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Traumatic axonal damage in the brain can be detected using β-APP immunohistochemistry within 35 min after head injury to human adults

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Immunohistochemistry staining for β-amyloid precursor protein (β-APP) is a sensitive method to detect early axonal damage in traumatic brain injury, which was previously estimated to be of minimum 60–90 min after head injury. We present seven cases of well-documented post-traumatic survival of 35–60 min where β-APP detects early axonal damage. Cases were selected from routine work where documentation about survival is judged to be accurate. These are divided into three groups: group 1: severe head injury ($n = 7$) with documented survival between 35 and 60 min. Group 2: severe head injury ($n = 4$) with documented survival of less than 30 min. Group 3: cases ($n = 4$) where death was not due to head injury but survival is documented between 45 and 109 min. The brains were fixed in formalin for 4 weeks and six regions (frontal lobe with anterior corpus callosum, parietal lobe with deep white matter, basal ganglia with posterior limb of internal capsule, cerebellum with white matter and middle cerebellar peduncle and pons with basis pontis and superior cerebellar peduncle) were sampled. All blocks were stained for haematoxylin and eosin and β-APP and selected ones for CD68, using antigen retrieval method. In group 1 sections revealed β-APP immunoreactivity in forms of small globules and granules and occasionally as thin and short filaments. These were detected in the pons, corpus callosum, internal capsule and cerebral white matter, with some variation in localization and intensity. In groups 2 and 3 all the sections were negative for β-APP staining. None of the cases showed evidence of severe brain swelling, increased intracranial pressure, ischaemia or infection. Using the antigen retrieval method, β-APP immunohistochemistry can detect axonal damage within 35 min after severe head injury. These results may have an implication in the consideration of minimal survival time after traumatic head injury in medico-legal practice.

Keywords: antigen retrieval, β-amyloid precursor protein (β-APP), diffuse traumatic axonal injury, immunohistochemistry, survival time

Introduction

Blunt head injury is associated with acceleration and deceleration forces to the head. In addition to focal damage to the brain it produces shearing, traction and compression, which may damage axons and small blood

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vessels. The axonal injury (AI) often involves specific supra- and infratentorial sites although the distribution and intensity of axonal damage vary from case to case and are related to the type and severity of impact [1,2]. The traumatic axonal injury (TAI) can be multifocal (mTAI) and in more severe cases diffuse [dTAI or traumatic diffuse axonal injury (tDAI)], involving specific infra- and supratentorial sites. It must be emphasized: not only trauma, but also ischaemia, hypoglycaemia and other metabolic imbalances, inflammation, drug and alcohol intoxication, and even ageing can cause AI [3].

Two mechanisms of AI are proposed: primary axotomy, which may occur at the time of injury in severe head trauma, and secondary, which occurs over a period of hours (h), or perhaps minutes (min), after the injury. Recent evidence suggests that secondary axotomy is the main, and in most cases the only pathomechanism [4]. There is focal damage to the axonal cytoskeleton, which results in impaired or blocked axoplasmic transport with subsequent accumulation of material proximal to the damaged segment [5]. The axonal swellings and varicosities are probably reversible as long as mitochondrial structures remain intact [4].

AI resulting from head trauma can be diagnosed and graded only by histological examination of the brain with wide sampling [2]. As the presence of AI can be an indicator of vitality at the time of injury and is often a consequence of head injury, its detection – particularly in cases with short or uncertain survival time – is of great medico-legal significance [2].

Haematoxylin and eosin (H&E) stains and silver impregnation visualizes damaged axons 24 and 15–18 h after injury respectively [6]. CD68, glial fibrillary acidic protein immunohistochemistry (IH) detects reactive changes days after injury. IH for chromogranin A, synaptosomal associated protein, cathepsin D, ubiquitin has also been used to highlight affected axons several hours after injury; however, the results are conflicting and they are definitely less reliable markers [7], as are the 68 kDa neurofilament protein [8] and neurone-specific enolase (NSE) [9]. The most sensitive and specific tool is β-amyloid precursor protein (β-APP) IH, which is capable to detect positive axon even after shorter survival [10].

β-APP is a ubiquitous membrane glycoprotein produced in the cell body. It plays a physiological role in cell adhesion and endogenous neuroprotection in response to injury [11] and the amyloidogenic proteolytic pathway is related to beta-amyloid accumulation, plaque formation and Alzheimer's disease [12]. β-APP is transported in vesicles by fast anterograde axoplasmic transport as it has synaptic function and accumulates proximal to the site of AI [5]. Therefore, β-APP IH detects damaged axons, however, is *not* specific for a traumatic aetiology. A recent study demonstrated that with appropriate sampling and examination of H&E-stained sections and β-APP IH can determine the cause of axonal pathology in most cases [13]. There is a noted correlation between the size of axonal β-APP positivities and survival time [14]; however, it does not enable a precise timing of injury [15].

