Severe Diffuse Axon Injury in Chronic Alcoholic Rat Medulla Oblongata Following a Concussion Blow

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Abstract — Aims: We investigated the axonal morphological changes and expression of both tau protein and β-APP following concussion to the medulla oblongata, in a rat model of chronic alcoholism. Methods: Fifty-nine male Sprague-Dawley rats were randomly divided into EtOH, EtOH-TBI and control groups (water group, water-TBI group). To establish chronic alcoholic rats, rats were intragastrically given edible spirituous liquor twice daily. Rats also received a blow on the occipital tuberosity with an iron pendulum. Morphological changes and expression of tau and β-APP proteins in the medulla oblongata were examined. Results: (a) Nerve fibre thickening and twisting were observed in alcoholic rats, with nerve fibre changes becoming more significant following a concussion blow, which leads to some nerve fibres fracturing. (b) Transmission electron microscopy revealed that the nerve fibre myelin became loosened and displayed lamellar separation, which became more significant following concussion. (c) The integral optical density (IOD) sum value of β-APP of the EtOH-TBI group was lower than that in the EtOH group (P<0.05); the Tau IOD sum value of the EtOH-TBI group was higher than that in the EtOH group (P<0.05). Conclusion: (a) Chronic alcoholism caused nerve fibre and neuronal morphology damage in the rat medulla oblongata, with structural damage becoming more significant following concussion. (b) Concussion changed the expression of β-APP and tau protein in chronic alcoholic rat medulla oblongata, suggesting that chronic alcoholism can lead to severe axonal injury following a concussion blow. (c) The effect of chronic alcoholism may be synergistic the concussion blow to promote animal injury and death.

INTRODUCTION

In recent years, the consumption of alcohol has gradually increased. Chronic alcoholism produces serious damage to the central nervous system (Harper, 2009). An extremely wide range of brain structures can be affected by alcoholism. It has been shown that the brain weight of long-term drinkers is significantly lower than that of non-drinkers (Harding et al., 1996), and the brain weight reduction is largely due to the loss of the white matter rather than the cortical tissue (de la Monte, 1988). Furthermore, long-term alcoholic drinking can cause hippocampal sclerosis, which often occurs in conjunction with epilepsy (Wilkinson et al., 1971). At the same time, hippocampal-dependent learning is impaired by alcohol in a dose-dependent fashion (Melia et al., 1996). Studies also show that attention and information processing can be damaged due to alcohol-related noradrenergic locus coeruleus lesions, preceded by reductions of noradrenaline and its metabolites in cerebrospinal fluid (McEntee and Mair, 1990). Thiamin (vitamin B1) deficiency evoked by excessive alcohol consumption can give rise to cerebellar atrophy. In alcohols with Wernicke–Korsakoff syndrome the basal ganglia, nucleus basalis and raphe nuclei have been found to be damaged (Harper, 1998). Neurofibrillary tangles, which can lead to neurodegeneration, are often found in the nucleus basalis among chronic alcohols (Cullen and Halliday, 1995).

Alcohol has a significant impact on the prognosis of traumatic brain injury (TBI). Animal experiments show that ethanol increases brain oedema and mortality in a rat model of brain contusion through effects on AP-1 (activator protein-1, a transcription factor pathway in cell survival) or COX-2 expression (Katada et al., 2009). In animal models of combined TBI and haemorrhagic shock, alcohol reduces survival time, impairs hemodynamic response and produces a decline in cerebral tissue perfusion (Zink et al., 1998). Compared with non-drinking patients, trauma patients admitted to the hospital with acute drunkeness have lower blood pressure and Glasgow coma scores (GCS) as well as increased incidence of cardiac arrest and post-traumatic mortality (Hadjizacharia et al., 2011). Toxicity from alcohol has adverse effects on hemodynamic responding and respiration and increased mortality to the detriment of the prognosis of patients with TBI (Zink et al., 1993; Tien et al., 2006). Acute alcohol intoxication exerts a significant impact on brain trauma. However, the impact of chronic alcoholism on TBI has rarely been reported. Because of the existence of pathological brain changes in chronic alcoholics, pathological consequences of head trauma are more complex and unpredictable in this population.

