytes (arrows). Diaminobenzidine × 260

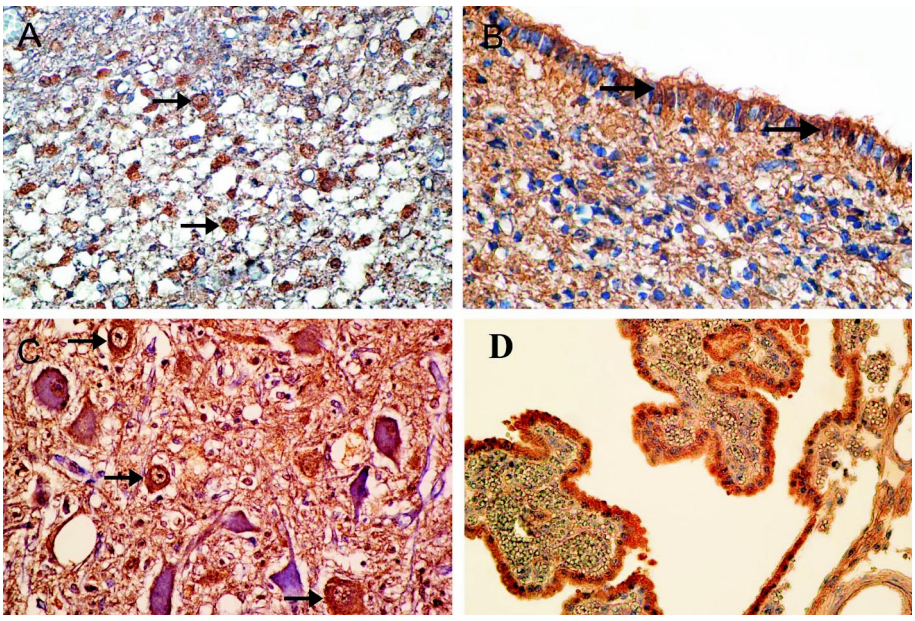

Fig. 5. Marked Hsp70 immunoreactivity in copper deficient lamb brains. (A) Cerebral cortex, Hsp70 immunoreactivity in neurons (arrows) and astrocytes. Diaminobenzidine × 260. (B) Hypothalamus, Hsp70 immunoreactivity of the ependymal cells (arrows) of the ventricle system and astrocytes and their processes. Diaminobenzidine × 260. (C) Medulla oblongata, Hsp70 immunoreactivity in both cytoplasm and nucleus of neurons (arrows) and astrocytes. Diaminobenzidine × 260. (D) Choroid plexus of the fourth ventricle, Hsp70 immunoreactivity in epithelial cells. AEC. × 180

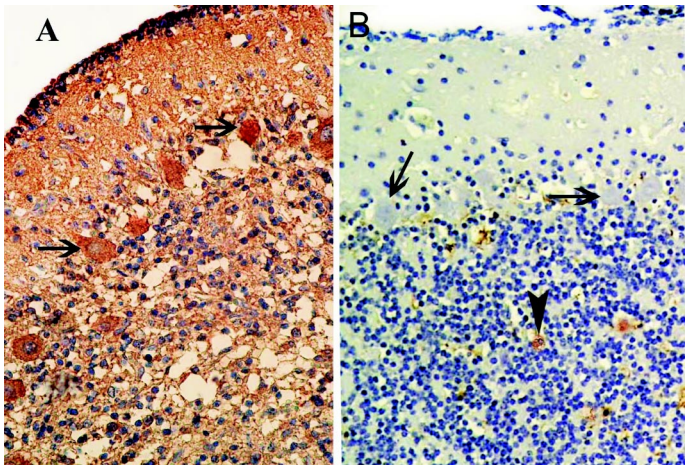

Fig. 6. Hsp70 immunoreactivity in the cerebellum. (A) Marked Hsp70 immunoreactivity in the Purkinje cells (arrows), astrocytes and their processes in the copper deficient lamb. AEC × 460. (B) Weak Hsp70 immunoreactivity in the astrocytes (arrow heads). No immunoreactivity in the Purkinje cells (arrows). AEC. × 460

found in the astrocytes while sparse and light labeling was seen in the cerebral neurons and more diffuse but weak reactions were present in the polymorphic neurons. The astrocytes of the cerebrum (grey and white matter), medulla oblongata, thalamus and hypothalamus were labeled weak to moderate for both MT and Hsp70 (fig. 4 D). In the cerebellum, weak to moderate MT labeling occurred particularly within the Bergmann's glia and Golgi type II cells of the granular layer. Hsp70 labeling of the cerebellar astrocytes was either absent or sparse and intensity varied from weak to moderate and the pia mater exhibited generally mild to moderate staining (fig. 6 B). Labeling of the ependymal cells was absent for the MT and it was weak to moderate for the Hsp70.

