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Laboratory Study

Neuropathological changes in a lamb model of non-accidental head injury (the shaken baby syndrome)

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ABSTRACT

Non-accidental head injury (NAHI), also termed the "shaken baby syndrome", is a major cause of death and severe neurological dysfunction in children under three years of age, but it is debated whether shaking alone is sufficient to produce brain injury and mortality or whether an additional head impact is required. In an attempt to resolve this question, we used a lamb model of NAHI since these animals have a relatively large gyrencephalic brain and weak neck muscles resembling those of a human infant. Three anaesthetised lambs of lower body weight than others in the experimental group died unexpectedly after being shaken, proving that shaking alone can be lethal. In these lambs, axonal injury, neuronal reaction and albumin extravasation were widely distributed in the hemispheric white matter, brainstem and at the craniocervical junction, and of much greater magnitude than in higher body weight lambs which did not die. Moreover, in the eyes of these shaken lambs, there was damage to retinal inner nuclear layer neurons, mild, patchy ganglion cell axonal injury, widespread Muller glial reaction, and uveal albumin extravasation. This study proved that shaking of a subset of lambs can result in death, without an additional head impact being required.

1. Introduction

Caffey[1,2] recognised subdural haematoma, retinal haemorrhages, and long bone fractures as being suggestive of inflicted head injury in infants and young children, usually perpetrated by a parent or carer. This concept has now evolved into a constellation of lesions (acute encephalopathy, and subdural and retinal haemorrhages) referred to as non-accidental head injury (NAHI). Child abuse of this type is also commonly termed the "shaken baby syndrome" or "shaken-impact syndrome", the latter by those who contend that, in addition to manual shaking, an additional head impact is required to produce these lesions.[3,4]

Since one of the major controversies in NAHI is whether shaking alone is sufficient to injure the brain or whether an additional head impact is required, we used a lamb model in this pilot study to determine whether shaking alone could produce lesions resembling those found in human NAHI. Lambs have a relatively large gyrencephalic brain and weak neck muscles similar to a human infant and are, therefore, an appropriate model for human NAHI. Prior to the development of this ovine model of NAHI, there was no satisfactory biomechanical model in which to investigate the pathogenesis of NAHI.[5]

2. Materials and methods

2.1. Experimental protocol

Nine anaesthetised (isoflurane) and ventilated lambs were manually grasped under the axilla and vigorously shaken with sufficient force to snap the head back and forth onto the chest, similar to head motions believed to occur in human NAHI. In addition to this acceleration/deceleration of the head, there was also considerable lateral and rotational head movement. Each lamb was shaken in this manner 10 times of 30 seconds duration over a 30 minute period, then placed quietly in the sphinx position for six hours under anaesthesia. No head impact occurred. Four control lambs were not shaken, but otherwise subjected to the same experimental protocol. In addition to being ventilated, animals were titrated against regular blood gas measurements to maintain a normal blood gas

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Table 1
Neuronal and axonal amyloid precursor protein (APP) immunopositivity in a lamb model of non-accidental head injury

Lamb No.	Body weight[1] (kg)	Neuronal APP score[2]	Axonal APP score[3]	Time to death (hours)
Shaken lambs – subset I				
1	12.0	49	10	6
2	11.0	56	13	6
3	10.5	37	6	6
4	10.0	74	12	6
5	10.0	61	15	6
6	8.5	71	15	6
Shaken lambs – subset II				
7	6.0	66	31	5
8	5.5	75	30	2
9	5.0	58	26	3
Control lambs				
1	10.5	3	0	6
2	9.5	2	0	6
3	9.0	2	0	6
4	5.0	2	0	6

Statistical analyses were conducted using an unpaired *t*-test.
[1] Body weights were significantly different ($p < 0.001$) between the two subsets of lambs.
[2] Neuronal APP expression was not significantly different ($p > 0.05$) between the two subsets.
[3] Axonal injury was significantly greater ($p < 0.001$) in the lower body weight lambs.

profile, and mean arterial blood pressure was measured from a fluid-filled, arterial catheter inserted into the right femoral artery.

