



A morphological study of diffuse axonal injury in a rat model by lateral head rotation trauma

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Abstract

Morphology in diffuse axonal injury (DAI) by lateral head rotation was investigated. SD rats were divided into injury ($n = 9$) and sham ($n = 3$) groups. A device was used to produce lateral rotational acceleration of the rats' heads. At different survival times three rats were killed for light and electron microscopic examination of the brain tissue. Sagittal sections were made from medulla oblongata and immunolabelled for NF68. At post-traumatic 30 min, NF68 immunolabelling showed a small number of swollen and irregular axons. Ultrastructurally slightly-separated myelin lamellae and disorderly arranged neurofilaments occurred. At 2 and 24 h axonal damage became more severe. Increases in immunolabelled axonal swellings, disconnected axons and axonal retraction bulbs appeared. EM provided evidence of myelin separation, peri-axonal spaces, blank areas in axoplasm, loss of microtubules, peripheral accumulation of mitochondria and clumped neurofilaments for DAI. A tendency was noted for greater labelling with NF68 as axonal damage increased. The disorderly arrangement of NFs occurred at early stage of post-traumatic axonal changes.

Key words: Diffuse axonal injury (DAI); morphological study; animal model; NF68.

Introduction

The pathogenesis of brain diffuse axonal injury (DAI) in humans has attracted much attention because of its high morbidity and mortality. Based on many studies, it had been hypothesized that the structural damages in neurofilaments (NFs) mainly result from the degeneration of NFs' protein subunits (*i.e.* proteolysis) and appeared prior to the occurrence of axonal swelling and disconnection (Adams *et al.*, 1989; Levi *et al.*, 1990; Adams *et al.*, 1991; Slanzinski and Johnson, 1994; Bennett *et al.*, 1995; Gentleman *et al.*, 1995). It was also suggested that

the initiation of immunological activity of multiple protein subunits in NFs might account for the degeneration of these subunits (Povlishock, 1992; Gennarelli, 1993; Graham *et al.*, 2000). Furthermore, some authors thought that the immunological activity of the lower molecular weight NF protein subunits was activated and enhanced at a very early stage after brain injury (Schlaepfer, 1987; Kampfl *et al.*, 1997; McCracken *et al.*, 1999). These hypotheses need to be further investigated and elucidated in more animal models of diffuse axonal injury (DAI).

In our previous study using silver staining, DAI was produced in a rat model by lateral rotational acceleration of the head, which caused the most severe damages in the brainstem, especially in the medulla oblongata (Xiao-sheng *et al.*, 2000). In recent years it has been well documented that NF68 is an NF protein subunit with a lower molecular weight of 68 kDa, and the immunohistochemical staining for NF68 is more specific than the traditional method with silver impregnation in morphological research on neuronal axons (Grady *et al.*, 1993; Ross *et al.*, 1994). Hence, we used the NF68 immunohistochemical techniques in this study to investigate the microscopic and ultramicroscopic changes in the same rat model of DAI in order to gain deeper insight into the pathogenesis of human DAI.

Methods and materials

ANIMAL GROUPING AND MODEL PRODUCTION

Twelve healthy male Sprague-Dawley rats weighing 250 to 300 g were randomly divided into injury and sham group ($n = 9$, $n = 3$, respectively). The rat model by lateral head rotation was produced as our previous publication (Xiao-sheng, 2000). All rats