Our previous study confirmed that there is a high incidence of subarachnoid haemorrhage (85%) and a high mortality rate (60%) in chronic alcoholic rats following a minor induced head injury (Wang et al., 2011). Death of rats in response to this injury occurs within minutes, with no gradual post-traumatic change, suggesting that the reason for rapid death is severe damage to the medulla oblongata. However, there is no direct evidence for this. In the current study, we observed the morphological changes, as well as tau protein and beta-APP (β-APP) expression in the medulla oblongata, to explore the possible mechanism of injury and death in chronically alcoholic rats following a concussive injury.
MATERIALS AND METHODS

Animals
Fifty-nine adult male Sprague-Dawley (SD) rats weighing 270–330 g were used in our study. All animal procedures were approved by the Animal Care and Use Committee of Shantou University Medical College (authorization number: SUMC2012-7). Prior to experimentation, animals were quarantined for 2 weeks to adapt to local conditions. All animals had free access to water, were fed a standard diet and were kept at room temperature (20–25°C) with a relative humidity of 55% and a 12-h light/dark cycle.

Establishment of a rat model of alcoholism and TBI
Twenty-nine rats were randomly chosen to receive intragastric administration of edible wine (56% alc/vol) twice a day, at 9 a.m. and 4 p.m., for 4 weeks. The dosage of wine was 8 ml/kg in the first 2 weeks and 12 ml/kg in the last 2 weeks. Two hours after the last intragastric administration, EtOH-TBI animals received a concussive injury by a self-made strike instrument, as previously described (Wang et al., 2011; Lai et al., 2013). Briefly, after rat limbs were restrained and the angle of the head fixed at 45°, a custom-made iron pendulum was used to strike the occipitalia of the skull in order to model a concussive injury. As the blow was mild, no skull fracture was observed, and the energy that was absorbed by the rat skull was ~2.5 J. Ten rats (EtOH group) received intragastric administration of edible wine the same way as the EtOH-TBI group but without a concussive injury. Twenty control rats received the same intragastric administration, in which distilled water was used in place of wine, of which 10 rats were given concussive injury (water-TBI group), and 10 remained without concussive injury (water group). Electrocorticographic and respiratory monitoring, using a BL-420E system (Taimeng Technology Ltd, Chengdu, China), was performed during the injury procedure.

Design of the study
All animals were observed for 2 h after strike and then killed by overdosing with sodium pentobarbital (Sigma Ltd, St. Louis, MO, USA). Brains were isolated and were either frozen at −80°C freezer (SANOY Ltd, Japan) or fixed in 10% formalin, then slit along the cerebral longitudinal fissure, dehydrated and paraffin-embedded.

Serial sections of 4 μm thickness were prepared along the sagittal plane. The sections in the lateral 1.9–2.4 mm of the rat brain were collected for toluidine blue, Bielschowsky’s silver staining, and immunohistochemical staining of tau and β-APP. Five fields of view in the rat medulla oblongata were chosen at high magnification to observe the histopathological changes under a biological microscope (Leica, Germany), and integral optical density (IOD) sum value of tau and β-APP were analysed using Image-Pro Plus 6.0 (Media Cybernetics, Carlsbad, CA, USA).

Transmission electron microscopy
At the same time, 1 mm × 1 mm × 2 mm tissue samples in the medulla oblongata of rats at the lateral 1.9–2.4 mm level from the centre were also taken and fixed in 2.5% glutaraldehyde, embedded, sectioned and stained, and observed with a JEM-1400 transmission electron microscope.

Statistical analysis
Data were analysed using the SPSS software 17.0 (SPSS Ltd, Chicago, IL, USA) and Excel 2003 (Microsoft Ltd, Washington, DC, USA). All values are shown as the mean ± SD. Statistical analyses were performed by ANOVA, followed by an independent sample T-test, with P < 0.05 being considered as statistically significant.

RESULTS

Clinical manifestation
After a 4-week intragastric administration of alcohol, rats in the EtOH-TBI and EtOH groups presented clinical manifestations of chronic alcoholism, including the gradual emergence of rough hair, lessened food intake and body weight, reduced activity, and limb weakness. Rats of the water and water-TBI groups were in good condition concerning both diet and activity, and they displayed a continual increase in body weight.

After being struck, rats in the water-TBI group presented with mouth breathing, stiffness of the limbs and corneal reflex disappeared, but they recovered after only a few seconds. However, EtOH-TBI rats showed limb rigidity, upturned tail and convulsions, with some rats dying in a few minutes after appearing to breathe deeply and then slowly, followed by cyanosis of the lips and limbs.

