Marginally Low Copper Causes Lesions of the Midbrain in Animal Models

The Implications for Man

J Joseph[1], T Alleyne[1], A Adogwa[2]

ABSTRACT

Serum copper levels must be maintained between very strict limits for the maintenance of good health. High levels have recently been linked to Alzheimer's disease while low levels during pregnancy cause enzootic ataxia (swayback disease) in offspring. In this study, we investigated the prolonged effect of serum copper that was maintained at and around 0.5 ppm, the level presently regarded as safe. Pregnant sheep and rabbits in the last trimester (1–4 weeks) of pregnancy were treated with the copper chelator, ammonium tetrathiomolybdate (ATM). Treatment was continued until the young were one month old at which time the animals were sacrificed. Serum copper levels of the parents and offspring were monitored by atomic absorption. The difference spectra (400–630 nm) was examined and SDS PAGE was used to evaluate the protein composition of the brain mitochondria. The anatomy of the midbrain was also studied. Although the young sheep and rabbits from the ATM-treated mothers showed no visible signs of disability or swayback disease, the midbrain of those young animals with serum copper between 0.3–0.9 ppm showed evidence of vacuolation, cavitation and chromatolysis. In contrast, the difference spectra and the protein composition of the brain mitochondria from these animals were all normal. These results suggest that although animals may appear normal and exhibit some normal biochemical markers, serum copper in the region of 0.5 ppm may not be safe for some breeds of sheep or rabbits. It is possible that a similar situation applies to man.

Deficiencia Marginal de Cobre Como Causa de Lesiones del Mesencéfalo en Modelos Animales

Implicaciones para el ser Humano

J Joseph[1], T Alleyne[1], A Adogwa[2]

RESUMEN

Los niveles séricos cúpricos tienen que ser mantenidos dentro de límites muy estrictos, si se quiere tener una buena salud. Los altos niveles de cobre han sido asociados recientemente con la enfermedad de Alzheimer, mientras que los niveles bajos durante el embarazo causan ataxia enzoótica (swayback) en la descendencia. En este estudio investigamos el efecto prolongado del cobre sérico mantenido a 0.5 ppm ó alrededor de 0.5 ppm – el nivel considerado seguro actualmente. Ovejas y conejas preñadas, en el último trimestre (1-4 semanas) de gestación, fueron tratadas con el quelante del cobre conocido como tetratiomolibdato de amonio (TM). El tratamiento continuó hasta que las crías tuvieron un mes, momento en el que los animales fueron sacrificados. Los niveles séricos cúpricos de los progenitores y la progenie fueron monitoreados mediante absorción atómica. Se examinaron los espectros de

From: [1]Biochemistry Unit, [2]School of Veterinary Medicine, Faculty of Medical Sciences, The University of the West Indies, St Augustine, Eric Williams Medical Sciences Complex, Uriah Butler Highway, Champs Fleurs, Trinidad and Tobago.

Correspondence: Dr T Alleyne, Biochemistry Unit, Faculty of Medical Sciences, St Augustine, The University of the West Indies, Eric Williams Medical Scieces Complex, Uriah Butler Highway, Champs Fleurs, Trinidad and Tobago. Fax: (868) 662-1873, e-mail: trevor.alleyne@gmail.com.

diferencia (400– 630 nm). Se usó la técnica de SDS-PAGE para evaluar la composición proteica de las mitocondrias cerebrales. También se estudió la anatomía del mesencéfalo. Aunque la anatomía de las crías de ovejas y conejas madres tratadas con TM no mostraron señales visibles de discapacidad o enfermedad swayback, el mesencéfalo de estas crías con cobre sérico entre 0.3–0.9 ppm, mostró evidencias de vacuolación, cavitación y cromatolisis. En contraste con ello, los espectros de diferencia y la composición proteica de las mitocondrias del cerebro de estos animales, fueron todos normales. Estos resultados sugieren que aunque los animales puedan parecer normales y presentar marcas bioquímicas normales, el cobre sérico en el rango de 0.5 ppm, puede no ser seguro para algunas crías de ovejas y conejos. Es posible que una situación similar se aplique al ser humano.

