Annexin A1 conveys neuroprotective function via inhibiting oxidative stress in diffuse axonal injury of rats

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Research Article

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Abstract

Purpose

Diffuse axonal injury (DAI) is a critical pathological facet of traumatic brain injury (TBI). Oxidative stress plays a significant role in progress of DAI. Annexin A1 has been demonstrated benefit for recovery of neurofunctional outcomes after TBI. However, whether annexin A1 exhibits neuronal protective function through modulating oxidative stress in DAI remains unknown.

Methods

Expression of annexin A1 was evaluated via real time PCR and western blot analysis in rat brainstem after DAI. Neurological effect of annexin A1 following DAI through quantification of modified neurologic severity score (mNSS) was compared between wild-type and annexin A1-knockout rats. Brain edema and neuronal apoptosis, as well as expression of oxidative factors and inflammatory cytokines was analysed between wild-type and annexin A1 deficiency rats after DAI. Furthermore, mNSS, oxidative and inflammatory cytokines were assayed after timely administration of recombinant annexin A1 for DAI rats.

Results

In brainstem of DAI, the expression of annexin A1 remarkably increased. Ablation of annexin A1 increased mNSS score and brain water content of rats after DAI. Neuron apoptosis in brainstem after DAI was exaggerated by annexin A1 deficiency. In addition, annexin A1 deficiency significantly upregulated level of oxidative and inflammatory factors in brainstem of DAI rats. Moreover, mNSS decreased by annexin A1 treatment in rats following DAI. Expression of oxidative and inflammatory molecules in rat brainstem subjected to DAI inhibited by annexin A1 administration.

Conclusions

These results demonstrated that annexin A1 exhibits neuronal protective function in the progression of DAI mainly dependent on suppressing oxidative stress and inflammation.

Introduction

Diffuse axonal injury (DAI) is a pivotal and common pathological facet of traumatic brain injury (TBI) (Gerber et al., 2013), which is a public health issue with an high rate of mortality, co-morbidities, and long-term disabilities (Frati et al., 2017). DAI is caused by sudden rotated acceleration and deceleration leading to immediate tearing of axonal cytoskeleton throughout white matter (Mu et al., 2022). The development of DAI is not only due to primary axotomy from mechanical forces, but also from secondary axotomy as a result of a progressive molecular and cellular cascade of pathologic alteration after initial traumatic...
damage. As the axonal transport is disrupted, pathological gradual accumulation of transport products causes axonal swelling (Kelley et al., 2006), cerebral axonal disconnection (Graham et al., 2020), and Wallerian degeneration, which finally leads to cognitive, motor, and sensory deficits (Huang et al., 2018). Nevertheless, since some axons are not permanently injured in early process, only their function is disrupted, so researchers regard this as a potential time-window for therapeutic intervention.

Initial events that contribute to progression of DAI include oxidative stress, inflammation, apoptosis and so on (Robinson, 2021). In the early process post trauma, oxidative stress is confirmed as playing a pivotal role in the genesis of DAI that is linked to the effects of a plethora of molecular and cellular changes (Frati et al., 2017). Excess intracellular Ca\(^{2+}\) influx caused by mechanical disruption induces the generation of reactive oxygen species (ROS) in the mitochondria, triggering oxidative stress into the axons (Davis and Vemuganti, 2022). Numerous production of free radicals in the axons leads to peroxidation and toxicity of lipid, protein, and DNA resulting in cellular and tissue degeneration (Palmieri et al., 2021). In addition, oxidative stress initiates and exacerbates neuroinflammation contributing to the mechanism of cell apoptosis, which can also further promote ROS (reactive oxygen species) generation. Importantly, the involvement of cytokines in neuroinflammation such as TNF-α, IL-1β, IL-6, and adhesion molecules (ICAM-1) have been demonstrated in the damaged diseases of central nervous system (Yang and Zhou, 2019). Furthermore, oxidative stress can directly disrupt the balance between neurotransmitters, leading to excitotoxicity and neuronal cell death. Thus, inhibiting oxidative stress in DAI may potentially improve multiple aspects of the response of the brain to trauma.