β-APP has been generally thought not to be detectable by means of IH earlier than 1 h after traumatic brain injury (TBI) [16], although sporadic reports of earlier detection emerged recently (see *Discussion*). We present seven cases with well-documented survival times of less than 60 min and with a minimum of 35 min where using antigen retrieval techniques subtle but consistent immunopositivity was detectable. There was no β-APP immunoreactivity neither in the head-injured patients with less than 30 min survival nor in non-head injured control subjects with survival times of 45–109 min.

Materials and methods

Material and design of the study

Because accurate timing of TBI was a prerequisite for the validity of this study, we felt that the best way is to prospectively collect medico-legal cases where we can obtain reliable information before conducting full neuropathological examination of the brain. We have studied more than 150 cases of head trauma for a period of almost 2 years and selected consecutive cases where the information regarding timing were judged to be reliable. All these cases had undergone extensive forensic examination and detailed police investigation for medico-legal purposes. We then grouped our selected cases into three groups, depending on survival time and diagnosis.

Group 1 contains seven cases of severe head injury and had survived between 35 min and 1 h. In these cases, the cause of death was determined as severe head injury by general autopsy and brain examination. Other natural

Table 1. Clinical and pathological findings in group 1 (head injury survived 35–60 min after head trauma)

No.	*Age and sex*	*History*	*Autopsy findings*	*Skull fracture*	*Cause of death*	*Survival time; source of info*	*Brain weight*	*Brain swelling*
1	43 F	Assault, punched and kicked	Deep scalp bruises and facial injuries	Numerous skull vaults	Head and facial injuries	< 60 min – police witness and hospital records	1313 g	Mild
2	57 M	Assault, punched and fell backward on hard object	Bruises over right eye	No	Severe head injury	55 min – CCTV, police and ambulance records	1572 g	No
3	80 M	Assault, punched and fell forward on his head	Abrasion and bruises of head, face, shoulder, chest	Ring fracture around foramen magnum	Severe brainstem injury	45 min – police, ambulance and hospital records	1695 g	No
4	33 M	Assault, punched and kicked including swinging punch	Bruises in face and head	No	Severe head injury	50 min – police, ambulance and hospital records	1382 g	Mild
5	41 M	Fall from 3 m height	Severe bruising on scalp	Skull fracture in occipital bone passing to foramen magnum	Severe head injury	< 35 min – reliable witness, ambulance and hospital records	1394 g	Mild
6	25 M	Assault, severe punching and kicking to head and face	Severe facial injuries	No	Head facial injuries	< 60 min – CCTV and ambulance records	1648 g	Moderate with flat gyri
7	66 M	Assault, punched and fell to the floor rendering him unconscious	Severe bruising at back of head	Fractured occipital bone	Severe head injury	35 min – reliable witness, ambulance and hospital records	1330 g	No

info, information; ICP, increased intracranial pressure; APP, β-amyloid precursor protein; IH, immunohistochemistry; F, female; ant, anterior; post, posterior; int, internal; –ve, negative; M, male; CCTV, closed circuit television; SAH, subarachnoidal haematoma; IVH, intraventricular haematoma; mid cbl ped, middle cerebellar peduncle; EDH, extradural haematoma.

and unnatural causes were excluded. The group contains one female and six males ranging from 25 to 80 years old. Six of the cases had a history of assault with punching and kicking and including three who had fallen on their heads, with severe impact. One case had fallen from a 3-m height and had skull fracture in different location. The cause of death was concluded after forensic, autopsy and neuropathological examination as severe head injury in five cases and as severe head and facial injuries in the other two cases (for more details see Table 1).

Group 2 contains four cases with survival times of less than 30 min (one died almost immediately) with severe head injury. All were male with age range of 30–66 years. Two had skull fractures and one had extensive fracture of the facial skeleton. Their injuries resulted from assaults with punching and kicking to head and face, in one case with strangulation. All showed extensive and deep bruising to the head and face region (Table 2).

Group 3 is a control group of four heterogeneous cases where the cause of death was not TBI and survival times were between 45 and 109 min. They were all males and ranged in age from 24 to 36 years. One was stabbed to the neck, two collapsed after heavy drinking, one collapsed for unknown cause. None had skull fracture (Table 3).