West Indian Med J 2007; 56 (6): 482

INTRODUCTION

In the last few years, it has emerged that it may be absolutely vital to maintain dietary copper between very strict limits. Recent research on rabbits has demonstrated that an excess of copper in the diet precipitates Alzheimer's-like characteristics (1, 2). On the other hand, extensive studies have shown that a deficiency of dietary copper causes enzootic ataxia, also known as sway back disease (SD) in a wide range of animals including man (3–5).

Studies conducted over the years have suggested that for man and other animals daily serum copper levels ought to be maintained above 0.5 ppm; below this level is considered clinically low and particularly dangerous to the health of developing fetuses (6). Evidence suggests that SD occurs in offspring if the pregnant mother experiences prolonged copper deficiency, serum copper < 0.5 ppm, during the last half of pregnancy (7, 8). It appears that the low copper status affects the efficiency of cytochrome c oxidase (COX) and several other copper containing enzymes of the developing young (9) precipitating a wide variety of symptoms (10).

Partly because of their sensitivity to copper (11) and their agricultural and economic importance, sheep have been the focus of study with regards to SD. In sheep, SD is known to occur in two forms: the congenital or neonatal form and the delayed form (12). In the congenital form of the disease, the symptoms are apparent at birth and the helpless lambs do not normally live long enough to be weaned (13). The delayed form, however, is subtler. Initially, lambs show no signs of disease but at some point within the first four months of life, instability and other signs of SD begin to appear (14). Pathologically, both forms of SD are characterized by lesions of the brain stem, spinal cord and the cerebellum (6, 15), and both forms are fatal.

In an effort to better understand the effects of copper deficiency on offspring, a number of groups, including ours (16), have used the chelator, ammonium tetrathiomolybdate (ATM), to induce copper deficiency in pregnant sheep. Almost without exception, the objective of those previous studies was to investigate the effects of copper levels that were below the 0.5 ppm level considered to be clinically critical (17). An objective of the present study is to re-investigate the long term effects of copper levels that were maintained at and around the threshold presently regarded as safe.

There was a second objective of this study. Previously almost all studies on the use of ATM to induce copper deficiency in offspring were conducted on sheep. However, sheep, because of their relatively large size and long gestation period, are expensive to maintain and slow to yield results. Therefore, the second objective was to introduce a cheaper, simpler animal model. Rabbits because of their small size, non-aggressive nature and high rate of reproductivity seemed ideal. In the work presented here, we employed ATM to induce low and marginally low copper levels in pregnant sheep and rabbits and investigated the effects on the offspring. Specifically, we examined the anatomy of the midbrain and some biochemistry of the brain mitochondria.

SUBJECTS AND METHODS

All animal experiments were approved by the Ethics committee, The University of the West Indies, St Augustine.

Induction of Copper Deficiency in Sheep: Four black-belly sheep, in the last 4–6 weeks of pregnancy and one in the last two weeks of pregnancy were treated with ATM as previously described (16) to induce copper deficiency in their offspring. No ATM was administered to five pregnant sheep of the same breed (controls). Each ewe produced a single lamb.

Induction of Copper Deficiency in Rabbits: A modified procedure to that previously used for sheep (16) was employed to induce copper deficiency in the offspring of pregnant rabbits. A total of 20 pregnant rabbits were employed in the study. Of these, ten (eight New Zealand white and two of mixed breed) were used as controls and another ten (eight New Zealand white and two of mixed breed) were used for the induction of copper deficiency. To induce the copper deficiency, ATM at 0.5 mg/kg was administered twice daily, commencing as soon as pregnancy was confirmed. This was usually at the end of the first week. For the first four days of treatment, the ATM was administered as intramuscular injections after which it was given as subcutaneous injections. The subcutaneous injections were continued after the birth of the young and up until the neonates were weaned. The ATM-treated rabbits produced 21 offspring while the control rabbits (not injected), produced 14 young ones.

The offspring of both the control and ATM-treated rabbits and sheep were sacrificed at the age of one month.