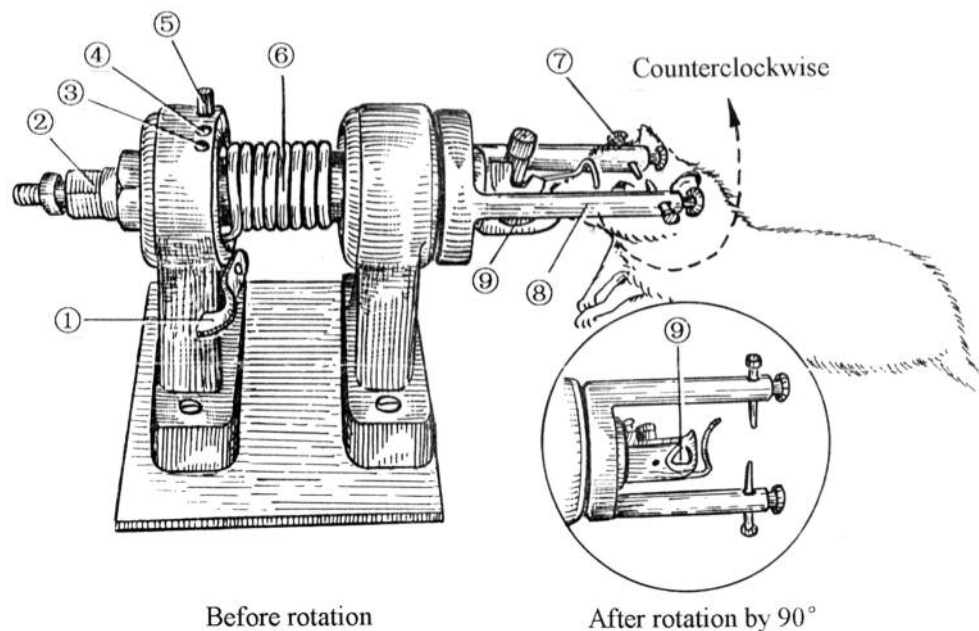


FIG. 1. — Diagram of the device for inducing brain injury by lateral rotational acceleration of the head.
 ① trigger; ② reset nut; ③ rotational angle-determined hole at 0°; ④ rotational angle-determined hole at 30° and its bolt; ⑤ rotational angle-determined hole at 30°; ⑥ driving spring; ⑦ ear pin; ⑧ horizontal arm; ⑨ anterior tooth hole.

were anesthetized under peritoneal injection of 1% sodium pentobarbital (35 mg/kg). The rat head was horizontally secured to the rotation device by two lateral ear bars, a head clip and an anterior teeth hole, with its body at 20° oblique to the top of the laboratory table (Fig. 1). For the injury group, after its trigger was pressed downwards, the device rapidly rotated the rat head through a 90° angle laterally (*i.e.* in the coronal plane). The head rotation was finished in less than 2.09 ms with an angular velocity in excess of 761 rad/s and the angular acceleration in excess of 1.87×10^5 rad/s² (Xiao-sheng *et al.*, 2000). At 30 min, 2 h and 24 h post head rotation, three rats from the injury group were sacrificed as a subgroup for histological processing. The sham rats were killed for histological processing at 2 h after being freed from the injury device.

HISTOLOGICAL PROCESSING

Sample preparation

At various survival times as scheduled, the injured and sham rats were injected peritoneally with 1% sodium pentobarbital (45 mg/kg), exsanguinated by transcardial perfusion with normal saline until the circulating effluent became transparent, and then fixed transcardially with 4% paraformaldehyde and 2% glutaraldehyde in PBS for 60 min, with the perfusion pressure maintained at 90–95 mmHg and the temperature at 37°C. The rats were decapitated

and their brains were removed immediately and bisected along the sagittal fissure. The half-brains were immersed in 4% paraformaldehyde and 2% glutaraldehyde in PBS for post-fixation for 4 h and kept in 30% sucrose in PBS overnight at room temperature.

Section preparation

Sections of 40 µm were cut at a site 1 mm from the midline surface of the half-brain with a vibratome and were preserved in 0.01 mol/L PBS. The sections were incubated with a mouse anti-NF68 monoclonal antibody (diluted 1:400, Sigma Co.) for 12–16 h at room temperature, and then reincubated in biotinylated antimouse immunoglobulin G (diluted 1:200) for 4 h at room temperature. The sections were then transferred to the avidin-labelled peroxidase-antiperoxidase complex (PAP, diluted 1:100) at room temperature, and then placed in the dilution of DAB and H₂O₂ in Tris.Cl for 15–25 min depending on the progress of the reaction as observed under a light microscope (LM). Each 30 ml of 0.05 mol/L Tris.Cl contained 5 mg of DAB and 3 µl of 3% H₂O₂. All sections were rinsed in 0.01 mol/L PBS (pH 7.2–7.4) between the above procedures. Finally, the sections were osmicated and dehydrated. The samples were cut from the medulla oblongata at each section and flat embedded by an Epon 812 between two plastic slides. After fixation, the plastic slides were removed and the