Methods

In all the cases, the brains were referred from practising forensic pathologist and the brain examination was

High ICP	*Intra-cranial haem-orrhage*	*Contusion*	*Intra-cerebral haematoma*	*Corpus callosum haem-orrhage*	*Brainstem haem-orrhage*	*Ischaemia*	*APP immuno-positivity*	*CD68 IH*
No	No	No	No	No	Small in lining of 4th ventricle	No	Ant & post corpus callosum, int capsule, white matter	–ve
No	Mild SAH and mild IVH	Mild frontal and temporal	No	No	No	No	Ant & post corpus callosum, int capsule, pons, white matter	–ve
No	SAH	No	No	Small	Extensive	No	Ant & post corpus callosum, pons, white matter	–ve
No	Mild SAH	Mild frontal and temporal	No	Small	No	No	Ant & post corpus callosum, mid cbl ped, int capsule, pons	–ve
No	Diffuse SAH	Mild frontal occipital	Small in thalamus	Small	Small in dorso-lateral quadrant	No	Ant & post corpus callosum, mid cbl ped, int capsule, pons	–ve
No	No	Gliding contusion	No	Small	No	No	Ant & post corpus callosum, int capsule, pons	–ve
No	Thin SAH and very thin patches of EDH	Mild frontal and temporal lobes	Small pinpoint in parietal and occipital White matter	No	No	No	Ant & post corpus callosum, mid cbl ped, int capsule, pons	–ve

thought to be important to the police investigation. The cases were accepted and entered in the departmental records and only examined when the circumstances of death, history of events, timing and hospital notes were provided by the investigating police department and forensic pathologist.

The brains were fixed for 3–4 weeks and examined externally and cutting 0.7-cm-thick coronal sections and recording all the details. Then, a full set of tissue blocks were taken for histology [17], including: (i) frontal lobe with parasagittal white matter and anterior part of corpus callosum; (ii) basal ganglia and thalamus where full length of posterior limb of the internal capsule was present; (iii) parietal lobe with deep white matter; (iv) splenium (posterior part of corpus callosum); (v) cerebellum with white matter and middle cerebellar peduncle; (vi) temporal lobe with hippocampus; (vii) pons (including the superior cerebellar peduncle).

All blocks were stained for H&E and examined microscopically. IH for β-APP in all groups and the microglia/macrophage marker CD68 (clone PGM1) in group 1 were performed. For β-APP detection, 7-μm paraffin sections on slides coated with 3-aminopropyltriethoxysilane were used. Sections were dewaxed in xylene, rehydrated in 99% industrial methylated spirit and endogenous peroxidase activity was blocked by incubation for 30 min at room temperature (RT) in 200-ml methanol containing 5-ml H_2O_2. Sections were washed well in running tap water and placed in TRIS-buffered saline (TBS) pH 7.8 for a few minutes and subsequently microwaved in a plastic

Table 2. Clinical and pathological findings in group 2 (head injury survived less than 30 min after head trauma)

No.	*Age and sex*	*History*	*Autopsy findings*	*Skull fracture*	*Cause of death*	*Survival time/(min) source of info*	*Brain weight*	*Brain swelling*	*High ICP*	*Intracranial haemorrhage*	*Contusion*	*Intracerebral haematoma*	*Corpus callosum haemorrhage*	*Brainstem haemorrhage*	*Ischaemia*	*APP*
1	48 M	Assault – punched and kicked	Periorbital haematoma Lacerations to face; Lacerations and bruises to scalp	Fracture in anterior fossa	Facial and head injury and strangu -lation	< 30 min – reliable witness, police and ambulance records	1381 g	Mild	No	Mild SAH	Small in frontal lobe	Small pinpoint in parietal lobe	Small in anterior and posterior corpus callosum	No	No	–ve
2	34 M	Assault – punched twice and fell on his back	Bruises and lacerations to the face; Bruises in the occipital scalp	Facial fracture; occipital bone fracture	Severe head injury	< 30 min – police, ambulance and hospital records	1641 g	Mild	No	Small SDH; mild SAH	Mild in frontal and parietal lobes	Multiple pinpoint in white matter and pons	No	Small around ventricul ar lining	No	–ve
3	66 M	Assault – three separate blows to head and strangu- lation	Three scalp bruises and lacerations	No	Head injury and strangu - lation	Probably < 30 min – police and ambulance records	1299 g	No	No	No	No	No	No	No	No	–ve
4	30 M	Assault – punched and kicked to head and face	Bruises deep in the scalp; Facial injuries	None but extensive facial fractures	Severe head and facial injury	< 30 min almost immediately – reliable witness and ambulance records	1321 g	No	No	No	No	No	No	No	No	–ve

info, information; ICP, increased intracranial pressure; β-APP, β-amyloid precursor protein; –ve, negative; M, male; min, minutes; SAH, subarachnoidal haematoma; SDH, subdural haematoma.