Recent studies have demonstrated that annexin A1 (AnxA1) is a 37-kDa calcium-dependent phospholipid-linked protein that is known to involve in oxidative stress, inflammation and immune response in multiple conditions. Knockdown of AnxA1 enhanced the oxidative stress and inflammation in cigarette smoke extract (CSE)-induced bronchial epithelial cells, a chronic obstructive pulmonary disease in vitro model (Yu and Zhang, 2022). In addition, AnxA1 peptide Ac2-26 significantly suppressed the activity of NADPH oxidase and malondialdehyde to decrease oxidative stress, lung injury and epithelium apoptosis in acute respiratory distress syndrome rats (Ju et al., 2023). Besides, in the kidney following ischemic injury, AnxA1 limited ROS production and increased antioxidant enzymes to abrogate oxidative stress and attenuate tubular cell death, which finally preserves the kidney against the ischemia (Suliman et al., 2021). Furthermore, Ac2-26 significantly ameliorated neuronal apoptosis, reduced the volume of cerebral infarct and improved the neurological function of mice on cerebral I/R (ischemic/reperfusion) injury (Xu et al., 2021).

On the basis of these observations and previous studies, rescuing axons not permanently damaged in early process is of great significance for DAI therapeutic intervention. Importantly, exogenous recombinational AnxA1 improved neuron functional outcomes through ameliorating inflammation and BBB (blood brain barrier) disruption in mice after an animal model for TBI [19]. In addition, given that focal lesions of DAI mainly involve in the midline of brain including brainstem and corpus callosum. Thus, in this project we further set out to investigate the effect of AnxA1 on neuronal function after DAI, and whether AnxA1 conveys its neuroprotective role via modulating the oxidative stress in the early
process of DAI. The study will provide key insights into the role of AnxA1 and its modulation mechanism in early development of DAI.

**Methods**

**2.1. Animal**

Sprague-Dawley rats (male, weight 250-300g, 8–10 weeks of age, n = 14), AnxA1 knockout (AnxA1<sup>−/−</sup>) and wild-type (AnxA1<sup>+/+</sup>) rats were purchased from Experimental Animal Center of Xi’an Jiaotong University (Shaanxi, China). The animals were housed and fed in a room under SPF conditions, and kept at 20°C to 23°C under 12:12-h light-dark cycles. All experimental procedures involving animals were in strict agreement with the Guidelines for the Care and Use of Laboratory Animals from the National Institutes of Health. All animal studies were approved by the Institutional Animal Care and Use Committee of the Medical College of Xi’an Jiaotong University.

**2.2 Rat model of diffuse axonal injury (DAI) and treatment**

A DAI rat model was established using a lateral head rotation device as described in previous studies (Pang et al., 2016)(Li et al., 2013)(Smith et al., 1997). After weighing, all rats were anesthetized with an intraperitoneal injection of ketamine (100 mg/kg) and xylazine (10 mg/kg). Head of rat was positioned and fixed horizontally in rotating injury device by two lateral ear bars, a head clip and an anterior teeth hole, with body of 30° oblique to the laboratory table. After the trigger was started, the injury device rapidly rotated the rat head, involving sudden acceleration and deceleration, through a 90° angle laterally in the coronal plane. Primary coma was tested in all injured rats. Control group (sham group) only underwent same anesthesia and fixation to device without injury. Rats died by injuries were excluded (One out of 10 modeled rats were excluded) and would be replaced by new one. Rats were held in an electric blanket to maintain body temperature until complete recovery from anesthesia. Administration of human recombinant AnxA1 (rhAnxA1) (R&D Systems, Minneapolis, MN) was performed through the tail vein at 30 mins after DAI.

**2.3 Neurological assessment**

Neurological dysfunction was assessed through the modified neurological severity score (mNSS) test at different time points (1, 3, and 7 days post-DAI) (Garcia et al., 1995). mNSS test is comprised of motor function, sensory function and reflexes. Function was graded on a scale from 0 to 18 points. A higher score implies worse neurological impairment (normal score is 0; maximal deficit score is 18). mNSS was applied to evaluate the neurological deficits of rats before sacrifice by two researchers were blinded to group information (AnxA1<sup>+/+</sup> group and AnxA1<sup>−/−</sup> group).

**2.4 Evaluation of brain edema**

Brain water content was measured at 1, 3, and 7 days after DAI between AnxA1<sup>+/+</sup> group rats and AnxA1<sup>−/−</sup> group rats(Wang et al., 2020). Rat brain was dissected as soon as possible after sacrifice. The
entire brain was divided into injured hemispheres, cerebellum, and brainstem. When the cerebellum were removed, the left cerebral tissue was weighed immediately to obtain the wet weight. Then brain tissues were incubated at 72°C to dry for 3 days to re-weigh the dry weight. At last, brain water content was analysed according to \[\frac{(\text{wet weight} - \text{dry weight})}{\text{wet weight}} \times 100\%\].