Table 1

The density value of ARBs and the percentage of obviously-labelled axons in all labelled axons post injury (M $\pm$ SD)

Time	n	Density value of ARBs	Percentage of OBAs*
30 min	6	0.400 $\pm$ 0.115	20.32 $\pm$ 6.87
2 h	6	3.433 $\pm$ 0.423	42.68 $\pm$ 7.59
24 h	6	6.000 $\pm$ 0.683 ^a	55.70 $\pm$ 9.64 ^b

*obviously-labelled axons, ^{a, b} t-test, $p < 0.05$

embedded sections were prepared for light microscopic observation. The positive sites were identified and cut into smaller samples (1 mm $\times$ 0.5 mm) for electron microscopic examination. The ultrathin sections were stained with 2% uranyl acetate and 0.2% lead citrate and examined under transmission EM (JEM-2000EX, Japan). As a control, small portions of the sections were incubated in the blood serum from normal mice instead of the serum containing the NF68 monoclonal antibody.

Results

LIGHT MICROSCOPIC OBSERVATION

At each time point for the injured rats there were six half-brain blocks (three Left and three Right) ready for section preparation. Sections from each half-brain block were observed under LM (magnification $\times 400$) and the average number of axonal retraction bulbs (ARBs) in five consecutive visual fields ($0.256 \times 10^5 \mu\text{m}^2$ each) was calculated as the density of ARBs for the corresponding brain block. The identification of ARBs was based on the simultaneous observation made by two experienced researchers. Besides the ARBs, the percentage of obviously-labelled axons under LM was also taken into account. These two experienced researchers sorted all the labelled axons using the terms “mild”, “moderate”, or “obvious” to describe the staining intensity. The percentage of the obviously-labelled axons in all labelled axons was averaged across five consecutive visual fields ($0.256 \times 10^5 \mu\text{m}^2$ each, magnified by 400) for any section in each half-brain block.

At 30 min after injury, a small proportion of neuronal axons in the medulla oblongata were NF68⁺, in which some axons were swollen and crooked. The enlarged axons showed cylinder expansion along their longitudinal axis. The ARBs were rarely seen at this time point (Fig. 2A). At 2 h the NF68⁺ axons increased and showed much stronger staining. The axons were significantly swollen and crooked. Many ARBs were observed where the NF68 staining was prominent and a dense immuno-

reactive core was also observed (Fig. 2B). At 24 h there were more neuronal axons strongly stained with NF68 among which there was much axonal swelling and many ARBs occurred (Fig. 2C). We also observed dense immunoreactive cores within the ARBs. The sham control showed a linear array of axons, slightly stained with NF68, and not accompanied by axonal swelling, axonal circuitry and ARBs (Fig. 2D).

The density value of ARBs and the percentage of obviously-labelled axons in all labelled axons were averaged across six half-brain blocks at each of the three time points, and summarized in Table 1.

ELECTRON MICROSCOPIC OBSERVATION

At 30 min, there was mild separation at some sites of the myelin lamellae in some neuronal axons. The axon became locally enlarged because of such separation. Many axoplasmic NFs were apparently NF68⁺ stained and unevenly expanded, while relatively normal NFs were still present. The electron dense dots due to the immunological reaction were found on these enlarged NFs along their longitudinal axes. Axoplasmic NFs exhibited a disordered arrangement. Adjoining enlarged NFs actually formed compacted bundles. Many blank areas were observed underneath the axolemma. The microtubules in tubular form or in the transversely- or obliquely-sectioned plane were clearly seen, and the mitochondria, though in small numbers, still maintained their central distribution (Fig. 3A).

At 2 h the separation of myelin lamellae was more obvious. There were vacant spaces between the outer surface of the axolemma and the inner aspect of the myelin sheath, which were described as “periaxonal spaces” (Jafari *et al.*, 1997). The blank areas were present at the outer part of the axoplasm, and the mitochondria were scattered peripherally and were decreased in number. It should be noted that microtubules were rarely seen. Many immunoreactive black dots were observed reflecting the labelled NFs while some relative normal NFs still existed (Fig. 3B). On transverse sections at a higher magnification, ten electron dense dots and ten unlabelled