Copper Analysis: Blood samples (~5 ml) were collected from the pregnant sheep at two weekly intervals, commencing at least one month prior to the start of the ATM treatment. At least two blood samples were taken from each offspring, one of which was at the time of sacrifice. For the rabbits, a single blood sample from the offspring was obtained at the time of sacrifice. Serum was isolated from the various blood samples by a method of centrifugation as previously described (16).

Serum samples obtained from the various experimental and control animals were diluted five fold with deionized water and their atomic absorption read for copper on a Varian Spectr AA300 absorption spectrometer. For each serum sample, the instrument automatically took three readings and calculated the average value. For each offspring from the sheep, two to three serum samples were obtained at specific intervals while a single sample was obtained from the offspring of the rabbits. The copper content of the serum samples was deduced from standard curves.

***Preparation of Standard* Curves:** A standard solution containing 100.00 ppm copper was diluted twenty fold and the latter used for serial dilutions. The serial dilutions usually ranged from 0.10–2.00 ppm but had to be extended beyond this on one occasion. All dilutions were made using deionized water. Atomic absorption data were only retained for use when the blank, comprising deionized water only, gave consistent absorbance readings of zero during the course of the recordings (Fig.1).

Histological Analysis: Midbrain samples of offspring from the controls and the ATM-treated sheep and rabbits were prepared for histological examination using standard procedures. Slides were stained with haematoxylin-eosin.

Mitochondrial Preparation: Mitochondria were prepared from the brain and liver of offspring of both the untreated controls and ATM-treated sheep and rabbits as described, elsewhere (18).

SDS-PAGE: Brain and liver mitochondria from the offspring of the controls and the ATM-treated sheep and rabbits were depleted of membrane by treatment with 2% sodium cholate. The protein compositions of the corresponding groups of mitochondria were then compared by SDS-PAGE on 18.7% gels as previously described (18). The Coomassie blue stained Gels were further analysed on a Bio Rad 620 video densitometer.

Results

Preliminary investigations showed that when the rabbits were given a dose similar to that administered to the sheep (*ie* 1 mg/kg twice daily) high serum bilirubin resulted. At half of this dose which was well tolerated by the pregnant rabbits, a total of 21 live offspring were produced. One of the two mixed breed experimental rabbits developed depigmentation during the ATM-treatment. For the sheep, a total of ten lambs, five from the controls and five from the ATM-treated ewes were obtained.

The mean copper levels of the offspring from the ATM treated rabbits was 0.76 ± 0.23 ppm which was just above half that of the 1.03 ± 0.5 ppm observed for the controls (Table 1). The lowest value observed for the 'ATM offspring' was 0.5 ppm (Fig. 1) and over 60% of this group had serum copper levels which were # 0.7 ppm.

Table 1: Serum copper levels of offspring from ATM-treated rabbits and normal rabbits used as controls

ATM Rabbits	Cu Level (ppm)	Control Rabbits	Cu Level (ppm)	ATM Lambs	Cu Level (ppm)
AR1	0.8	NR1	0.7	L1E5	0.3
AR2	0.8	NR2	0.7	L2E5	0.4
AR3	0.9	NR3	0.8	L4E5	0.4
AR4	0.7	NR4	1.1	L1E2	0.9
AR5	0.8	NR5	0.7	L2E11	1.0
AR6	0.7	NR6	0.8		
AR7	0.7	NR7	0.6		
AR8	1.0	NR8	0.6		
AR9	0.7	NR9	0.7		
AR10	0.5	NR10	1.7		
AR11	0.5	NR11	0.7		
AR12	0.5	NR12	1.3		
AR13	0.6	NR13	2.4		
AR14	0.6	NR14	1.2		
AR15	0.6	NR15	1.4		
AR16	0.7				
AR17	0.6				
AR18	0.6				
AR19	1.3				
AR20	1.2				
AR21	1.2				

Control Lambs	Cu Level (ppm)
C1	1.2
C2	1.2
C3	1.0
C4	1.0
C5	0.9

Columns 1 and 2; one month old rabbits born to ATM-treated mothers
Columns 3 and 4; one month old controls, *ie* rabbits born to untreated mothers
Columns 5 and 6 (top); one month old lambs born to ATM-treated mothers
Columns 5 and 6 (bottom); one month old controls, *ie* lambs born to untreated mothers. Cu = copper

