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Laboratory Study

Diffuse neuronal perikaryal amyloid precursor protein immunoreactivity in an ovine model of non-accidental head injury (the shaken baby syndrome)

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ABSTRACT

Non-accidental head injury ("shaken baby syndrome") is a major cause of death and disability in infants and young children, but it is uncertain whether shaking alone is sufficient to cause brain damage or an additional head impact is required. Accordingly, we used manual shaking in an ovine model in an attempt to answer this question since lambs have a relatively large gyrencephalic brain and weak neck muscles resembling a human infant. Neuronal perikaryal and axonal reactions were quantified 6 hours after shaking using amyloid precursor protein (APP) immunohistochemistry. Neuronal perikaryal APP was widely distributed in the brain and spinal cord, the first time such a diffuse neuronal stress response after shaking has been demonstrated, but axonal immunoreactivity was minimal and largely confined to the rostral cervical spinal cord at the site of maximal loading. No ischaemic-hypoxic damage was found in haematoxylin and eosin-stained sections.

1. Introduction

In Western industrialised countries, traumatic head injury is the leading cause of death and disability in infancy and childhood[1] and inflicted head injury comprises almost 25% of all head injuries in children less than 2 years of age admitted to hospital.[2]

Caffey first identified a causal link between shaking and infant subdural and retinal haemorrhages, and long-bone fractures.[3,4] Subsequently, the neuropathological triad of subdural and retinal haemorrhages and acute encephalopathy was termed the "shaken baby syndrome" (SBS).[5] It has also frequently been designated "non-accidental head injury" (NAHI)[6] or, by those who contend that a head impact is a precondition for the development of this lesion complex, the "shaken-impact syndrome".[7] However, none of these lesions is pathognomonic of inflicted head trauma.[8]

Death occurs in 10% to 40% of patients with NAHI.[9] The most severe presentation is that of the collapsed, apnoeic baby or one showing severe respiratory distress, but there may be more subtle and non-specific signs, including lethargy, irritability, seizures, vomiting and inappetence. Survivors frequently experience chronic neurological problems such as cognitive and behavioural disturbances, cerebral palsy, blindness and epilepsy.[10,11]

One of the dominant controversies in the SBS is whether a head impact is necessary to produce pathology or whether shaking alone is sufficient to injure the brain. Its resolution is frequently impeded by difficulty eliciting the circumstances surrounding these injuries due to denial or obfuscation by the perpetrators.[12] A recent survey of the literature spanning three decades[13] revealed that abuse was admitted in <20% of cases and that there was evidence of cranial impact in 80%. Other studies[14–16] have concluded that head impact is not essential. Furthermore, impact of the head with a soft surface may not produce contact injuries, but sufficient angular deceleration may be generated to damage neural tissue due to the sudden deceleration of the head.[17] Biomechanical studies have shown that head impact generates a very much higher loading than shaking.[18] However, while some[7] argue that shaking is insufficient to injure the brain, others[19] contend that shaking alone cannot be excluded as sufficient cause.

There is currently no satisfactory biomechanical model in which to investigate the pathogenesis of SBS or potential therapeutic intervention strategies.[20] Accordingly, since sheep have a relatively large gyrencephalic brain resembling that of humans, we used neonatal lambs to examine neuronal perikaryal and axonal changes in the brain resulting from shaking alone. Neuronal perikaryal reactions and axonal injury in these brains were detected using amyloid precursor protein (APP) immunohistochemistry.

2. Materials and methods

2.1. Experimental protocol

Seven anaesthetised and ventilated, 7–10-day-old lambs were manually grasped under the axilla and vigorously shaken with enough force to snap the head back and forth onto the chest, similar to the actions believed to occur in the SBS.[4,17] This shaking also re-

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sulted in considerable lateral and rotational head movement. Each lamb was shaken in this manner regularly (10 times of 30 seconds duration) over a 30-minute period, then placed quietly in the sphinx position for 6 hours under anaesthesia. There was no head impact. Three, age-matched control lambs were not shaken, but otherwise subjected to the same experimental protocol.

Lambs were maintained under anaesthesia until killed by perfusion fixation of the brain with 4% paraformaldehyde containing 0.02% heparin. Brains remained *in situ* for 2 hours and were then immersed in 10% buffered formalin for 7 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 and paraffin-embedded. Eyes were routinely processed for light microscopy.

2.2. Immunohistochemistry

Axonal 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 at a dilution of 1:3000, stained with 3,3-diaminobenzidine tetrahydrochloride (DAB, Sigma-Aldrich, St Louis, MO, USA) using avidin-biotin peroxidase (Vector ABC kit; Vector Laboratories, Burlingame, CA, USA) and counterstained with haematoxylin. An APP-positive control brain, and a negative control with the primary antibody excluded, accompanied each staining procedure. Duplicate sections were stained with haematoxylin and eosin (H&E).