### 2.5 Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL)

Apoptosis of brainstem at 1, 3 and 7 days after DAI was tested by TUNEL assay using In Situ Cell Death Detection Kit (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. After rats were euthanatized, the brains were collected and fixed in 4% PFA solution. The fixed brain tissues were embedded in paraffin. 5µm paraffin-embedded sections were de-paraffinized, rehydrated, and permeabilized. Permeabilized brain sections were incubated with the TUNEL reaction mixture solution in 37°C incubator for 1h avoiding the light. Sections were counterstained with hematoxylin for 1min after washing under running water. Sections were dehydrated in graded ethanol, cleared in xylene, and coverslipped with fluorescent mounting medium (Invitrogen). At last, sections were imaged under the microscope (Carl Zeiss Microscopy LLC, Thornwood, NY). Apoptotic cells in the brainstem, identified by a dark brown stain, were counted by using Adobe Photoshop software with two independent researchers blinded to group information.

### 2.6 Measurement of superoxide anion for brainstem

Superoxide (\(O^{2-}\)) anion is one form of reactive oxygen species, which is main contributor to trigger oxidative stress. Production of superoxide anion in brainstem homogenates was measured according to the approach of Babior et al. (1973), based on reduction of cytochrome c. After sacrificed the rats, brainstem was dissected immediately under surgical microscope. Brainstem tissues were homogenized in freshly prepared Tris-KCl buffer (1.15% KCl and 0.05 M Tris, pH = 7.4), using a Potter-Elvehjem homogenizer fitted with Teflon pestle. The 2 mL reaction mixtures contained 25 µL of homogenate and 0.05 mM cytochrome c in Tris-KCl buffer (pH = 7.4). Reaction mixtures were incubated at 37°C for 15 min, and then tubes were placed on ice to terminate reactions. The mixtures were centrifuged at 700 \(\times\) g for 10 min, and the supernatant fractions were collected for subsequent spectrophotometric measurement at 550 nm under a Spectronic-20 spectrophotometer. After averaging the measurements and background subtraction, the values were normalized by protein concentration for each brainstem sample.

### 2.7 Quantification of glutathione in brainstem tissue

Antioxidative substance total glutathione (GSH) in brainstem tissue was assayed using the Glutathione Fluorometric Assay Kit (BioVision, China) according to the manufacturer's protocol. Fresh obtained brainstem tissues were sonicated in ice cold glutathione assay buffer. De-protonation was conducted using perchloric acid followed by centrifugation at 13,000 g \(\times\) 2 min. Reduced glutathione in the supernatant was assayed using o-phthalaldehyde (OPA), which reacts specifically with reduced GSH (not GSSG), with generation of fluorescent signal. Samples and standards were read on a fluorescence plate reader equipped with Excitation/Emission at 340/420 nm using a microplate reader (BioVision, China).
2.8 Real-time PCR

Total RNA was extracted from fresh brainstem tissue of rats with RNeasy-Kit (Qiagen, Valencia, MD) according to the manufacturer’s protocol. RNA samples were quantified by measurement of optic absorbance at 260 nm and 280 nm in a spectrophotometer. RNA concentration result showed high purity with a 260 nm/A280 nm ratio > 2. First stranded cDNA was synthesized from 1 µg total RNA with PrimeScript™ RT reagent Kit (Takara Biomedical Technology, Beijing, China) in a final reaction volume to a total of 20 µL. Procedure of real-time PCR was conducted with the SYBR green Master Mix Kit (Thermo Scientific, Waltham, US) and run on standard Real-time PCR System (Applied Biosystem, Waltham, US). Rat Annexin A1 forward 5’-CCTACCCTTCCTCTCAATC-3’ and reverse 5’-CATCTGTTCCAAGTCCCT-3’; Rat Nox2 forward 5’-CTGCCAGTGTGCTGGAATCT-3’ and reverse 5’-TGTGAATGGCCGTGTGAAGT-3’; Rat IL-1β forward 5’-AGCTTCAGGAAGGCAGTGTC-3’ and reverse 5’-TCAGACAGCAGAGGCATT-3’; Rat TNF-α forward 5’-GCTCTTCTTCATTCTGCTT-3’ and reverse 5’-TGGGAACTTCTCATCCCTTTG-3’; Rat ICAM1 forward 5’-GCCTCTTCTCATCCCTGCTT-3’ and reverse 5’-TGGGAACTTCTCATCCCTTTG-3’; GAPDH forward 5’-CAACGGGAAACCACCCCATACCA-3’ and reverse 5’-ACGCCAGTAGACTCCACGCAT-3’. GAPDH was regarded as housekeeping gene. Real-time PCR was conducted using a LightCycler (Roche) at an annealing temperature of 60°C with 40 cycles. The analysis of data was done by GraphPad Prism and Microsoft Excel. In order to gain fold change induction of mRNA level expression, the \(2^{-\Delta\Delta Ct}\) approach was used as described by Schmittgen et al., 2008.