For the sheep, none of the control lambs had mean serum levels below 0.5 ppm. This was also the case for the pregnant ewes prior to the treatment with ATM (Table 1) (16). For the four lambs produced from the ewes that were treated with ATM for 4–6 weeks, one had serum copper level of 0.3 ppm, two had serum copper level of 0.4 ppm and the

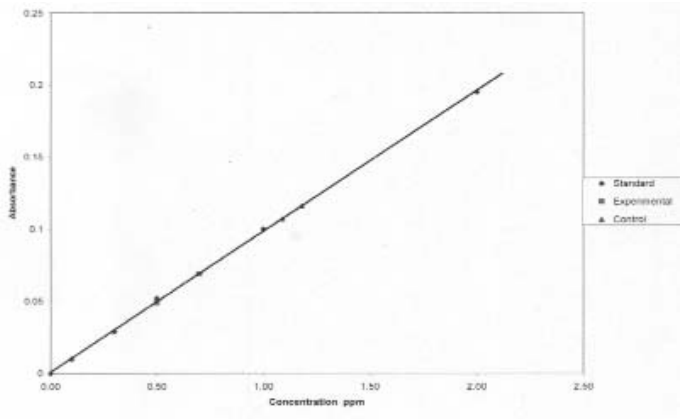

Fig. 1: Standard curve for copper content determined. A standard curve 0.10 ppm – 2.00 ppm copper, prepared by serial dilution of a stock solution, is shown (u). Also shown is the copper content of blood from two control rabbits (Δ) and two offspring (□) whose mother was treated with ATM, a copper chelator, during pregnancy.

other 0.9 ppm. The lamb produced from the ewe treated with ATM for the shorter period of two weeks had a copper serum of 1.0 ppm.

Except for the expected post mortem autolytic changes, the midbrain of the control lambs appeared normal, Fig. 2a.

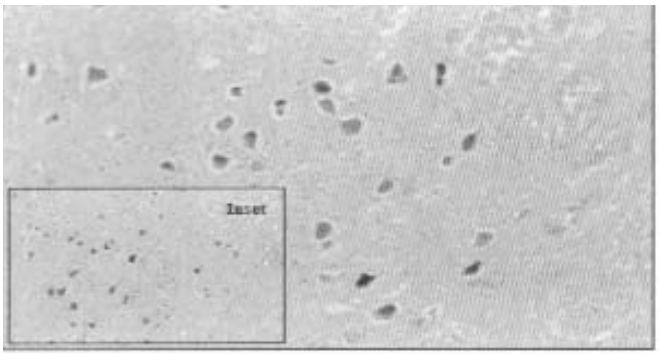

Fig. 2a: Typical section of midbrain of control lambs with normal serum copper (*ie* 1.0 – 1.5 ppm). Note the absence of vacuolation and cavitation in the tissue of this animal (x100).

In contrast, four of the five lambs from the ATM-treated ewes (the four with serum copper between 0.3 and 0.9 ppm) showed histological lesions that were similar to those observed for field lambs suffering with SD (Fig. 2b, inset). In all four cases, there was evidence of vacuolation, cavitation and chromatolysis (Fig. 2b). For the fifth lamb with serum copper of 1.0 ppm, there was no evidence of vacuolation, cavitation and chromatolysis. In fact the midbrain of this lamb exhibited histology that was identical to that of the controls (Fig. 2a, inset). The situation was the same for the rabbits. For the offspring from the ATM treated rabbits, in addition to expected autolytic changes, the midbrains showed evidence of vacuolation, cavitation and chromatolysis (Fig. 3a): The midbrains of the control rabbits were normal (Fig. 3b).