2.3. Morphometry

Neuronal perikaryal and axonal reaction was assessed using a semi-quantitative grid system. This method produces a detailed topographical overview of the neuronal cell body and axonal response. 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 cervical spinal cord was analysed in a similar manner. 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 reactive granules occupying at least 50% of the neuronal perikaryon resulted in a positive score. 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, producing an APP score ranging from 0 to 100. The APP reaction was independently assessed by two pathologists, blind to whether the lambs had been shaken or were controls. These data were statistically analysed by an analysis of variance (ANOVA) and Student's *t*-test.

This project was approved by the Animal Ethics Committee of the Institute of Medical and Veterinary Science, Adelaide.

3. Results

At necropsy, the only significant macroscopic finding was focal subdural haemorrhage, confined to a 1 × 1 cm area, in two shaken lambs (lambs 1 and 5). Microscopically, neuronal perikaryon APP immunoreactivity (Fig. 1) was widely distributed (Table 1), including cerebral cortical neurons, cerebellar Purkinje cells and brainstem neurons. The neuronal perikaryal APP reaction in the treated group (mean ± standard deviation, 43.3 ± 15.8) was higher than that of the control group (2.3 ± 0.6) ($p < 0.01$, ANOVA and Student's *t*-test). There was no evidence of neuronal loss in areas showing increased APP expression. By contrast, there were very

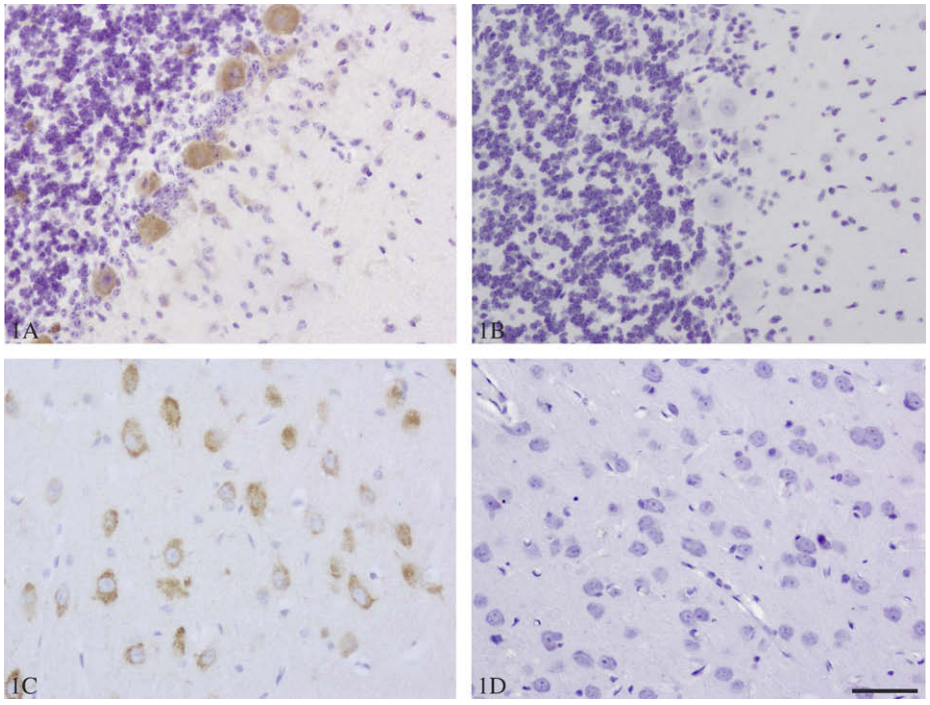

Fig. 1. Amyloid precursor protein (APP) immunostaining of (A, B) the cerebellar and (C, D) cerebral cortices showing (A) numerous immunopositive (brown) Purkinje cells and (C) cortical neurons in shaken lambs, while these neurons are immunonegative in (B, D) control lambs. Bar = 50 μm.

Table 1
Percentage of grid squares containing amyloid precursor protein-positive axons and neurons

Lamb No.	Axonal reaction	Neuronal reaction
Control		
1	0	2
2	0	2
3	0	3
Shaken		
1	6	76
2	6	38
3	8	45
4	3	41
5	3	44
6	2	28
7	3	31

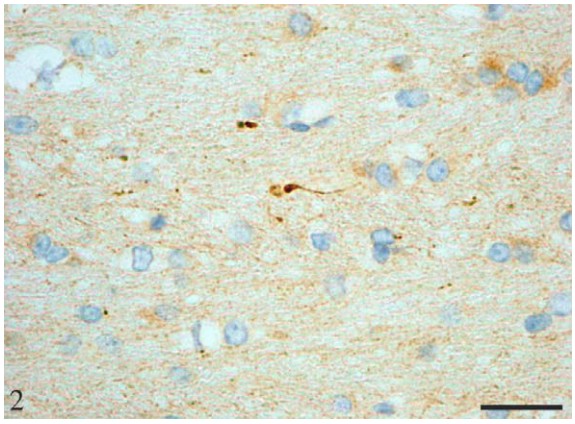

Fig. 2. Amyloid precursor protein (APP) immunostaining showing only a few APP-positive (brown) axons in the brainstem of a shaken lamb. Bar = 50 μm.