During the experiment, a subset of three lower body weight lambs (mean 5.5 kg, range 5.0–6.0 kg) died unexpectedly at two, three and five hours after the last episode of shaking, without reaching the designated six-hour post-shaking period. By contrast, the higher body weight lambs (mean 10.3 kg, range 8.5–12.0 kg) survived for six hours post-shaking, until killed by perfusion fixation of the brain (Table 1).

Lambs were maintained under anaesthesia for the full duration of this experiment, without ever regaining consciousness, until killed by perfusion fixation of the brain with 4% paraformaldehyde containing 0.02% heparin. Brains remained *in situ* overnight and were then removed and immersed in 10% neutral buffered formalin for seven days. Rostral cervical spinal cord and both eyes (including optic nerves) were also collected. Brains and cords were sectioned into 5-mm whole coronal slices, paraffin-embedded, and 6-μm sections were cut and stained with haematoxylin and eosin (H&E). Eyes were routinely processed for light microscopy.

2.2. Immunohistochemistry

Axonal injury and neuronal perikaryal reaction in these brains was evaluated using amyloid precursor protein (APP) immunohistochemistry. Brain sections were incubated overnight with a monoclonal antibody to APP (clone 22C11, gift from Professor Colin Masters, Mental Health Research Institute, University of Melbourne) at a dilution of 1:1000 using a standard streptavidin-biotinylated immunoperoxidase technique. Polyclonal antibodies to glial fibrillary acidic protein (GFAP; Dako, Glostrup, Denmark) at a dilution of 1:40,000 and ionised calcium-binding adaptor molecule 1 (Iba1) at a dilution of 1:50,000 (Wako Pure Chemical Industries, Osaka, Japan) were also used as immunomarkers for retinal Muller glial cells and microglia, respectively. In brief, sections were dewaxed using xylene and rehydrated through an alcohol series, and antigen retrieval was performed using citrate buffer (pH 6). Slides were allowed to cool and washed twice in phosphate buffered saline (PBS) (pH 7.4), then endogenous peroxidase activity was quenched. Non-specific proteins were blocked using normal horse serum for 20 minutes. The following day, the sections were given two washes in PBS and then a biotinylated anti-mouse secondary antibody (Vector Laboratories, Burlingame, CA, USA) was applied for 60 minutes at room temperature. Following two PBS washes, the slides were incubated for one hour at room temperature with a streptavidin-conjugated peroxidase secondary antibody (Pierce, Pasadena, CA, USA). Sections were then visualised using diaminobenzidine tetrahydrochloride (DAB), washed, counterstained with H&E, dehydrated, cleared, and mounted on glass slides. Positive and negative controls were included in these protocols. Extravasation of endogenous albumin was detected using a goat polyclonal antibody directed against albumin (Cappel, Westchester, PA, USA) at a 1:250 dilution for 30 minutes. Sections were then washed in PBS. No antigen retrieval was necessary for the albumin antibody.

2.3. Morphometry

Neuronal perikaryal reaction and axonal injury were assessed using a semi-quantitative grid system, which produced a detailed topographical overview of these morphological changes. This morphometric system was concordant with that used in a previous study using this lamb model.[6] A transparent graticule comprised of 4-mm grid squares, each with a unique reference number, was placed over each section. On average, there were 10 coronal slices of the double hemispheres and seven of the cerebellum and brainstem producing, in total, approximately 1100 grid squares representing the entire surface area of the brain sections. The graticule had reference marks so correct alignment could be made with the underlying slide and independent evaluation of brain sections conducted. A central and peripheral reference point was made on each glass slide and these were then matched up with corresponding reference points on the transparent graticule. The detection of any APP immunostaining of axons in a grid square or APP immunoreactive granules occupying at least 50% of the neuronal perikaryon resulted in a positive score, although it is acknowledged that this methodology does not assess the total burden of APP immunopositivity within a given grid square. Axonal injury was only assessed in white matter as axons were sometimes difficult to distinguish from APP-positive dendrites in grey matter. The number of positive grids was then summed and the percentage of APP-positive grids for neuronal cell bodies and axons calculated, yielding a total APP score. The APP reaction was independently assessed by two pathologists, blind to whether the lambs had been shaken or were controls. The distribution of albumin extravasation was also assessed in brains and spinal cords. Statistical analysis of lamb weights and neuronal and axonal APP scores was performed using an unpaired *t*-test.