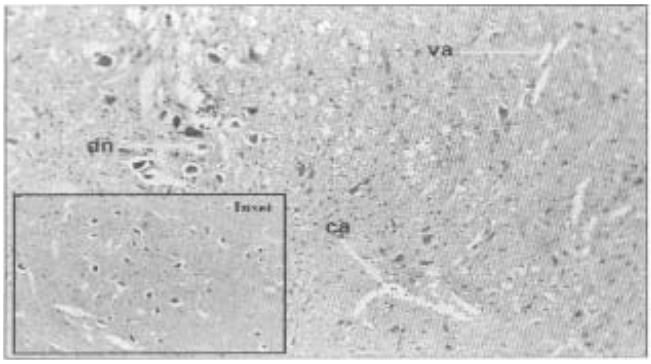

Fig. 2b: Comparative histopathology of the midbrain of lambs
The figure presents a typical haematoxylin-eosin stained section of the midbrain of lambs with marginally low (0.5 – 0.7 ppm) serum copper. The lambs were the offspring of ATM-treated ewes. The arrows highlight the presence of (dn) neuronal degeneration, (va) vacuolation and (ca) cavitation. These lesions are pathognomonic of SD. Inset: typical section of midbrain of SD lamb showing similar pathology (x100).

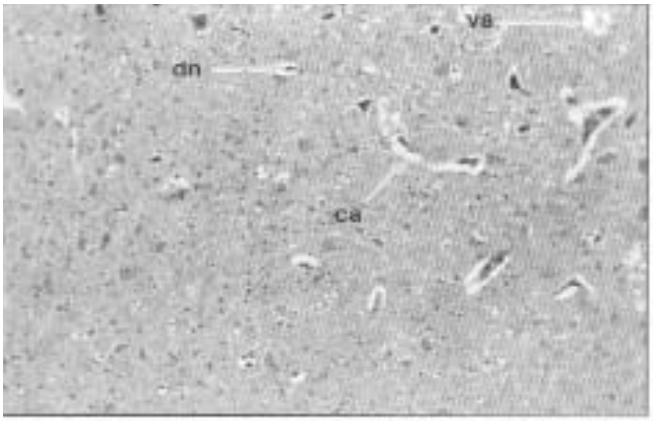

Fig. 3a: Comparative histopathology of the midbrain of young rabbits.
The figure presents a typical haematoxylin-eosin stained section of the midbrain of one month old rabbits with marginally low (0.5 – 0.7 ppm) serum copper: The young rabbits were the offspring of ATM treated ewes. The arrows highlight the presence of (dn) neuronal degeneration, (va) vacuolation and (ca) cavitation. These lesions are pathognomonic of SD (x100).

SDS-PAGE showed that for the rabbits, the protein composition of the brain mitochondria was different to that of the liver mitochondria. The main differences occurred between 14–30 KDa, Figure 4a, lanes 3 and 4. Also, for young rabbits produced from mothers treated with ATM, the protein composition of their liver mitochondria was different from that seen for the controls whose mothers received no ATM. The 'ATM' offspring showed decreased protein concentration in the 14–20 KDa region, Figure 4a, lanes 4 and 5. In the brain, the situation was quite different. For both the sheep, Figure 5, and the rabbits (Fig. 4a, lanes 2 and 3 and Fig. 4b) the mitochondria of the 'ATM' offspring and the controls appeared to be identical.

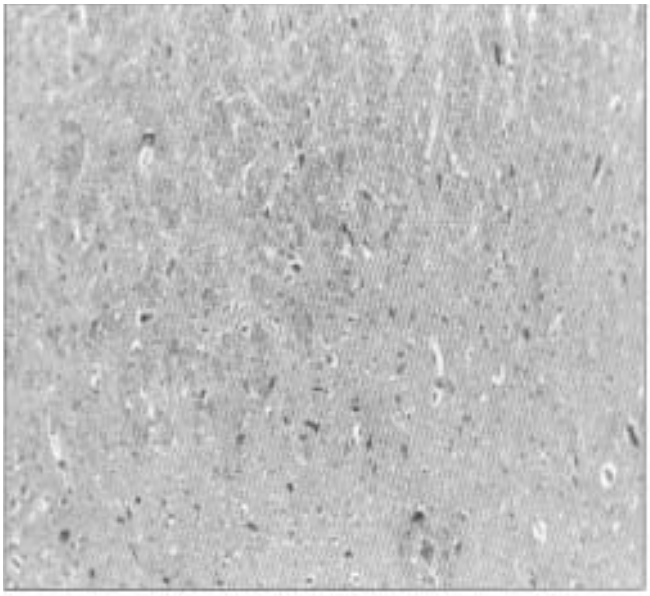

Fig. 3b: Typical section of midbrain of controls *ie* one-month old rabbits with normal serum copper (*ie* 1.0 – 1.5 ppm). Note the absence of vacuolation and cavitation (x100).