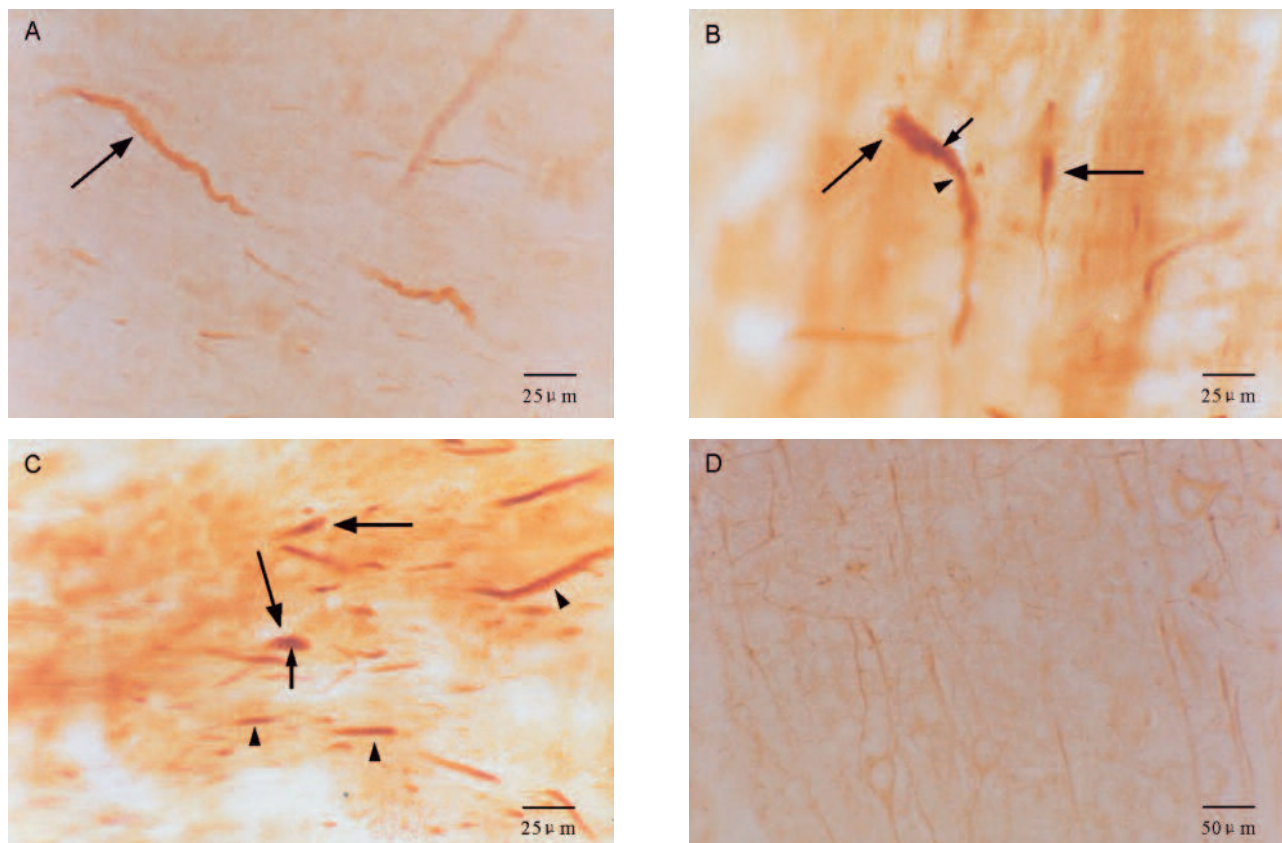


FIG. 2 (A-D). — Light micrographs of flat embedded sections of the medulla oblongata from rats with different survival time post head rotation, and sham rats, showing NF68 immunohistochemistry.

A. At 30 min, some axons are immunoreactive and are swollen and crooked. The swollen axon (long arrow) shows cylinder expansion along its longitudinal path with a much different diameter. No marked ARB can be observed. Magnification $\times 400$; B. At 2 h, the strongly stained axons increase and manifest higher immunoreactivity. These axons are significantly swollen and crooked. The disconnected axons form ARB (long arrow) at their proximal ends where the NF68 staining is prominent and the dense immunoreactive core (short arrow) is visible within the ARB. The local narrowing (arrowhead) reflects the non-uniform enlargement along the longitudinal axis of the axonal cylinder. Magnification $\times 400$; C. At 24 h, there are more obviously-stained axons among which many show axonal swelling (arrowhead) and ARBs (long arrow) occur. The dense immunoreactive core (short arrow) is shown. Magnification $\times 400$; D. The sham rat shows a linear alignment of neuronal axons, slightly stained with NF68, and not accompanied by axonal swelling, axonal circuitry, or ARB. Magnification $\times 200$.