This project was approved by the Animal Ethics Committees of SA Pathology and the University of Adelaide.

3. Results

There was no hypoxia or sustained hypercarbia or hypocarbia in any animal.

3.1. Neuropathology

At necropsy, the only significant macroscopic finding was mild, focal, subdural, approximately 1 cm × 1 cm, haemorrhage in lambs 3, 6, and 8. Microscopic subarachnoid haemorrhage of mild degree was infrequently found in both subsets of lambs, but was more common in the lower body weight group, particularly where albumin extravasation was marked. Subdural haemorrhage was only

assessed macroscopically. In addition to dying before the designated six-hour post-shaking period (at two, three and five hours), microscopic examination of the brains and spinal cords of three lower body weight lambs revealed some substantial differences compared to a subset of older and heavier, surviving animals. This finding was not anticipated when designing the experimental protocol. Body weights of heavier lambs were significantly ($p < 0.001$) different from those of lighter animals.

Neuronal APP expression (Fig. 1) in the two subsets of lambs was similar in magnitude ($p > 0.05$) and widespread distribution (Table 1), and resembled that found in a previous study.[6] However, there was a significant difference ($p < 0.001$) between the two shaken groups with respect to axonal injury (AI). Total APP AI scores in the higher body weight group (lambs 1–6) were substantially lower than those in the younger lambs (lambs 7–9) (Table 1). Injured axons in the former were few in number, often single, and randomly distributed in hemispheric white matter, brainstem and rostral cervical spinal cord. However, APP immunoreactive axons in these older lambs were more common at the craniocervical junction (CVJ) at the site of maximal impact loading during shaking. AI in the younger subset of lambs was particularly severe in the hemispheric white matter (Fig. 2), but also common in brainstem white matter tracts and at the CVJ (Fig. 3). The distribution of AI (as a percentage of the total burden of AI) in the hemispheric white matter, brainstem and rostral cervical spinal cord was, respectively, in: lamb 7 – 75%, 20% and 5%; in lamb 8 – 60%, 30% and 10%; and in lamb 9 – 60%, 20% and 20%. No APP immunopositive axons were found in control lambs and neuronal APP expression was minimal (Table 1).

Some axons in the cervical spinal cord at the site of maximal stress during shaking, and to a much lesser degree in the caudal brainstem, were embraced by cells expressing APP (Fig. 3). These cells were Iba1-immunopositive microglia and, although a very small number were found in control brains, they were much more numerous in shaken lambs. Moreover, although an occasional APP-positive axon was clasped by these cells, most embraced axons did not express APP.

In view of hypoxic oedematous brain swelling being a prominent feature in a landmark study of human NAHI,[7,8] we decided to evaluate albumin extravasation in these shaken lambs. The neuroanatomical distribution of immunostained albumin in the parenchyma was heterogeneous in both subsets of lambs and multifocal to diffuse, but was frequently found in the cerebral hemispheres (grey and white matter) (Fig. 4A), cerebellar folia white matter (Fig. 4B), brainstem (Fig. 4C) and cervical spinal cord (Fig. 4D). However, in general, albumin leakage was greater in a given region, and more consistently present, in the lower body weight lambs. The total area of albumin extravasation in the brain was 30%, 40% and 20% in lower body weight lambs 7, 8 and 9, respectively, while that in the heavier lambs ranged from 5% to 15%.