The histopathology of affected lambs in the present study was similar to what has previously been described in lambs with swayback (1, 18). The disease has been described very well in earlier studies (1, 8, 10, 11, 18). Lambs that were severely affected showed gross cerebral cavitations of varying severity. PCR and ELISA analysis revealed that all of the samples were negative for the bluetongue, border, and Akabane virus. If these viruses had been present during pregnancy they may have caused porencephaly even though they were not present at the time of examination. They would not have caused neuronal lesions in spinal cords and brain stem nuclei such as the red nucleus nor would they have caused lesions in the white matter of the spinal cord. The neurological signs, distribution and nature of gross and histopathological lesions and low liver copper concentrations were consistent with a diagnosis of congenital copper deficiency in the lambs of the present study.

The present investigation is the first to compare immunohistochemical expression of MT I-II and Hsp70 associated with copper deficient lamb brains and normal lamb brains. There is much current interest for the regional and cellular localization of MT in mammalian brains since this information may help in the interpreting the neural functions of MT. MT is expressed in the mammalian brain and its synthesis can increase in this tissue following treatment with glucocorticoid hormones, bacterial endotoxins and ischemia (5, 19).

The present study exhibited modest MT immunoreactivity located within astrocytes, pia mater, choroid plexus and ependyma in the normal lamb brains. Similar observations have been made in normal sheep (4), and in adult mice but not in adult rats (14). The study also showed that was an increased MT immunoreactivity in these sites in copper deficient lamb brains. It is interesting to note that increased MT expression was also evident in copper enhanced sheep brains in the same sites (4). Similarly, cadmium administered rat brains exhibited increased MT expression in the vascular endothelial, ependymal and subependymal cells (14). Yamasaki et al. (19) observed an increased immunoreactivity for MT in the CNS after ischemic brain damage using rats. Based on evidence that MT is an endogenous antioxidant (17), the present results suggest that expression of MT may be important step in protective degenerative processes following brain damage. In this respect, the induction of MT is possibly related to cellular protective mechanisms. There is substantial evidence that the induction of MT is neuroprotective, based on the study of transgenic mice over expressing MT (7).

Astrocyte processes encircling blood vessels constitute a functional syncytium to facilitate molecular exchange throughout the glial compartment. The transport function of astrocytes between the blood and cerebrospinal fluid gives them an essential role in the monitoring of intracerebral metal transport and the storage of metal ions (12). Consistent MT immunopositivity of the cell body and their processes in this study has indicated the functional importance of this protein and its multifunctional aspects. Additionally, it suggests that not only does it have a transport and storage function (12), but it also sequestrates copper (4) as well as functioning as a free radical scavenger in reactive astrocytes indicating that MT is a major neuroprotective protein within the brain. The present results provide further evidence that MT plays an important role in the cellular response to neuronal injury.

Induction of Hsp70 protein occurs within 24 h following injury in experimental models (3, 15) and the present study showed an increase in Hsp70 in brain tissue samples from Cu deficient lambs. Brain injuries in rats (3), humans (16) and lambs (present study) result in an increase in Hsp70 protein expression, including astrocytes and neurons in the brain cells. The enhancement of Hsp70 protein expression was particularly prominent in and around the lesions. This

is possibly due to increased cellular stress genes and/or an elevation of the quantity of stress proteins. Human (16) and rodent studies (3) have not been able to determine if the increase in Hsp70 is sufficient enough to afford cellular protection following traumatic brain injury; although Yenari et al. (20) showed that over-expression of Hsp70 using viral vectors could protect neurons in rat models of stroke and epilepsy. Similarly, cells expressing Hsp70 were more resistant to apoptosis induced by hyperglycemic cerebral ischemia in the rat (13).

In conclusion, the present study suggests that Hsp70 expression in astrocytes and neurons may possibly indicate that they are less susceptible to the consequences of cell stress factors and can thus be exploited to selectively increase their survival in copper deficiency. The results also provide further evidence that MT may also play an important role in the cellular response to neuronal injury in copper deficiency.

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