Table 3 Clinical and pathological findings in group 1 (non-head injury cases survived more than 35 min)

No.	Age and sex	History	Autopsy findings	Skull Fracture	Cause of death	Survival time/(min) source of info	Brain weight	Brain swelling	High ICP	Intracranial haemorrhage	Contusion	Intracerebral haematoma	Corpus callosum haemorrhage	Brainstem haemorrhage	Ischaemia	APP
1	35 M	Found unconscious after heavy drinking	Mild bruises in scalp and forehead	No	Unascertained, possibly aspiration	109 min – police and hospital records	1438 g	No	No	No	No	No	No	No	No	–ve
2	28 M	Stabbed to neck	Stab injury	No	Blood loss	91 min – ambulance and hospital records	1337 g	No	No	No	No	No	No	No	No	–ve
3	24 M	Collapsed after heavy drinking; hit his head	Mild bruises on scalp	No	Wernicke encephalopathy	45 min –	1270 g	No	No	No	No	No	No	No	No	–ve
4	36 M	Collapsed. Schizophrenia	Unremarkable	No	Unascertained	90 min – witness and hospital records	1489 g	Mild	No	No	No	No	No	No	No	–ve

info, information; ICP, increased intracranial pressure; β-APP, β-amyloid precursor protein; M, male; –ve, negative.

container containing 400-ml citrate buffer pH 6.0 on 'High' (800 W) for × 2 periods of 6 min in a Panasonic microwave. The excess TBS surrounding the sections was wiped and sections were ringed with vector pen. It was followed by incubation in 10% Dakocytomation Normal Rabbit Serum for 30 min at RT then the serum was drained off the slides.

Sections were incubated in monoclonal anti-β-APP IgG as primary antibody (Chemicon, Chandlers Ford, UK clone 22C11) at a dilution of 1:10 000 at 4°C. After two rinses in TBS for 5 min each change, the sections were incubated in 1:200 Dakocytomation rabbit anti-mouse IgG for 45 min at RT. Sections were washed in two changes of TBS for 5 min each change. For CD68 IH, the primary monoclonal antibody (Dako, Ely, UK clone PGM1) was applied overnight at 4°C in a 1:100 dilution. After rinsing in TBS sections were incubated with biotinylated rabbit anti-mouse IgG (1:200, Dako) in TBS for 45 min at RT. After extensive rinsing sections were incubated with preformed avidin–biotin horseradish peroxidase (ABC-HRP Complex, Dako) complex for signal amplification. The reaction product was visualized using a solution of 10 mg 3,3′-diaminobenzidine tetrahydrochloride (DAB, Sigma, Gillingham, UK) as chromogen dissolved in 20-ml TBS. The solution was filtered and 10 µl of 30% H_2O_2 was added to it. The sections were incubated for 10 min at RT. Standard control experiments were performed by omission of the primary antibody and resulted in no detectable cellular immunolabelling (data not shown). Thereafter they were washed in running tap water for 5 min then counterstained in Harris' haematoxylin. Sections were dehydrated in graded alcohols, cleaned in xylene and mounted in Ralmount. The sections were examined using high magnification (× 200, × 400) to assess the immunoreactivity of β-APP and CD68. Due to the fact that the deposits were infrequent and scattered, a quantitative or semiquantitative assessment was not possible.

Results

Group 1 (severe head injury survived by 30–60 min; *n* = 7) (Table 1)

The brain weights were 1313–1695 g. The cause of death was head injury with brain damage therefore all qualified for severe TBI. There was mild brain swelling in three out of the seven cases and moderate swelling in one but none

showed evidence of increased intracranial pressure or brain herniation. Subarachnoid haemorrhage and intraventricular bleeding were described in four and one cases respectively. One case showed small extradural haematoma of few millilitres in volume. There were four cases with mild contusions in the frontal, temporal, parietal or occipital lobes. Small haemorrhages in the corpus callosum were present in four. There were two brains with small brainstem haemorrhages in the dorsolateral quadrant and lining of fourth ventricle respectively, and one presented with extensive brainstem haemorrhage. All cases lacked histological evidence of ischaemia based on absence of shrunken, eosinophilic neurones in the cerebral cortex, hippocampal layers and Purkinje cells of cerebellum and lack of marked widespread overexpression of β-APP in neurones. Regarding β-APP examination, all cases showed scattered deposits as small globules, sometimes with separated or attached fine filaments (Figure 1). The deposits were seen with clear background and were consistent with early axonal disruption. They were different from those of ischaemic AI, described earlier as ill-defined areas of deposits with linear and geographical pattern and coarse granular background [13,18]. However, early ischaemic disruption is also possible, although no evidence of ischaemia was present in our cases. Most of the deposits had uniform dense staining and none had granular or faint staining as seen in nonspecific or old axonal damage. The β-APP deposits were seen in multiple places in each case to include the anterior and posterior parts of corpus callosum, white matter of parietal lobe, internal capsule, middle cerebellar peduncle and white matter tracts of pons with variable intensity and location for each individual. They were present in the white matter tracts and not adjacent to the foci of haemorrhages in many cases. However, some foci were seen near small capillaries. In two cases (No. 3 and 5), there were small haemorrhages both in the corpus callosum and brainstem; a picture consistent with grade III diffuse AI according to the grading of dTAI performed to Adams *et al*. [6], while in two other cases (No. 4 and 6) there were small haemorrhage in the corpus callosum only therefore with the axonal damage detected by β-APP, fulfilling the criteria of grade II dTAI. The remaining cases showed brainstem haemorrhage (No. 1), contusions (No. 2 and 7) and white matter haemorrhages (No. 7), in addition to the widespread β-APP positivity. The CD68 staining showed no staining or over-expression, particularly in areas with β-APP staining.