In some brainstem nuclei, and usually bilaterally symmetrical, numerous "dark neurons" (Fig. 5) were scattered among neurons of apparently normal appearance in these well-perfused brains. These dark, shrunken and hyperchromatic neurons with elongated and irregular ("corkscrew") dendrites were also randomly distributed in small numbers in the cerebral cortex, central grey matter and cerebellum, but not in the hippocampus. However, cytoplasmic shrinkage and hypereosinophilia with nuclear hyperchromasia or pyknosis ("red neurons") were not found in the cerebral cortex

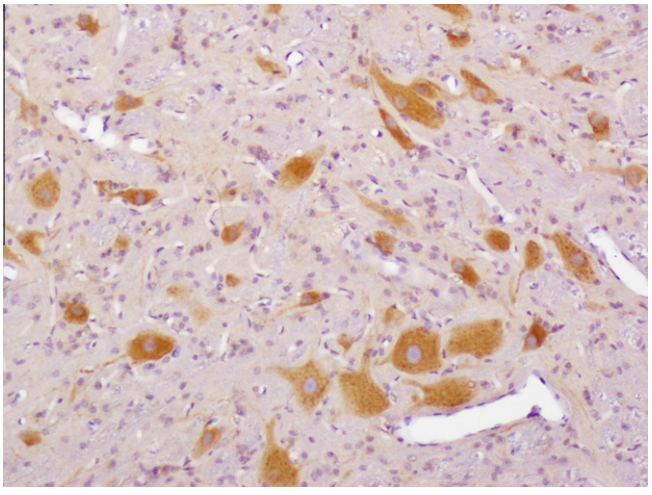

Fig. 1. Immunohistochemistry of brain sections showing marked neuronal amyloid precursor protein (APP) immunopositivity in cervical spinal cord neurons (original magnification, ×10).

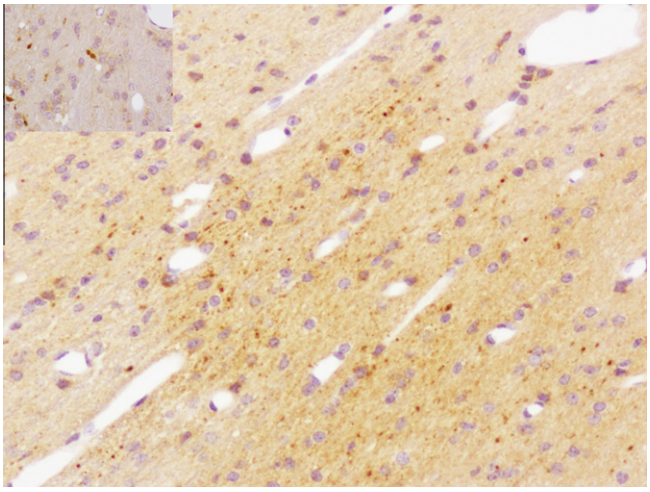

Fig. 2. Immunohistochemistry of a brain section from a younger (lower body weight) shaken lamb (subset II) showing numerous amyloid precursor protein (APP)-immunoreactive, injured axons in the hemispheric white matter (higher power in inset, original magnification, ×40) (original magnification, ×10).

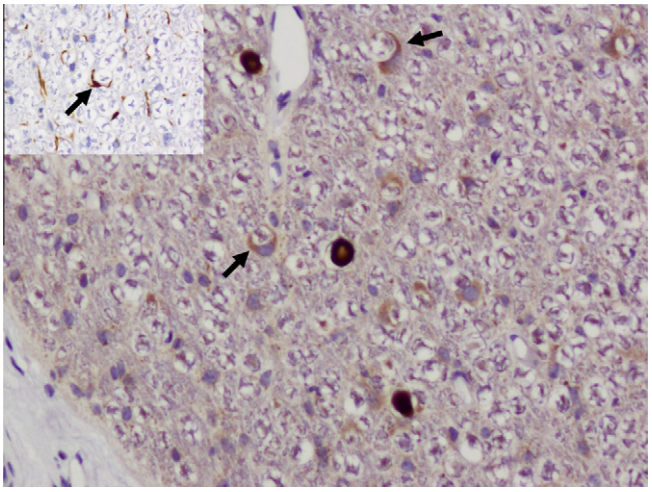

Fig. 3. Immunohistochemistry of a brain section from a younger (lower body weight) shaken lamb (subset II) showing amyloid precursor protein (APP)-immunopositive swollen axons in the rostral cervical spinal cord. Some axons are also embraced by APP-expressing cells (arrows), identified as Iba1-immunopositive microglia (in inset) (original magnification, ×20).