2.9 Western blotting

Proteins of brainstem tissue were extracted using Laemmli sample buffer (BioRad Laboratories, Hercules, CA). The protein concentration was determined by Bio-Rad DC protein assay kit. Equal amounts of protein were applied to 10% SDS-PAGE and transferred to Hybond ECL nitrocellulose membrane (GE Healthcare). The membranes were incubated with anti-TNF-α (Abcam), monoclonal anti-IL1-β (Cell Signaling Technology), and anti-ICAM-1 (Cell Signaling Technology) respectively. β-actin (Cell Signaling Technology) was used as the loading control. Secondary antibodies were detected by the SuperSignal West Pico. All bands intensities were quantitated by Image J.

2.10 Statistical analysis

Results were expressed as the mean ± SEM. Prism software was used for statistical analyses. Significant differences between samples were analyzed with two-tailed Student’s t test. \(P<0.05\) was considered as statistically significant.

Results

3.1 Endogenous annexin A1 molecule was activated in rat brainstem after DAI
Previous studies showed a significant increase of the AnxA1 level in injured cortex of mice at different time points after controlled cortical impact model, an animal model for TBI (Liu et al., 2021). In addition, DAI is characterized by focal lesions in the midline of the brain such as the brainstem and corpus callosum. Therefore, in the first set of experiments, we investigated the involvement of AnxA1 in brainstem after DAI induced by TBI.

As shown in Fig. 1A, an obvious increase of AnxA1 mRNA level in rat brainstem at different time points (1 day, 3 days and 7 days) post DAI was observed compared to sham control. Besides, western blotting results showed that DAI remarkably increased AnxA1 protein levels in brainstem compared to normal control brainstem (Fig. 1B). Quantification of western blotting band intensities were consistent with band picture results (Fig. 1C). These mRNA and protein data suggested that AnxA1 may be an important regulator in the pathogenesis of DAI.

### 3.2 Annexin A1 deficiency exacerbated neurological outcomes and brain edema of rats after DAI

The above results demonstrated obviously increased expression of AnxA1 in DAI brainstem, which revealed involvement of AnxA1 in pathogenesis of the DAI. In addition, given that the previous literature indicated exogenous AnxA1 administration improves the neurological outcomes following animal TBI model, we investigated the potential influence of AnxA1 deficiency on the neurological function for rats after DAI. Modified Neurological Severity Score (mNSS) system, a reliable and well-accepted approach for quantitative evaluation of neurological function, was applied to assess the degree of injury. As shown in the Fig. 2A, compared to wild-type rats, AnxA1 knockout rats showed higher score at 1 day, 3 days, and 7 days after DAI, suggesting that deficiency of AnxA1 sharply impairs the neurological function for rat subjected to DAI.

Brain edema, induced by DAI, is a critical pathological facet resulting in dysfunction of neuron. Therefore, brain water content was measured for DAI rats with AnxA1 deficiency at different time points. Figure 2B showed that the brain water content increased remarkably in $\textit{AnxA1}^{-/-}$ rats at day 3 and day 7 post-DAI compared to $\textit{AnxA1}^{+/+}$ rats post-DAI. This result indicated that AnxA1 protects the neuron against the DAI.