NFs were randomly selected and measured for their diameter. This showed that a NF varied between 8 and 9 nm in diameter and with an average of 8.6 nm [8.6 ± 0.3 nm as in mean (M) $\pm$ standard deviation (SD)], however, it changed to 9–11 nm (10.0 ± 0.6 nm) if labelled with NF68. The electron dense dots were clustered at a higher density, reflecting the closer alignment of the labelled NFs at this time point. The thickly labelled NFs tended to gather in “clumps”. Viewed integratively, there was a reduced inter-neurofilament spacing in injured axons, for which some authors used the term “NFs compaction” (NFC) (Jafari *et al.*, 1998; Maxwell, 1996; Maxwell *et al.*, 2003).

At 24 h the axonal myelin lamellae were markedly separated and locally disconnected. There were numerous and enlarged periaxonal spaces. The

number of mitochondria increased and still tended to be distributed peripherally. Microtubules were absent in the axoplasm. Though intact NFs were present, many NFs became enlarged and gathered in clumps. Seen transversely, the strongly stained NFs (in electron dense dots) and the intact NFs assembled more closely. Such a compacted alignment was demonstrated more clearly under higher magnification (Fig. 3C).

The neuronal axons of the sham animals had regular architectures. The myelin lamellae formed a spiral compact structure. The lamellae in the myelin sheath were shown more clearly under higher magnification (Fig. 3D, Fig 3E, Fig 3F). The axolemma adhered tightly to the inner layer of the myelin sheath. The intra-axonal NFs were intact, finely shaped, slightly stained, and distributed evenly in a