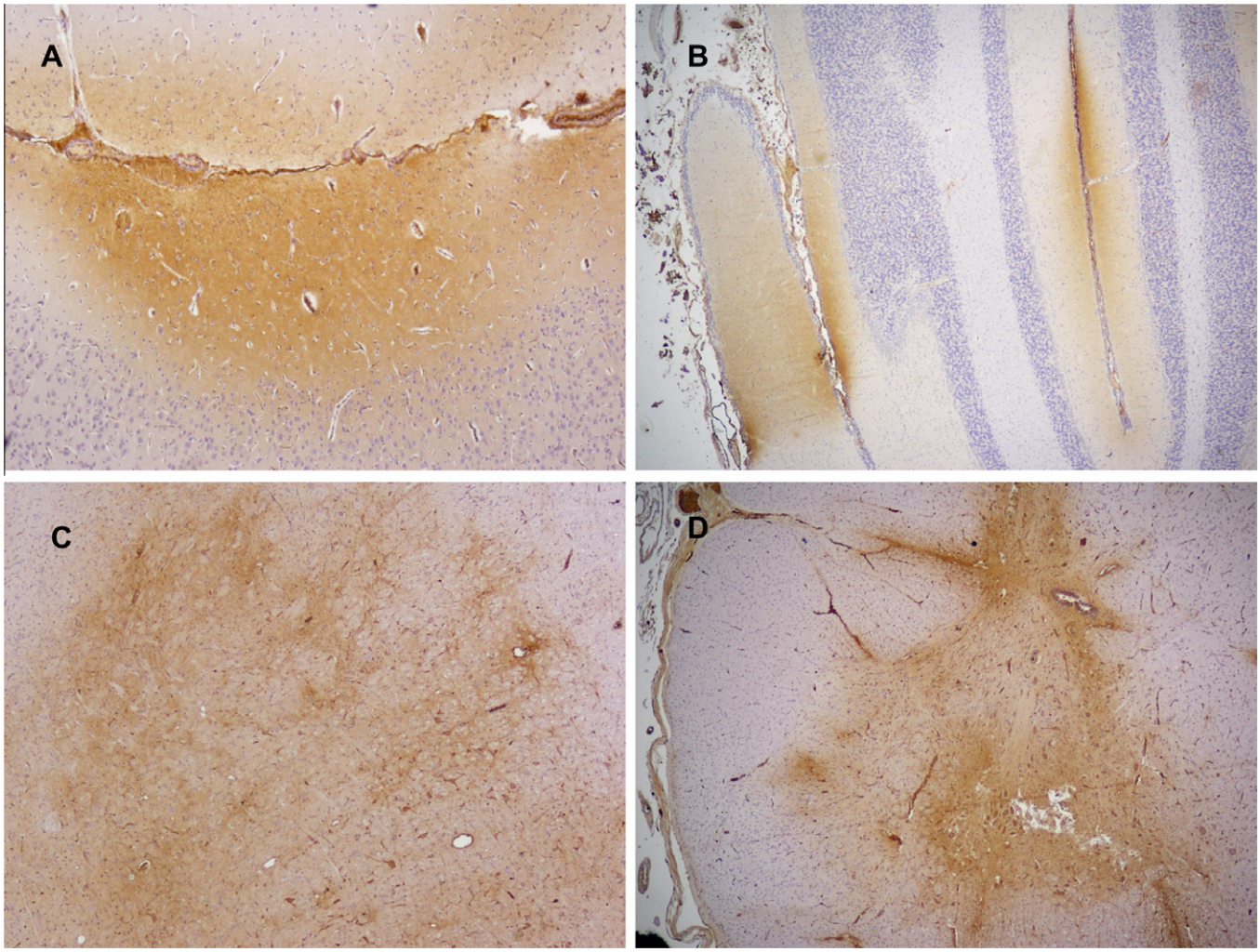

Fig. 4. Immunohistochemistry of brain sections from a younger (lower body weight) shaken lamb (subset II) showing diffuse albumin extravasation in: (A) the cerebral cortex, (B) cerebellar folia, (C) brainstem and (D) cervical spinal cord (original magnification, ×4).