Group 2 (cases of severe head injury that have survived less than 30 min; $n = 4$) (Table 2)

There were four cases in this group with recorded severe head injury, including three with facial injuries and one with strangulation. The brain weights were 1299–1641 g. Two had mild brain swelling without pathological evidence of increased intracranial pressure or brain herniation. Two had mild subarachnoid haemorrhage, two mild frontal or parietal contusion. One case presented with small haemorrhage in the corpus callosum and another one with small haemorrhage in the lining of fourth ventricle. None of the cases showed evidence of ischaemia, after careful examination of the neurones in the area of frontal and parietal lobe cortex, hippocampus or Purkinje cells of cerebellum. There were small haemorrhages in the corpus callosum (No. 1) and brainstem (No. 2), features seen in cases with dTAI of grade II and III (1) respectively, whereas the other two cases (No. 3 and 4) showed no haemorrhages in these areas. Case No. 2 had multiple small haemorrhages in the white matter consistent with diffuse AI. Cases 3 and 4 had severe facial and scalp injuries. Accordingly, all these cases displayed features, which may be regarded as severe head injury. In all cases β-APP stain was negative in all eight stained sections. This was confirmed by multiple light-microscopic examinations including using high-power magnifications.

Group 3 (control group, no head injury, survived 45–109 min; $n = 4$) (Table 3)

This pathologically heterogeneous group represented cases with non-head injury cause of death and reliable record of times of incidence and death. These cases served as a control group to the TBI cases of group 1. The survival time (45–109 min) was within the same range as of group 1. The brain weights ranged from 1270 to 1489 g. None of the cases showed skull fracture or had features of TBI such as contusions or internal bleeding. In two cases (No. 1 and 4) the cause of death was unascertained and brain on examination was normal. In one case (No. 2) the death was due to blood loss and the brain examination was unremarkable; there was no evidence of ischaemia and the patient died at 91 min post injury. A patient (No. 3) with history of chronic alcoholic abuse had Wernicke encephalopathy. None of the cases displayed histological evidence of ischaemia. β-APP was uniformly negative in all four cases of the group in all