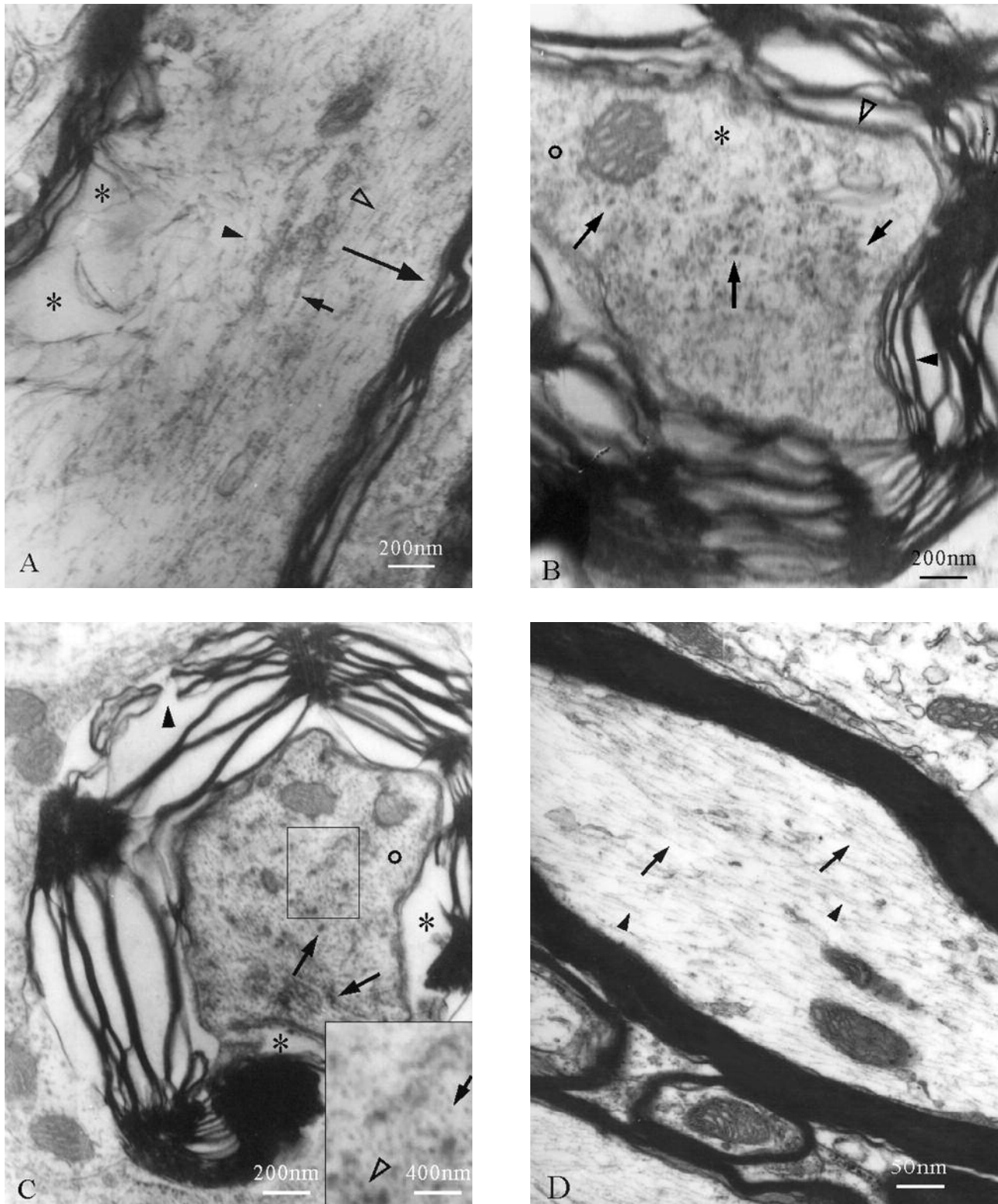
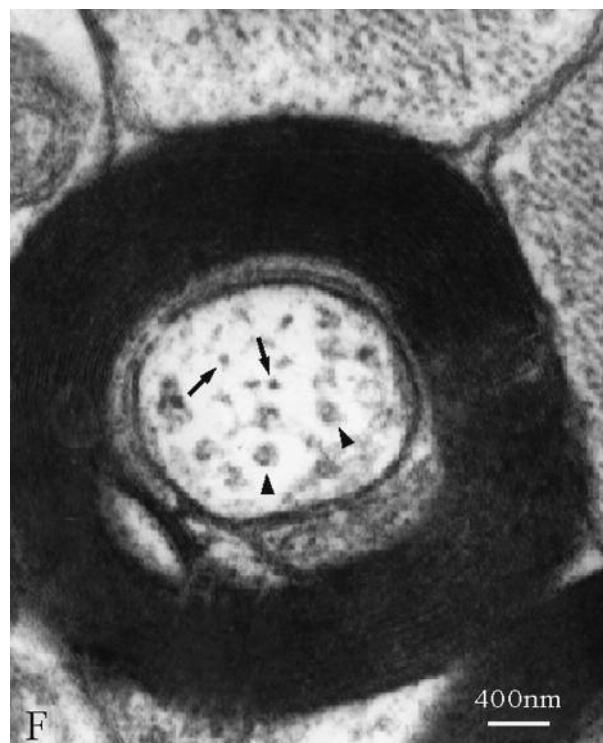


FIG. 3 (A-F). — Electron micrographs of ultrathin sections of the medulla oblongata from rats with different survival time post head rotation, and sham rats, showing NF68 immunohistochemistry.

A. At 30 min. There was mild separation (long arrow) at the axonal myelin lamellae. The axon became locally enlarged. Many NFs were apparently stained and evenly expanded (short arrow) while normal NFs (open triangle) were present. The electron dense dots were found longitudinally located on the thick NFs. The NFs exhibited a disorderly arrangement. The closer adjoining between the NFs formed a compacted bundle (black triangle). The microtubules in tubular form or in transversely- or obliquely-sectioned plane, and the