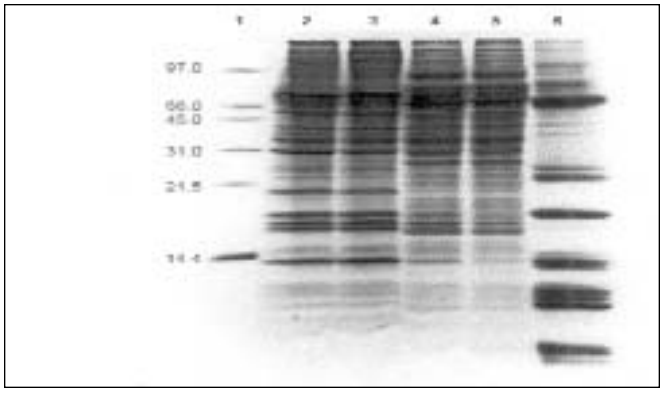

Fig. 4a: SDS-PAGE and densitometric analysis of brain and liver mitochondria from offspring of ATM-treated and normal rabbits.
The protein composition of mitochondria from the brain and liver of ATM-treated and control rabbits was compared by SDS-PAGE. Lane 1 contains low molecular weight standards. Lanes 2 and 3 contain brain mitochondria of offspring from control and ATM-treated rabbits respectively. Lanes 4 and 5 contain liver mitochondria of offspring from control and ATM-treated rabbits respectively. The subunits of bovine heart COX, used as markers, are shown in lane 6.

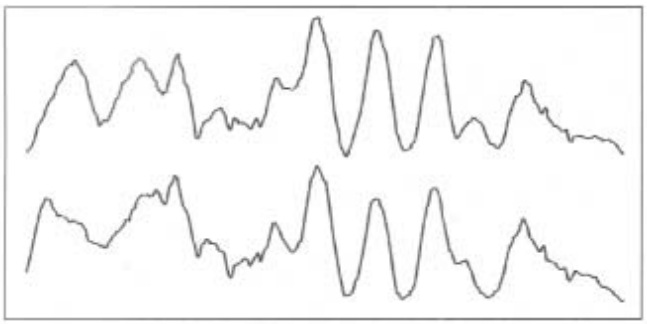

Fig.4b: Densitometric analysis of the SDS gel shown in a. Upper trace: scan of lane 3, brain mitochondria from the "ATM"-treated offspring. Lower trace: the scan of lane 2, brain mitochondria from offspring of control, 'normal' rabbits.

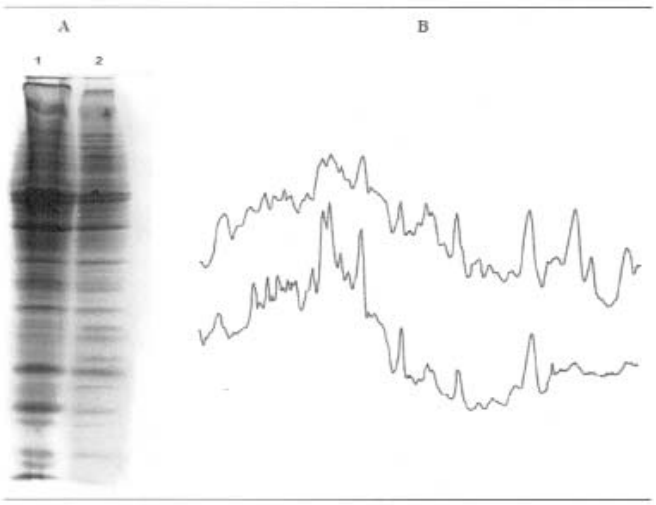

Fig. 5a: SDS-PAGE and densitometric analysis of brain mitochondria from the offspring of ATM-treated and normal ewes. The protein composition of brain mitochondria from the offspring of ATM-treated and control sheep was compared by SDS-PAGE. Lane 1 contains mitochondria of offspring from ATM-treated sheep while lane 2 contains mitochondria of offspring from normal, untreated, sheep.