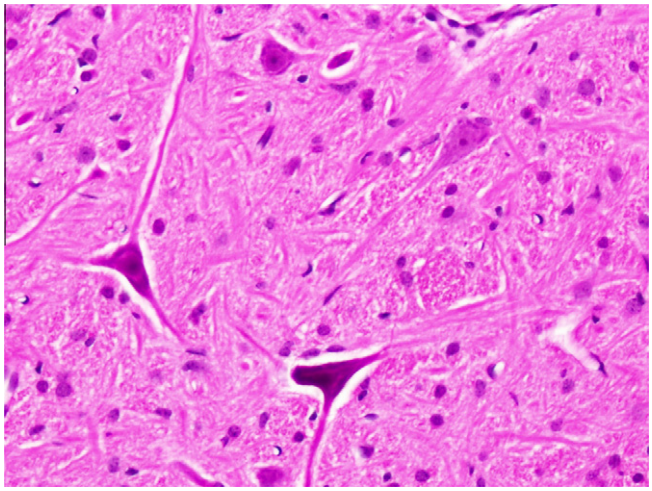

Fig. 5. Photomicrograph of a brainstem nucleus from a younger shaken lamb (subset II) showing "dark" neurons, with a few neurons of normal appearance (haematoxylin and eosin, original magnification, ×40).

(except for a single focal area in lamb 7), caudate–putamen, hippocampus or cerebellar cortex.

3.2. Ocular pathology

In shaken lambs, there was multifocal damage to inner nuclear layer neurons of the retina in the form of pyknotic nuclei (Fig. 6), sometimes surrounded by a narrow rim of hypereosinophilic cytoplasm and, less commonly, condensed chromatin caps against the nuclear membrane, suggestive of apoptosis. Mild, segmental, splitting of this layer was also evident. Many ganglion cells showed increased APP expression (but appeared normal on routine H&E sections) and some unmyelinated ganglion cell axons in the nerve fibre layer were APP immunopositive (Fig. 7). However, although the optic nerve is a continuation of ganglion cell axons into the brain, these axons becoming myelinated at about the level of the lamina cribrosa where they exit the globe through the sclera at the posterior pole, no APP-immunoreactive axons were found in this nerve in shaken lambs. GFAP immunostaining of Muller glial cells in retinas from control lambs was largely confined to the thick inner limiting membrane and ganglion cell and inner plexiform layers (Fig. 8A). However, in shaken lambs, these glia showed increased GFAP immunopositivity, their processes being thicker and more prominent. Moreover, their GFAP immunoreactive processes spanned more of the retina, extending through the inner nuclear and outer plexiform layers and abutting on the outer nuclear layer (Fig. 8B). The outer nuclear layer containing the cell bodies of photoreceptors appeared unaffected. While no haemorrhage was

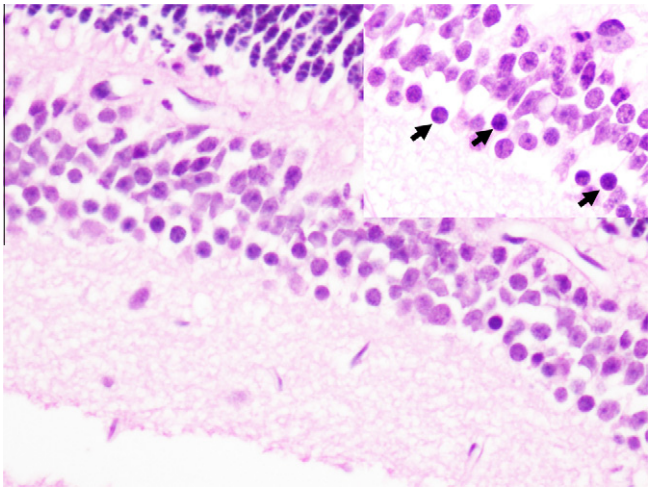

Fig. 6. Photomicrograph of the retinal inner nuclear layer neurons showing necrosis (arrows) (haematoxylin and eosin, original magnification, ×20; inset ×40.).