Immediately following the blow, a transient bradycardia and amplification of the QRS wave occurred in the water-TBI rats but returned to normal within 0.3 ± 0.16 s. A similar change took place in the EtOH-TBI group, although the recovery time was 1.7 ± 0.32 s, much longer than that for the water-TBI group (P < 0.05). The amplification of the QRS wave of rats that died after being struck in the EtOH-TBI group was more apparent, and the electrocardiogram (ECG) was observed as a straight line following an interval of 10 s to 1 min (Supplementary Fig. S1). In the EtOH-TBI group, 15 of 29 rats died after being struck (51.7%), which was significantly higher than that (0%) of the water-TBI group (P < 0.05).

Changes in morphology
As shown in Fig. 1, swelling and disconnection of nerve fibres were observed in the medulla oblongata of the EtOH-TBI group, water-TBI group and EtOH group, especially in alcoholic rats after injury. Size and staining of neurons and Nissl bodies were non-uniform in all groups except rats fed with water. In the EtOH-TBI group, mass aggregation of Nissl bodies as well as dark neurons was observed (Fig. 2).

Ultrastructural alteration of the medulla oblongata
Significant changes in the structure of neurons and nerve fibres were observed with transmission electron microscopy (TEM). Neurons of the water group rats presented with a uniform cytoplasm and nucleus and were rich in organelles. Neuron cytoplasm of the water-TBI group appeared less dense with unevenly distributed cellular organelles. Cell bodies and nuclei appeared pyknotic in both the EtOH-TBI group and EtOH group, with changes being more obvious after being
Fig. 1. Microphotographs of nerve fibres in the medulla oblongata of different treatment groups. (A) No apparent nerve fibre swelling was observed in the water group. (B) Nerve fibre swelling and distortion were observed in the water-TBI group. (C) Nerve fibre distortion and thickening were observed as well as local swelling and rupture in the EtOH group. (D) More severe nerve fibre distortion and rupture were observed in the EtOH-TBI group compared with the EtOH group. Bielschowsky’s silver staining, ×400.

Fig. 2. Toluidine blue staining. (A) Size and staining of the neurons and Nissl bodies were uniform in the water group. (B) Size and staining of neurons varied, with Nissl bodies disappearing (arrow) in the water-TBI group. (C) Size of neurons also varied with mass aggregation Nissl bodies (arrow) in the EtOH group. (D) Dark neurons (arrow) were observed in the EtOH-TBI group. Toluidine blue staining, ×400.
struck. Axonal swelling and layered myelin were observed in all groups except in rats fed with water where axons and myelin appeared compact and uniform. In addition, ruptured microfilaments and microtubules were observed in the EtOH-TBI group (Figs 3 and 4).

**Immunohistochemical microscopy**

**Tau IOD sum value**

Tau in normal rats is mainly present in axons and cytoplasm, appearing yellow to dark brown. The medulla oblongata tau IOD sum value of the EtOH group was reduced compared with...
that of water group \((P < 0.05)\), whereas the tau IOD value of the EtOH-TBI group was higher \((P < 0.05)\) than that of the EtOH group. However, the difference between the water-TBI group and water group was not significant \((P > 0.05)\) (Supplementary Fig. S2, Fig. 5).

**Beta-App IOD sum value**

\(\beta\)-APP in normal rats is mainly expressed in the cytoplasm. The medulla oblongata \(\beta\)-APP IOD sum value for the EtOH group was higher than that of the water group \((P < 0.05)\). The sum value of the EtOH-TBI group was reduced \((P < 0.05)\) compared with that of the EtOH group. Compared with the water group, the sum value of the water-TBI group was higher \((P < 0.05)\) (Supplementary Fig. S3, Fig. 6).

**DISCUSSION**

**Chronic alcoholism has a significant impact on the physiological functions of the rat**

Chronic alcoholic rats show signs of malnutrition such as loss of body weight, loose hair and diarrhoea. They also exhibit neurological signs such as withering, confusion and weakness. Long-term absorption of alcohol and its metabolites in rats decreases appetite, causes a decline gastrointestinal function, and the process of alcohol metabolism excessively consumes vitamins, all of which cause a deficiency of vitamin B1 (thiamine). Vitamin B1 deficiency can block glucose metabolism, causing energy reduction for nerve tissue and decreasing pentose phosphate metabolism, affecting the synthesis of phospholipids, and causing nervous system demyelination and axonal degeneration (Nakada and Knight, 1984; Zimitat et al., 1990), eventually causing irreversible damage to the nervous system.