Fig. 5b: Densitometric analysis of the SDS gel shown in A. Upper trace: the scan of lane 1. Lower trace: the scan of lane 2.

DISCUSSION

Of the five lambs produced from the ATM-treated ewes, the four with serum copper below 1.0 ppm all showed vacuolation and chromatolysis *ie* lesions normally associated with SD. For those offspring of the ATM-treated rabbits with the serum copper between 0.5 and 0.7 ppm, similar anatomical changes were also observed. None of these experimental animals *ie* neither the young rabbits nor the young sheep showed any outward signs of physical disability or SD. The only outward sign consistent with SD occurred in one of the adult mixed-breed rabbits for which low serum copper was induced; this animal exhibited depigmentation, a characteristic of SD.

The observation that anatomical alterations of the midbrain did not lead to any obvious effects on the physical ability of the young rabbits or lambs raises two issues. Firstly, the observation of lesions in these two groups of young animals appear to suggest that a serum copper of 0.5 ppm, currently regarded by many experts as the lower 'safe-threshold', is probably too low for some rabbits and some breeds of sheep. This latter conclusion is consistent with previous reports which indicated that some breeds of sheep

are more susceptible to low copper diets than others (19, 20). Moreover, if lesions of the type detected are asymptomatic in so far as physical activity is concerned, then it is possible that the cut-off threshold for other animals, including man, may also have to be reviewed. The possibility that some ethnic groups are more susceptible to low copper diets than others should also be considered.

The second issue is linked directly to the first. Although there is a need for a lot more research into this phenomenon, the absence of a correlation between outward physical indicators and anatomical lesions of the midbrain suggests that it may be worthwhile, as an interim precautionary measure, to screen pregnant mothers for copper in order to avoid exposing the developing fetus to low copper. In the absence of such screening, it is possible that children could be born with the type of midbrain lesions now reported. It would be of interest to explore the physiological significance of these lesions of the midbrain.

Given the diverse roles of the liver and the fact that the liver is the organ which is mainly responsible for drug detoxification, it is not unexpected that liver mitochondria from the ATM-treated rabbits had a different protein profile from liver mitochondria from the untreated controls. The observation that liver and brain mitochondria had different protein composition was also as expected, again reflecting the different roles of these two organs. More interesting was the apparent lack of differences in protein composition observed when the brain mitochondria from the ATM-treated rabbits were compared to the brain mitochondria of the controls. It is instructive, however, to await the isolation of purified COX and other mitochondrial proteins before final pronouncements are made, as differences at the level of a single enzyme subunit, could easily be masked by the vast sea of mitochondrial proteins. Notwithstanding this precaution, the fact that brain mitochondria from both the ATM-treated and the controls appear to have the same protein composition suggest that whatever else may be happening, the structure of COX is probably not affected by the level of copper deficiency induced.

Two unforeseen problems were encountered in the rabbit study. Firstly, there was some difficulty in obtaining adequate volumes of blood for the serum analysis. Secondly, when blood was taken during pregnancy, there was a very high incidence of spontaneous abortion. It is possible however, that both of these problems can be resolved by switching to microanalysis. Both in terms of time and cost of maintenance, the rabbit study was significantly cheaper than that of the sheep. In addition, the rabbits provided a much larger number of offspring for evaluation. We conclude, therefore, that although some refinement of the model is necessary, the ATM-treated rabbit as a model for studies on the effects of low serum copper appears to offer a good, cheap alternative particularly for those scientists who require fairly substantial quantities of tissue.