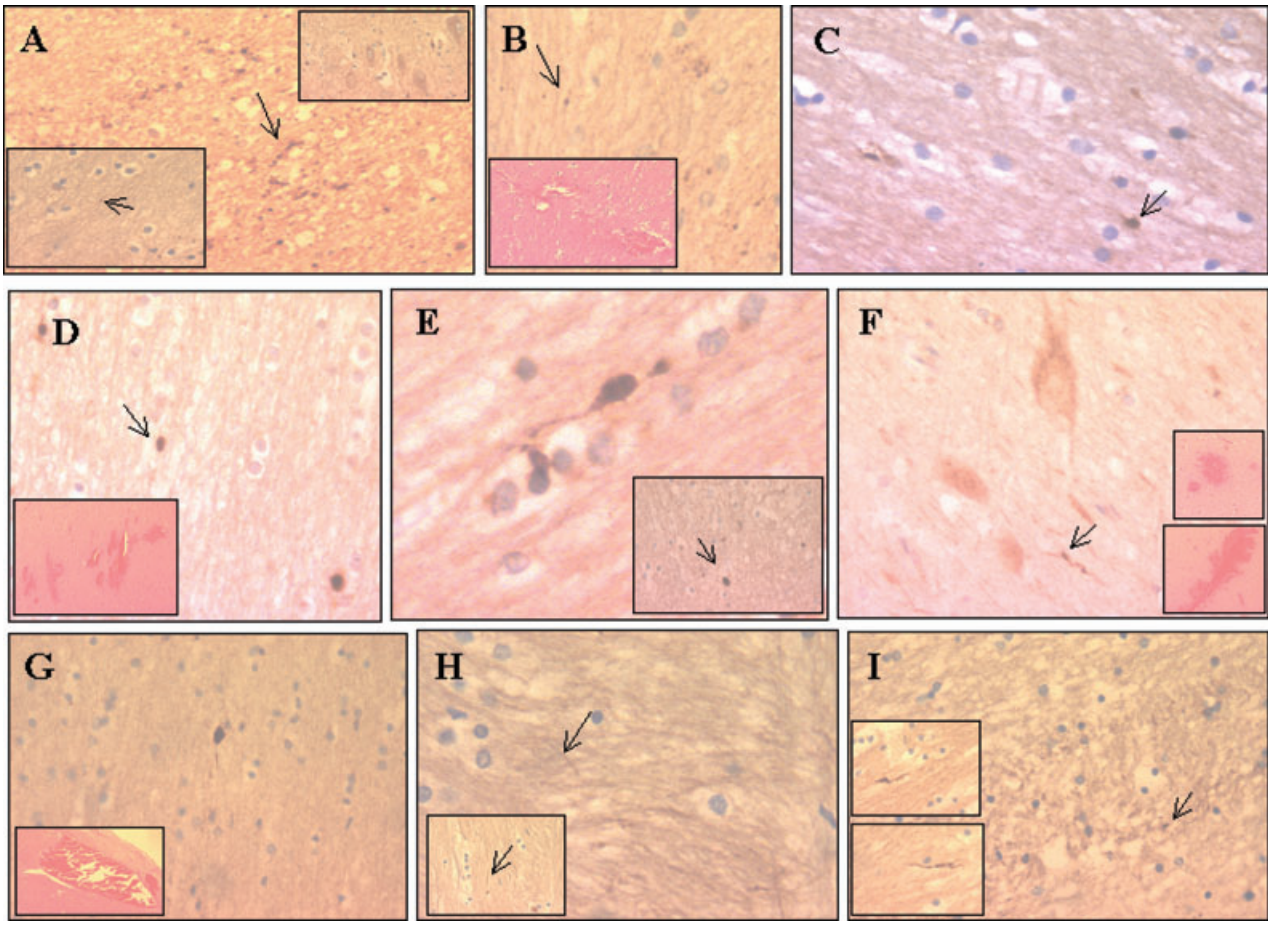

Figure 1. Histological appearances in group 1 (severe head trauma, survival time 35–60 min). For further details see text and Table 1. (**a**,**b**,**c**) Case no. 4. (**a**) β-APP was expressed in forms of small granules (arrow) adjacent to the superior cerebellar peduncle in the dorsolateral pons (main picture) and cerebellum (lower inset). There were axonal beads in the central tegmentum (upper inset) where neuronal cytoplasmic immunopositivities were also noted. (**b**) Occasional β-APP immunoreactive axons (arrow) and microscopic tissue-tear haemorrhages (inset) were seen in the splenium. (**c**) In the parasagittal frontal white matter few axonal β-APP immunoreactivities (arrow) were seen with a clear background. (**d**,**e**,**f**) Case no. 5. (**d**) β-APP immunoreactivity (arrow) and tissue-tear haemorrhages (inset) in the posterior corpus callosum. (**e**) Axonal beads in the frontal (main picture) and parietal (inset) parasagittal white matter. (**f**) Axonal (arrow) and neuronal immunoreactivity in the central tegmentum. Bilateral tissue-tear haemorrhages in the dorsolateral pons (insets). (**g**,**h**,**i**) Case no. 6. (**g**) β-APP immunopositivity (arrow) and tissue-tear haemorrhage (inset) in the anterior corpus callosum. (**h**) Thin filamentous immunoreactivities (arrows) in the pons (main picture) and internal capsule (inset). (**i**) Numerous small β-APP-positive axonal profiles in the parasagittal frontal white matter (arrow, main picture and insets).

regions, sampled according to the TBI protocol used in the other two groups.

A comparison and summary of major neuropathological findings in the different groups is shown in Table 4.

Discussion

In this study we have provided evidence that β-APP staining can detect early axonal damage due to head trauma in less than 60 and minimum of 35 min (group 1; severe TBI). However, when the survival time was less than 30 min (group 2; severe TBI) β-APP failed to show injured axons despite the fact that the death was due to severe TBI. The control cases (group 3) represented a heterogeneous group; none of the cases qualified for the diagnosis or cause of death of TBI. They showed uniform β-APP immunonegativity.

The cases were selected consecutively from more than 150 cases based on the diagnosis of presence or absence of head injury and, most importantly, the accurate timing of events and death. The timing was taken from reliable records by closed circuit television camera, of first call to

Table 4. Summary of neuropathological findings

	Group 1 *Severe head injury, survived 35–60 min*	*Group 2* *Severe head injury, survived < 30 min*	*Group 3* *No head injury, survived 45–109 min*
Brain weight	1313–1695 g	1299–1641 g	1270–1489 g
Brain swelling	4/7 (3 mild, 1 moderate)	2/4 (mild)	1/4 (mild)
Increased intracranial pressure	0/7	0/4	0/4
Intraventricular bleeding	1/7	0/4	0/4
Subarachnoid haemorrhage	4/7 (mild, patchy)	2/4 (mild)	0/4
Contusions	4/7 (mild)	2/4 (mild)	0/4
Small haemorrhage in corpus callosum	4/7	1/4	0/4
Haemorrhage in brainstem	3/7	1/4	0/4
Ischaemia	0/7	0/4	0/4
APP immunohistochemistry	7/7 (scattered; in anterior and posterior corpus callosum, white matter, middle cerebellar peduncle, pons)	0/4	0/4
CD68 immunohistochemistry	Negative	Not applicable	Not applicable

β-APP, β-amyloid precursor protein.

police or ambulance service for the incident or reliable witness with accurate timing. The time of death was taken from the ambulance service or hospital time of death certification.