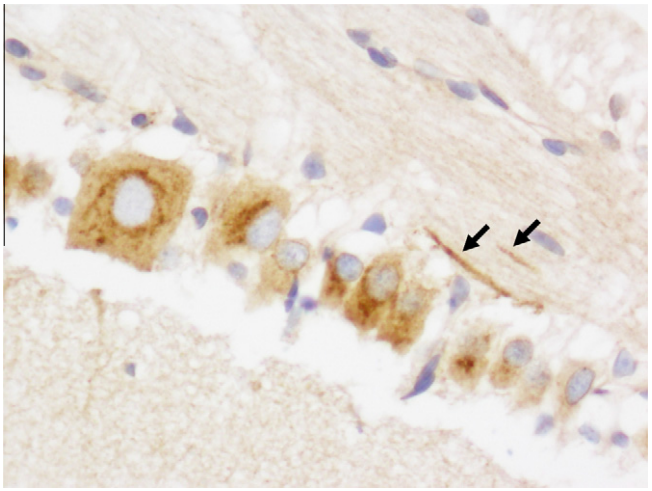

Fig. 7. Immunohistochemistry of shaken lambs showing amyloid precursor protein (APP)-positive, injured ganglion cell axons (arrows). Ganglion cells are also expressing APP (original magnification, ×40).

found in serial histological sections of retina, optic nerve or uvea (iris, ciliary body, and choroid), albumin extravasation was observed in the uvea, the vascular tunic of the globe, in shaken lambs. Ocular lesions in the higher body weight lambs were of a similar nature to those in lighter animals, but they were of much greater magnitude in the latter subset.

4. Discussion

The results of this study contribute to the debate on whether shaking alone is sufficient to cause brain damage in NAHI or whether an additional head impact is required. We have shown that shaking in a lamb model of NAHI caused death in a subset of lower body weight animals (5.0–6.0 kg), whereas lambs of higher body weight (8.5–12.0 kg) survived for six hours post-shaking until killed by perfusion fixation of the brain. This outcome in the younger lambs, which were delivered for experimentation earlier than anticipated, was unexpected as death did not occur in older lambs in this study, or in a previous experiment[6] using lambs of similar body weight to the heavier group reported herein. Support for the head from the cervical musculature during shaking was

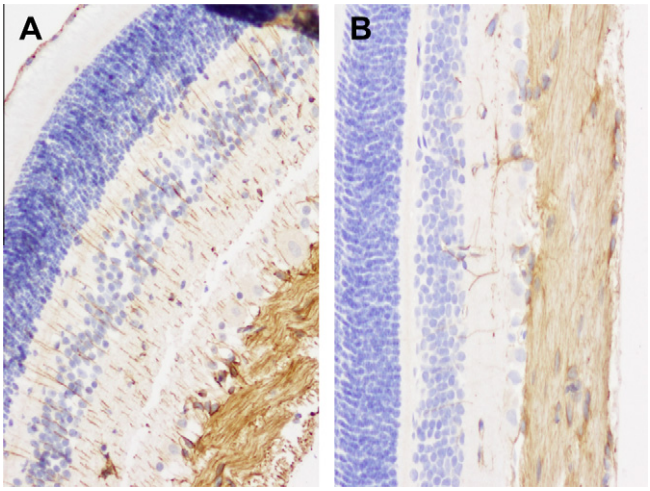

Fig. 8. Immunohistochemistry of the retinas of lambs showing that Muller glial cell glial fibrillary acidic protein (GFAP) immunopositivity is much more widely distributed in the retina of (A) a shaken lamb, with increased GFAP immunopositivity, and thicker and more prominent processes compared to (B) a control lamb, where staining was largely confined to the thick inner limiting membrane and ganglion cell and inner plexiform layers (original magnification, ×10).

probably less in the lower body weight lambs, particularly as neonatal lambs grow very rapidly. While this is an animal model of NAHI, the lamb nevertheless resembles a human infant in important respects in the present context, namely having a relatively large gyrencephalic brain and weak neck muscles supporting the head.