REFERENCES

1. Sparks DL, Schreurs BG. Trace amounts of copper in water induce b-amyloid plaques and learning deficits in a rabbit model of Alzheimer's disease. PNAS 2003; **100:** 11065–9.
2. Haas U, Sparks DL. Cortical Cathepsin D activity and immunolocalization in Alzheimer disease, critical coronary artery disease, and aging. Mol Chem Neuropathol 1996; **29:** 1–14.
3. Frank A. Mysterious moose disease in Sweden. Similarities to copper deficiency and/or molybdenosis in cattle and sheep. Biochemical background of clinical signs and organ lesions. Sci Total Environ 1998; **209:** 17–26.
4. Palmer AC, Blakemore WF, O'Sullivan B, Ashton DG, Scott WA. Ataxia and spinal cord degeneration in llama, wildebeeste and camel. Vet Rec 1980; **107:** 10–1.
5. Giovannoni G. Human swayback disease: expanding the spectrum of diseases associated with abnormal copper metabolism. J Neurol 2001; **248:** 707.
6. Howell JMcC, Pass DA, Terlecki S. Swayback lesions and vulnerable periods of development. In: Howell JMcC, Gawthorne JM, White CL, editors. Proceedings of the 4th International Symposium on trace element metabolism in man and animals (TEMA-4); Perth, Western Australia. Canberra: Aust Acad Sci; 1981; 289–300.
7. Kimberling CV. Jensen and Swift's diseases of sheep 3rd ed. Philadelphia, PA: Lea and Febiger; 1988.
8. Smith MC, Sherman DM. Goat medicine. Philadelphia, PA: Lea and Febiger; 1994.
9. Linder MC, Hazegh-Azam M. Copper biochemistry and molecular biology. [Review]. Am J Clin Nutr 1996; **63:** 797–811.
10. Fell BF. The pathology of copper deficiency in animals. In: Howell JMcC, Gawthorn JM, editors. Copper in animals and man Vol. II. Boca Raton: CRC Press; 1987; 1–27.
11. Barlow RM. Swayback. In: Martin WB, Aitken ID, editors. Diseases of sheep 2nd ed. Oxford: Blackwell Scientific Publications; 1991: 178–81.
12. McDonald PMcC, Edwards RA, Greenhalph JFD. Animal health. 4th ed. Essex: Longman Scientific and Technical; 1990.
13. Scott PR. Other nervous diseases. In: Martin WB, Aitken ID, editors. Diseases of sheep 3rd ed. Oxford: Blackwell Science Ltd. 2000: 228–39.
14. Linklater KA, Smith MC. Color atlas of diseases and disorders of the sheep and goat. Aylesbury: Wolfe Publishing; 1993.
15. Perlman M, Buchanan B, Sarkar B. Copper deficiency and timing of hypomyelination. In: Howell JMcC, Gawthorne JM, White CL, editors. Proceedings of the 4th International Symposium on trace element metabolism in man and animals (TEMA-4); Perth, Western Australia, Canberra: Aust Acad Sci; 1981; 302–4.
16. Joseph J, Alleyne T, Adogwa A, Campbell M, Neckles F. Copper deficiency in pregnant ewes: its influence on the course of the pregnancy and the health status of the offspring. J Caribb Vet Med Assoc 2002; **2:** 21–6.
17. Gordon AJ, Hill JL. The effect of injecting sheep with thiomolybdates. In: Howell JMcC, Gawthorne JM, White CL, editors. Trace element metabolism in man and animals. Proceedings of the Fourth International Symposium on Trace Element Metabolism in Man and Animals. (TEMA-4); Perth, Western Australia 1981: 557–60.
18. Alleyne T, Joseph J, Lalla A, Sampson V, Adogwa A. Cytochrome c oxidase isolated from the brain of swayback-diseased sheep displays unusual structure and uncharacteristic kinetics. Mol Chem Neuropathol 1998; **34:** 233–47.
19. Woolliams JA, Woolliams C, Suttle NF, Jones DG, Weiner G. Studies on lambs genetically selected for high and low copper status. II. Incidence of hypocurosison improved hill pasture. Animal Prod 1986; **43:** 303–17.
20. Wiener G. Relationships between swayback incidence and concentration of copper in the blood of sheep of different breeds. J Comp Pathol 1971; **81:** 515–20.