Gorrie and co-workers demonstrated APP immunoreactivity in paediatric TBI cases with 35–45 min survival [19]. Our data confirm that β-APP is detectable between 35 and 60 min and in our knowledge these are the first reported adult cases. Earlier reports on human cases demonstrated AI in TBI with survival times of 1.5–2 h by means of β-APP and NSE IH respectively [1,9,20]. In a rat model of brain trauma β-APP was detectable in swollen axons by IH as early as 30 min following impact acceleration TBI [21] and in rat needle stab brain injury [5] respectively. In a sheep impact TBI model widespread AI was detectable by β-APP IH 1 h after trauma [22]. The 68-kDa neurofilament protein IH could detect in an experimental study axonal damage within 60 min [8].

The question inevitably arises: what could be the earliest possible time point to detect AI by means of IH techniques? There are some data available in the literature, which could enable a fair assumption of the minimum time window required to accumulate sufficient amounts of β-APP in damaged axons. It is probable that an increased amount of transported β-APP is required in order to accumulate in the damaged axons in a quantity that enables IH detection. It is known that widespread up-regulation of β-APP mRNA and antigen expression was detected in neuronal cell bodies by *in situ* hybridization as early as 30 min after head impact in an ovine TBI model [23]. In contrast, at 15 min no significant changes were noted and the resting β-APP production was at very low level [23]. These findings gained support from subsequent reports, indicating that the mRNA of the β-APP binding protein FE65 started to increase significantly at 30 min and peaked at 1 h after TBI in rats [24], detected by real-time polymerase chain reaction method. In our cases β-APP immunoreactivity of neuronal cell bodies were observed (Figure 1**f**) suggestive of a similar early increase of β-APP production. The speed of axonal transport is another potentially relevant factor. β-APP is transported in the axons with a speed of 5–7 μm/s (0.30–0.42 mm/min) [25]. However, the velocities of anterograde axoplasmic transports decreased with age in experimental animals [26,27] and were also influenced by axonal size. Given that the same occurs in humans, β-APP may concentrate earlier and in greater quantity in younger people. An early paper reported the appearance

of β-APP immunopositivity in axons following needle stab injury in rats as early as 30 min after the trauma adjacent to the lesion [5]. These data suggest a minimum survival of approximately 30 min after TBI required for detection of β-APP, which is in concert with our present findings in humans.

The axonal positivities in our cases were infrequent, scattered and predominantly in forms of small beads and granules, consistent with previous reports on early β-APP detection emphasizing a correlation between survival time and diameter of immunopositive axonal profiles [14]. However, small filamentous positivities of apparently non-disrupted axons were also noted (Figure 1**h**). In a rat ultrastructural study small nodal and paranodal swellings of nondisconnected fibres were described, which may represent axonal responses with a different pathobiology [20]. Furthermore, electron microscopic studies revealed that at early time points the transported vesicles in axons appeared as linear arrays, beginning to aggregate at the site of AI [25]. Our findings of thin filamentous axonal positivities may represent these axonal populations. The diffuse background staining also deserves some discussion. This was detectable also in cases without evidence of AI (data not shown), however, appeared more prominent in TBI cases. Stone and his colleagues described similar features in rodents and speculated that the this background staining may either be related to the increased secretion of N-terminus fragments as a consequence of TBI-associated excitotoxicity and at least some of the immunopositivity may reflect the nonpathobiological β-APP content in the neuropil [25].

The β-APP is known to detect early AI and disruption in fast axonal transport well before full trans-section of axons occurs (axotomus). Therefore, it is important to emphasize here that the AI detected by β-APP may not be permanent and a degree of reparative process may occur resulting in restoration of axonal function. One should always be careful not to over-interpret multifocal early axonal damage as evidence of diffuse AI without other features like haemorrhages in the corpus callosum and brainstem. It is equally important not to assume that the cause of death was traumatic brain damage based upon multifocal mild β-APP deposits only, without further evidence of severe head injury. It is quite possible that very early deposition of β-APP, of minimum 35 min, is related to the severity of the head injury and, more specifically, to the severity of the early axonal disruption. Five cases had evidence of severe (grades II and III) dTAI with haemorrhage in the corpus callosum and/or brainstem, one with multiple haemorrhages in the white matter and two with facial skull fracture. Further work may be required to find out if this early deposition of β-APP could also occur in mild or moderate head injury. However, this may be a difficult task on practical grounds.