In a large study of human NAHI,[7,8] there was macroscopic and/or microscopic evidence of craniocervical injury in about one-third of cases, which could have lead to apnoea and subsequent hypoxic brain swelling. Apnoea was the presenting sign in 75% of the cases in Geddes et al.[7,8] The AI in the brainstem of shaken lambs, and at the CVJ, together with marked neuronal "dark cell" change in some brainstem nuclei, may have resulted in apnoea and cardiorespiratory arrest. Although "dark cell" change is a common artefact in immersion-fixed brains, these lamb brains were perfusion-fixed and "dark neurons" were scattered among neurons of normal appearance. Dark neurons in perfusion-fixed material are now considered to represent an early, reversible morphological change, and do not indicate cell death. Rather, cell membranes and cytoplasmic organelles are intact and no permanent damage ensues.[9,10]

AI in the young lambs that died after shaking was much greater and more widely distributed in the brain compared to the older and heavier lambs, being particularly severe in the hemispheric white matter, but also substantial in the brainstem and at the CVJ. The AI in human NAHI was regarded by Geddes et al.[7,8] and Shannon et al.[11] as of ischaemic or vascular, rather than traumatic, origin, with global hypoxic brain damage found in up to 85% of NAHI brains. However, since evidence of hypoxic–ischaemic injury was limited to a single, focal cerebral cortical area in one lamb in the present study, and continuous physiological monitoring of these ventilated animals ensured that no hypoxic episodes supervened, the neuropathological changes are more likely due to mechanical deformation. The Iba1-immunoreactive, axon-embracing microglia found at the CVJ were presumably an early indicator of AI at the site of maximal impact loading during shaking since microglia respond more rapidly than any other neuroparenchymal element to changes in their microenvironment and function as intrinsic sensors to insults.[12]

Neuronal perikaryal APP immunopositivity was widely distributed in the brain, spinal cord and retinal ganglion cells of this, and a previous study,[6] and probably represented a non-specific, acute stress response to trauma.[13,14]

In lamb brains, especially in those that died, there was widespread, multifocal to diffuse, albumin extravasation in the cerebral hemispheres, cerebellar folia, brainstem and rostral cervical spinal cord. The cause of this blood-brain barrier breakdown was not precisely determined, although diffuse albumin leakage found in cerebral and cerebellar cortices could have been due to differential movement between the superficial brain and the skull during shaking, resulting in mechanical deformation of the vasculature.

The observed retinal damage in shaken lambs may also have been caused by mechanical deformation from rotational/translational and acceleration/deceleration forces. It was characterised by multifocal injury to inner nuclear layer neurons, widespread Muller glial reaction, and increased APP expression in ganglion cells, with injury to some of their axons. Albumin extravasation was also found in the uveal tract. Optic nerves, however, appeared to be unaffected, possibly because they are more securely tethered than the retina.

Although it is debated whether shaking alone can generate impact loading sufficient to cause brain damage consistent with NAHI or whether an additional head impact is required,[15,16] our finding that shaking alone resulted in death in a subset of younger lambs is evidence that a head impact is not always needed.

The pathological and biomechanical aspects of this paediatric disorder remain controversial and intermittently undergo revision.[17] Mechanisms of brain injury may also vary between individual NAHI cases,[18] a reliable history of the precise circumstances surrounding the abuse is usually lacking,[19] and any wrongdoing is frequently denied by the perpetrator. The absence of any external evidence of head trauma also does not necessarily negate a diagnosis of NAHI for, if the head is impacted against a soft surface, substantial brain damage may still be sustained from rapid angular deceleration of the head. Furthermore, since the lesions found in NAHI are not pathognomonic for inflicted head trauma, a pathological diagnosis of NAHI should be concluded with caution, unless there is other corroborating evidence of abuse or a convincing admission by the perpetrator.[4]

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