mitochondria, were observed in central distribution. There were blank areas (star) in the axoplasm. Bar = 200 nm; B. At 2 h. Myelin lamellae separation (black triangle) was more obvious. Periaxonal spaces (open triangle) were clearly observed. The blank areas (star) existed at the outer part of the axoplasm and the mitochondria were scattered peripherally. Immunoreactive black dots (long arrow), reflecting the labelled NFs and the relative normal NFs (open circle), coexisted. The thickly labelled NFs tended to gather in "clumps". Viewed integratively, there was a reduced spacing between axons. Bar = 200 nm; C. At 24 h. The axonal myelin lamellae were markedly separated and locally disconnected (black triangle). There were numerous and enlarged periaxonal spaces (star). The number of mitochondria increased and still tended to be distributed peripherally. The microtubules were absent from the axoplasm, though the intact NFs (circle) were present. Many more NFs became enlarged and gathered in clumps (long arrow). The highly stained NFs (in electron dense dots) and the intact NFs assembled more closely. Such a compacted alignment among highly-stained NFs (open triangle) and intact NFs (short arrow) was demonstrated more clearly in the rectangular field visualized with higher magnification as at the lower right top. Bar = 200 nm, Bar = 400 nm for the lower right top; D. Sham rat. Axonal longitudinal section. The myelin lamellae are composed of compacted structure. The axolemma adhered tightly to the inner layer of the myelin sheath. The intact NFs are finely shaped and thinly stained. An even and linear distribution parallel to the long axis of the axon can be seen for NFs (long arrow). The other axoplasmic structures including mitochondria and microtubules (black triangle) are normal in number, form, and alignment. Bar = 50 nm; E. Sham rat. Axonal longitudinal section. The densely-compacted lamellae are shown under higher magnification. The NFs (short arrow) and microtubules (black triangle) exhibit different diameters and a linear distribution. Bar = 200 nm; F. Sham rat. Axonal transverse section. The spiral densely-compacted structure for myelin lamellae is shown under higher magnification. The NFs (short arrow) and microtubules (black triangle) exhibit different diameters and scatter in a uniform distribution. Bar = 400 nm.

linear orientation parallel to the long axis of the axon. The other axoplasmic structures including mitochondria and microtubules, etc. revealed normal numbers, forms, and alignments.

Discussion

Recent studies have attempted to investigate the events happening in DAI at its earlier stage, and the implication of the functional and structural changes of NFs was noted (Graham *et al.*, 2000; McCracken *et al.*, 1999; Maxwell *et al.*, 2003). In this study, we observed neuronal axons in medulla oblongata in a rat model of lateral head rotation. Our findings

suggested that NF68 immunolabelling can be detected at 30 min post-injury. Some axons exhibited reactive swelling and crookedness (Fig. 2A). The lack of ARBs at this time implied that there was an interval before the formation of these bulbs. The ARBs occurred after a period of time as a secondary pathological event. We reported that ARBs occurred as early as 6 h post head rotation with the same rat model using silver staining (Xiaosheng *et al.*, 2000). The underlying ultrastructural basis for the ARB included a spectrum of pathological changes, such as separation of myelin lamellae, formation of axoplasmic blank areas, and disorderly arrangement of NFs. The NFs were apparently NF68⁺ stained, evenly

expanded and seen clearly on transverse sections in the form of electron dense dots. The coexistence of intact NFs and immunolabelled NFs suggested that DAI was really composed of secondary events. The expanded NFs were more closely assembled than were the intact NFs. It should be noted that the microtubules and mitochondria still existed in this phase (Fig. 3A). As rat survival increased from 2 h to 24 h NF68⁺ staining of NFs and axonal collapse became more marked. Significantly swollen and crooked axons were seen with greater staining and density. The ARBs appeared and revealed a dense immunoreactive core within them (Fig. 2B). It was necessary to find the distinction between the intact NFs and the immuno-labelled NFs (Jafari *et al.*, 1998; Maxwell, 1996; Maxwell *et al.*, 2003). The thickly labelled NFs existed in "clumps" (densely-distributed) (Fig. 3B). At 24 h, NF68 staining was greatly enhanced and more ARBs with a dense immunoreactive core were developed (Fig. 2C). We concluded that the density value of ARBs and the percentage of the obviously-labelled axons increased gradually across different survivals. The above results made it clear that the severity of axonal skeleton damage and the degree of axonal NF68 immunological activity both increased at 24 h post injury. At 24 h, there was severe separation and local disconnection in myelin lamellae. The periaxonal spaces increased in number and in volume. More mitochondria appeared to accumulate peripherally. It has been noted that mitochondria are involved in the transportation of cellular energy; hence, the increase in mitochondria might be responsible for the repair and regeneration for injured axons. The axoplasm also lacked microtubules. Loss of axonal microtubules has been assumed to be related to influx of calcium into stretch-injured axons (Maxwell, 1996). Much more NFs became enlarged and gathered in clumps, however, intact NFs still existed. Seen transversely, there was a markedly reduced interfilament spacing between the NFs, and under higher magnification, such a compacted alignment was demonstrated more clearly (Fig. 3C). The intra-axonal clumped immunoreactive array of NFs was the ultrastructural reflection of dense immunoreactive core as observed under LM.