Hypoxic brain damage [28] and AI [29] is frequently present in fatal blunt head injury. However, hypoxia *per se* is not a usual cause of AI [30], and the AI in hypoxic brains can be better explained by vascular complication caused by raised intracranial pressure and brain herniation [30]. As the transport of β-APP is an active, energy-dependent process [31], it is reasonable to assume that it may accumulate in cases of severe metabolic compromise, such as hypoglycaemia, severe global hypoxia or brain swelling and infection. None of our cases in groups 1 and 2 showed pathological evidence of severe oedema, space-occupying lesion, increased intracranial pressure or ischaemia. We did the assessment and exclusion of histological evidence of ischaemia with detailed and careful examination of cortical layers in two regions of the cerebral cortex (the frontal and parietal lobe); the latter was taken from the watershed area between the anterior and middle cerebral artery territories of blood supply, where the neurones are particularly vulnerable to ischaemia. We also examined vulnerable neurones in the hippocampal formation and the Purkinje cells of cerebellum. There was faint β-APP immunopositivity in morphologically normal neurones in the cortex and pontine nuclei (Figure 1**a**,**f**). However, marked and widespread overexpression, indicative of significant ischaemia, was not a feature. The axonal β-APP positivities in group 1 cases were seen in forms of small globules, beads and fine small filaments. This was different from the pattern described in hypoxia-ischaemia and cases of increased intracranial pressure with ill-defined area of intense granular deposits of β-APP and sometimes with a geographical wavy pattern [18]. In the latter paper, the majority of primary hypoxic-ischaemic cases were not associated with β-APP immunoreactivity. A more recent study concluded that detailed neuropathological examination of the brain according to published guidelines, including β-APP IH, allows determination of the cause of axonal pathology and differentiation of ischaemic and traumatic axonal damage in the vast majority of cases [13]. Early (less, than 1 h) β-APP deposition in axons has not been studied before; therefore, early β-APP deposition due to acute ischaemia in cases from group 1 still remains a possibility. However, we

consider it unlikely with lack of histological features of ischaemia and with evident signs of traumatic head injury. The pattern, distribution and intensity of β-APP immunoreactivity in group 1 cases also differed from the occasional deposits of nonspecific AI, sometimes seen in elderly or alcoholic patients. In the latter case, the globules were very occasional, mainly limited to one or two areas and showed medium-sized globules with faint and granular staining. For all the above reasons, we think that the β-APP deposits in our cases in group 1 were most likely due to traumatic disruption of axons. Furthermore, sections with β-APP-positive staining showed no over-expression of the macrophage/microglia marker CD68 (PGM1).

The early detection of β-APP could be also attributed to the type of antibody applied and immunohistochemical method we used. The IH protocol included two cycles of heat-induced epitope retrieval in citrate buffer and application of microwave. A monoclonal primary antibody targeting the N-terminus was applied and followed by ABC-HRP secondary complex formation and DAB as chromogen. About the same intensity of staining can be archived at a dilution of 1:5000 of the primary antibody for 1 h at RT. For negative controls primary antibody was omitted. Without antigen retrieval no immunoreactivity was detectable (data not shown), whereas the method we used demonstrated the accumulating β-APP in the affected axons as small beads and bulbs. Antigen retrieval was necessary to visualize APP in cases with short survival. Microwave has been proved to be superior to formic acid treatment or primary alcohol fixation [32]. Temperature-controlled (45°C) microwave treatment is probably the best method [20]. The mechanisms for the beneficial effects of MW are still incompletely understood. MW acts probably by its ability to unmasks epitopes obscured by cross-linking fixatives, probably via by both heat- and direct microwave effects [20,32].

The used monoclonal anti-β-APP antibody (Chemicon, clone 22C11) recognized amino acids 66–81 of the N-terminus of the pre-A4 molecule. It recognized all three isoforms of β-APP (immature ~ 110 kDa, sβ-APP ~ 120 kDa and mature ~ 130 kDa). This novel N-terminus antibody has high specificity and sensitivity, and can be used in high dilution. However, a better yield has been claimed with antibodies targeting the C-terminus of the β-APP molecule [21]. The C-terminus lies on the exterior surface of the transported vesicles, whereas the N-terminus intravesicularly [33]. It is also of interest that the C-terminal fragments of β-APP may be involved directly in damage related to TAI as they are neurotoxic and pro-apoptotic [34].

In conclusion, β-APP was detected in our seven TBI cases with minimum of 35-min survival time after severe head injury. This new minimum time limit may have implications on the medico-legal practice. We recommend the use of microwave antigen retrieval as a routine laboratory technique to detect diffuse axonal injury and meticulous examination of the slides with β-APP IH using high magnifications (× 200 and × 400).

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