few immunopositive axons (often <1% positive grids and never >2%) and they were usually single (Fig. 2) and randomly distributed in hemispheric and cerebellar white matter, midbrain, and brainstem (Table 1). However, APP-positive axons were more common, albeit still very limited in number, in the rostral cervical spinal cord at the site of maximal stress. No parenchymal haemorrhage or recognizable ischaemic-hypoxic damage was found in H&E-stained brain and spinal cords. Minor retinal haemorrhage was observed in two lambs (lambs 1 and 5) and no APP-positive axons were detected in optic nerves. In control lambs, no APP-positive axons were observed (and thus statistical analysis could not be performed) and neuronal APP expression was minimal (2–3% positive grid squares).

4. Discussion

The results of this study showed that vigorous whiplash shaking of lambs produces widespread neuronal perikaryal APP expression. This generalized upregulation of neuronal APP probably represents a non-specific, acute stress response to trauma[21,22] as it is unlikely that all these APP-immunoreactive neurons were irreversibly damaged. Rapidly developing and widely distributed neuronal perikaryal APP expression was also found in an ovine head impact model[23] due to upregulation of APP messenger RNA.[24] We believe this to be the first report of such a diffuse, acute phase neuronal reaction in the brain to shaking.

By contrast, axonal injury (AI) was minimal in these lambs, occurring as individual APP-positive fibres randomly distributed in the white matter, although it was minimally more common in the rostral cervical spinal cord at the site of maximal loading during shaking. The craniocervical joint may be particularly vulnerable in infants <3 months of age and damage at this site may provide a substrate for the common presenting signs of apnoea and respiratory distress.[25–27] Diffuse (multifocal) traumatic AI (dTAI) was not found in shaken lambs and, although a few studies[15,28,29] have reported diffuse axonal injury (DAI) in patients with SBS, it is generally accepted that dTAI occurs rarely in infants <1 year of age.[27,30]

Death is most patients with NAHI is now attributed to diffuse cerebral swelling resulting from global hypoxic brain damage.[16,26,30] In one large study, 75% of abused babies presented with severe apnea and most died from raised intracranial pressure (ICP) secondary to brain swelling.[25] Global hypoxic brain damage was commonly found upon microscopic examination and, although one-third had vascular AI related to increased ICP, dTAI was rarely observed. No identifiable ischaemic–hypoxic injury was found in shaken lambs.

Neuronal perikaryal APP expression in these shaken lambs was similar to that found in lambs, albeit somewhat older (4–5 weeks), impacted to different regions of the head with a humane stunner.[31] In the latter, neuronal APP upregulation was widely distributed and APP scores after temporal, frontal and occipital impacts were 40, 78 and 74, respectively. However, APP-positive axons in this ovine impact–acceleration model were also widespread, with APP scores of 12, 35 and 22, respectively, much higher than in the shaken lambs. Most impacted lambs also had contusions, unlike shaken lambs in the present study.

The response of a child's brain to an insult often differs from that of an adult.[1] A disproportionately large infant head, weak cervical muscles and a relatively large subarachnoid space permit significant differential movement of the immature brain within the skull during shaking[17] and the incompletely myelinated white matter and higher brain water content predispose the infant brain to shear injury.[32] The thin, pliable infant skull also transmits impact forces more readily to deeper brain structures.[33] Shaking is an acceleration–deceleration type of injury which is similar to whiplash, but of longer duration. It is the sudden deceleration of the brain that is believed to cause intracranial injury[17] and brain damage is accentuated if additional contact forces are applied to the head.

A small subdural haemorrhage was found in two shaken lambs, due to tearing of fragile bridging veins between the cortical surface and dural sinuses during shaking,[17] but retinal haemorrhages were minimal and only seen in two animals. However, subdural and ocular haemorrhage, although common in patients with NAHI, is not specific for trauma.[11]

There have been very few animal models attempting to replicate brain injury due to head shaking. Raghupathy and Margulies[34] and Raghupathi et al.[35] subjected neonatal pigs to head rotational acceleration in the axial plane. A single, mild rotational acceleration of the head produced AI in frontal lobes only, while consecutive rotations produced additional AI in parietal and temporal lobes, the corpus callosum, hippocampus and basal ganglia. Neuronal perikaryal changes were not described. Smith et al.[36] and Bonnier et al.[37] used a rotating shaker to produce more severe brain injury in neonatal rats and mice, respectively, but these are lissencephalic species and the high mortality rate (27%) in the Bonnier et al.[37] study suggested that rotational loading may have been much greater than occurred in lambs or piglets. The length of time from shaking to necropsy was also much greater in these rodent studies. Moreover, while the mechanical devices used in these studies facilitated reproducibility of shaking, they do not produce the type of head shaking believed to occur in real-world instances of human infant abuse.

In conclusion, this lamb model of the SBS showed widespread neuronal perikaryal APP immunoreactivity, consistent with an acute stress response, but minimal APP immunopositive AI.

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