Generally, it is considered that ARBs form over a longer period in humans than in animals, due to the disparity in brain mass. The formation of ARBs was reported to be as long as 12 h or more in humans (Grady *et al.*, 1993), and 6 h in swine (Ross *et al.*, 1994). Our findings indicated that ARBs were observed in rats at 2 h post injury. The different interval reflected the disparity of the anatomical structure between animal brains.

It is necessary to determine the early changes that occurred prior to the enlargement of axons and formation of ARBs, and analyze the relationship between them. Christman *et al.* (1994) reported that disorderly arrangement of NFs occurred as early as 6 h post injury in human DAI. The structure of NFs was further damaged at 12 h and the changes persisted at 88 h. In this present rat DAI model, with the exposure of immunological activity of NF68, the NFs lost their regular structure and linear orientation at 30 min, and blank areas emerged in the axoplasm. Blank areas suggested that either the dissolution of partial NFs or intra-axonal edema had developed, or both. At 2 h, there were obvious separations of myelin lamellae, and the axoplasmic mitochondria showed a peripheral accumulation towards the underneath of the axolemma. At 24 h axonal swelling was widely distributed and was associated with obvious lamellae separation, periaxonal spaces, and axoplasm dilation. Once the swollen axons became disconnected, the axoplasm leaked out and resulted in the formation and development of ARBs.

Hence, it is rational to speculate that intra-axoplasmic neurofilaments and organelles responded to mechanic injury earlier than myelin sheaths. Another fact that should be noted is that the axoplasm NF68 staining was much enhanced after injury, and as axonal structures became damaged, the NF68⁺ axons increased gradually both in staining intensity and in number. The enhancement of the immunoreactivity of NF68 in NFs along with the degradation of this subunit, might lead to gradual dissolution and disorderly arrangement of the NFs. Our findings showed that the immunological reactivity of NF68 in NFs was activated and enhanced at as early as 30 min after brain injury. At this time many morphological changes had happened to the axonal skeleton, including myelin separation, periaxonal spaces, axoplasmic blank areas, expanded caliber and disorderly alignment of NFs, which led to axonal swelling, axonal disconnection and formation of ARBs observed under LM. Posmantur, using Western blots, examined the protein level of NF68 in a rat model of traumatic brain injury and found an increase in the amount of NF68. This suggested the accumulation of NF68 breakdown products caused by pathologic activation of neuronal proteases such as calpain (Posmantur *et al.*, 1994). In one of our previous studies using a calcium cytochemical method and the same model as used in this study, fine calcium pyroantimonate precipitates were observed to be scattered on the separated lamellae and the axolemma at 2 h post injury, which implied that calcium overloading was related to the structural impairment of axons (Xiaosheng *et al.*, 2004).

Conclusions

A rat model of DAI by lateral head rotation and NF68 immunochemical staining were used to observe the pathological changes of neuronal axons under LM and EM. The enhanced detection of NF68 occurred as early as 30 min post-traumatically which was associated with the initiation of the process of damages in the structure of intra-axonal NFs. The ultrastructural changes seen under EM such as separation and disconnection of myelin sheaths, periaxonal spaces, peripheral accumulation of mitochondria, gradual loss of microtubules, blank areas in the axoplasm, disorderly arrangement and compacted assembly of NFs were responsible for axonal swelling, axonal disconnection, and formation of ARBs as observed under LM. Observation of these changes will help us obtain a deeper understanding of the events occurring during the initial period of DAI and aid in the development of effective strategies to treat human DAI at the very early stage.

Acknowledgments

This work was supported by the Shaanxi Provincial NSFC Grant (No. 2005C209). The authors acknowledge the assistance of Mr. Zhexi H in tissue preparation of the experimental animals.

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