After being struck, consciousness and reaction to external stimuli change quickly, but they are restored in the control animals, indicating that the blow did not cause serious lasting injury to the rat. However, in EtOH-TBI rats, QRS wave amplitudes in the ECG are enlarged, recovery time is prolonged and some rats with serious ECG abnormalities die rapidly, suggesting that the resistance to brain injury of chronically alcoholic rats is decreased with even a slight external force having the potential to lead to their death.

**Concussion blow leads to serious axonal damage in chronic alcoholic rat medulla oblongata**

Alcohol and lecithin combine and deposit in brain tissue, resulting in nerve cell degeneration and nerve fibre demyelination, gliosis and brain atrophy (Tuck and Jackson, 1991). Long-term excessive drinking leads to loss of brain grey matter, which is particularly evident in the frontal cortex (Pfefferbaum et al., 1995). Glia are lost or even absent in the cerebellum following chronic alcoholism (Rintala et al., 2001). In this experiment, Bielschowsky’s silver staining showed irregular thickening of the nerve fibres, some of which became ruptured in EtOH-TBI animals. The toluidine blue staining revealed neuronal damage, as indicated by the presence of ‘dark neurons’. Electron microscopy showed that the axonal fibres in the medulla oblongata of alcoholic rats were thick and disorganized, with the myelin partially disrupted, particularly in the EtOH-TBI group, where the damage in medulla oblongata nerve fibres and myelin was more severe. These findings further suggest that even a slight external force can lead to severe damage to nerve fibres following chronic alcoholism poisoning.

**Tau protein expression in experimental rat medulla oblongata**

The normal function of tau protein is to promote the composition and maintain the stability of microtubules and tau protein is involved in the maintenance of cell morphology, cell signaling and division. It is indispensable in axonal growth and the formation of neuronal polarity. Our current study found that the injury did not have an impact on tau protein expression in the medulla oblongata. Tau protein expression is reduced in chronic alcoholism rat medulla oblongata; however, it is recovered under the combined effect of alcoholism and the concussive injury. The reason may be that the axon structure is severely damaged by a minor strike in chronic alcoholism, blocking axonal transport, causing tau protein to accumulate at the location of axon injury. Tau protein accumulation leads to nerve damage: tau protein hyperphosphorylation not only competes with normal microtubule associated proteins (MAP) and affects microtubule formation but also promotes the separation of normal MAP with microtubules (Daly et al., 2000; Gong et al., 2000). Further, tau hyperphosphorylation causes normal axonal transportation system damage, leaves a variety of sediments deposited in the neurons and causes neuronal damage, ultimately leading to brain degenerative diseases.
\(\beta\)-APP expression in experimental rat medulla oblongata

\(\beta\)-APP expression is maintained at a very low level in normal neurons, but it can be increased after brain damage caused by mechanical injury (Bramlett et al., 1997) or ischaemia and hypoxia (Tomimoto et al., 1995). \(\beta\)-APP is a sensitive marker of axonal damage and fracture. Cytoskeleton disintegration caused by TBI is a major reason for \(\beta\)-APP accumulation in axons (Leclercq et al., 2002). Our study found that either chronic alcoholism or strike alone can raise \(\beta\)-APP expression in the medulla oblongata. Under the combined effect of alcoholism or strike alone can raise axonal damage, promote neuronal apoptosis and be abnormally cleaved into A\(\beta\) which can damage cell membranes, organelles and cytoskeletons, leading to neuronal apoptosis or necrosis (Golde et al., 1992; Haass et al., 1992; Suzuki et al., 1994). Through activation of GSK-3beta, A\(\beta\) promotes the hyperphosphorylation of tau protein (Takashima et al., 1996, 1998; Ferreira et al., 1997; Shea et al., 1997), causing microtubule damage and affecting neurotransmitter synthesis, transport, release and uptake which results in abnormal communication between nerve cells.

CONCLUSIONS

This study revealed that chronic alcoholism can cause axonal injury in the rat medulla oblongata, and a concussion blow can directly cause nerve damage, promote neuronal apoptosis and be abnormally cleaved into A\(\beta\) which can damage cell membranes, organelles and cytoskeletons, leading to neuronal apoptosis or necrosis (Golde et al., 1992; Haass et al., 1992; Suzuki et al., 1994). Through activation of GSK-3beta, A\(\beta\) promotes the hyperphosphorylation of tau protein (Takashima et al., 1996, 1998; Ferreira et al., 1997; Shea et al., 1997), causing microtubule damage and affecting neurotransmitter synthesis, transport, release and uptake which results in abnormal communication between nerve cells.

SUPPLEMENTARY MATERIAL

Supplementary material is available at Alcohol and Alcoholism online.

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