### 3.3 Annexin A1 deficiency significantly increased the apoptotic index in the rat injured brainstem

Apoptosis, a serious pathological process for neuronal injury, was demonstrated to significantly increase after DAI in previous study. So next the extent of apoptosis for DAI rats with AnxA1 deficiency was performed via TUNEL assay. The results showed that rats subjected to DAI in $\textit{AnxA1}^{-/-}$ group had more apoptotic cells and neuronal loss than the wild-type rats at 1 day, 3 days, and 7 days post-DAI, which implied that AnxA1 plays potential benefit for neuronal survival in the process of DAI (Fig. 3).
3.4 Annexin A1 deficiency increased DAI-stimulated oxidative stress in rat brainstem after DAI

The above results demonstrated an upregulation of AnxA1 in brainstem subjected to DAI and improved neuron survival in rats with DAI, which revealed that involvement of AnxA1 during pathogenesis of the axonal injury induced by TBI. Previous studies demonstrated that oxidative stress evoked by TBI boosts neuroinflammation and contributes to neuronal degeneration and cell death in the brain after injury. AnxA1 has not only been confirmed to exert neurological benefit, but also has been demonstrated to correlate with oxidative stress in several disease conditions, including a chronic obstructive pulmonary disease in vitro model induced by cigarette smoke extract (Yu and Zhang, 2022) and ischemic kidney injury (Suliman et al., 2021). Thus we set out to investigate the effect of AnxA1 on expression of oxidative stress factors in DAI. Quantification of the level of superoxide, a critical component of reactive oxygen species (ROS), was performed in brainstem tissues of rats with AnxA1 deficiency. Compared to wild-type rats, AnxA1−/− rats showed higher level of superoxide at 3 days after DAI (Fig. 3A). GSH is the major cellular antioxidant to counteract reactive oxygen species. At the 3 days after DAI, the GSH level robustly reduced in the brainstem of AnxA1−/− rats compared with AnxA1+/+ rats (Fig. 3B). NADPH oxidase 2 (Nox2), the primary producer of ROS, is the major contributor to oxidative stress. In AnxA1−/− rats, DAI brainstem generated significantly mRNA accumulation of Nox2 as compared to wild-type brainstem at the 3 days after DAI (Fig. 3C). These results implicated that the deficiency of AnxA1 in brainstem subjected to DAI exacerbates the induction of oxidative stress mediators.

3.5 Annexin A1 deficiency promoted inflammatory molecules expression in brainstem of DAI rats

In addition to the effects of AnxA1 on expression of oxidative stress molecules in DAI, we explored the influence of AnxA1 on inflammation after DAI. We evaluated the mRNA and protein levels of typical inflammatory cytokines, including TNF-α, IL-1β and ICAM-1, involved in process of DAI. The mRNA level of TNF-α, IL-1β and ICAM-1 significantly increased in the rat brainstem with AnxA1 deficiency at 3 days after DAI, when compared to wild-type DAI rats (Fig. 4A). Western blotting results showed that AnxA1−/− brainstem exhibited an upregulation in TNF-α, IL-1β and ICAM-1 protein levels at day 3 after DAI (Fig. 4B-D).

3.6 Recombinant annexin A1 downregulated oxidative stress and inflammatory factors in rat brainstem after DAI

Previous study revealed that human recombinant AnxA1 ameliorated inflammation and improved functional outcomes in mice subjected to TBI triggered by controlled cortical impact (Liu et al., 2021). Our study results indicated deficiency of endogenous AnxA1 exacerbated neuronal injury and aggravated the oxidative stress and inflammation in DAI. In order to investigate whether AnxA1 exerts its neuronal benefit through modulating the oxidative stress and inflammation, we further evaluated the effect of exogenous...
AnxA1 on the oxidative stress and inflammation after DAI. After modeling, animals were treated with a concentration of rhAnxA1 (1 µg/kg) by tail vein at 30 mins after DAI modeling for one time, and controls were injected with 0.9% NaCl. As shown in the Fig. 6A, levels of superoxide, pro-oxidative factor, in brainstem tissues significantly decreased in rat brainstem subjected to DAI with immediate rhAnxA1 administration, compared to DAI rats with 0.9% NaCl administration. Besides, rhAnxA1 treatment robustly increased level of antioxidant factor GSH in rat brainstem at the 3 days after DAI (Fig. 6B). Real time PCR results showed rhAnxA1 treatment significantly decreased Nox2 expression in DAI brainstem at the 3 days after DAI (Fig. 6C). In addition, the inflammatory molecules TNF-α and IL-1β downregulated by rhAnxA1 in brainstem subjected to DAI (Fig. 6C).

3.7 Recombinant annexin A1 protected neurological function for rats against DAI

In light of the above results that exogenous AnxA1 ameliorated oxidative stress and inflammation after DAI, we next investigated whether exogenous AnxA1 improves neuronal functional outcomes following the DAI. Recombinant annexin A1 was injected through tail vein at 30 mins after DAI modeling. mNSS was quantified for rats at different time points (1 day, 3 days and 7 days) after DAI. Compared to vehicle group DAI rats, treatment with rhAnxA1 led to a significant decrease in the score in treatment groups, especially at 3 days and 7 days after DAI (Fig. 7).

Discussion

TBI is a major source of health loss and disability worldwide. Globally, the annual incidence of TBI is variably estimated at 27 to 69 million. Many suffered individuals live with significant permanent disabilities, resulting in major socioeconomic burden (Capizzi et al., 2020). DAI, a significant pathological facet of TBI, is resulted from direct impact of the shearing forces, which cause disruption, retraction, edema of the axons. These alterations impede the normal transport of proteins and electrolytes that resulting in secondary axonal damage (Pavlovic et al., 2019). The degree of injury and the duration of loss of consciousness and coma directly correlate with the extent of axonal pathology. While most of the suffered brain appears structurally normal and only a minority of axons are affected even in severe TBI, DAI can cause collective impairment that leads to disruption of signal transfer and plays critical implications for neurological function (Angelova et al., 2021). Nevertheless, some axons are not permanently injured during the early period of DAI, and only their function is disrupted. Therefore, this early period is regarded as a optimal time-window for DAI therapy. According to this theory, timely administration is crucial to rescuing function of the axoplasmic transport.

Recent study indicated that imbalance between production and removal of ROS is disputed in the early time after DAI (Fesharaki-Zadeh, 2022), and further triggers and aggravates inflammation, apoptosis and calcium-mediated damage (Hakiminia et al., 2022). AnxA1 is demonstrated to suppress the oxidative stress in multiple diseases and exit neuronal benefit in nervous system conditions. In the current study, our objective was to gain insights into the potential effect of AnxA1 in the progression of DAI. In addition,
we had a particular interest in studying whether AnxA1 plays its role via modulating oxidative stress and inflammation. Together, current results clearly demonstrated that modulating AnxA1 may represent an extra approach of suppressing oxidative stress and inflammation, further to attenuate neurological impairment in the progress of DAI.

Recent literature demonstrated that endogenous AnxA1 obviously increased in injured cortex of mice at different time points after controlled cortical impact model, an animal model for TBI (Liu et al., 2021). Significant insights in therapy for axonal pathology have arisen through establishment of lateral head rotation device using suddenly acceleration and deceleration (Pang et al., 2016). Given that brainstem is vulnerable to the mechanical rotated acceleration damage (Sandhu et al., 2019), so we detected the AnxA1 expression in brainstem of DAI rat. In agreement with these previous results, our research results showed that both protein and mRNA level of AnxA1 upregulated in DAI brainstem, especially at the first day following DAI. Consider that the previous study results and current results, we can see that AnxA1 significantly increased and activated in early pathogenesis of axonal injury.

Neurological function is ultimate prognostic outcome for brain trauma. Neuronal benefit of AnxA1 is studied in various conditions, such as ischemic retina diseases (Hui et al., 2022), nociception in dorsal root ganglion neurons (Zhang et al., 2021) and epileptogenic processes (Gimenes et al., 2019). Therefore we established AnxA1 knockout rat and evaluated the neurological effect of AnxA1 on rat after DAI. The results demonstrated that AnxA1 deficiency aggravated neurological dysfunction at 3 days and 7 days after DAI, which is consistent with previous research data that AnxA1 has neuroprotective effect.

Brain edema, induced by axonal mechanical damage, is critical pathological element contributed to dysfunction of neuron. DAI can also induce brain edema, and uncontrolled brain edema increases intracranial pressure and lead to brain herniation, which results in poor prognosis (Castaño-Leon et al., 2022). The edema was more serious, and the extent of axonal injuries was more severe (Andriessen et al., 2010). In our study, brain water content results showed that AnxA1 deficiency increase brain edema at 3 days and 7 days after DAI, which indicated that AnxA1 significantly attenuates the brain edema. In our study, measurement of brain water content also indicated AnxA1 beneficial effect on brain edema which might contribute to the neuroprotective properties of AnxA1. In addition, consider that dysfunction of axonal transport could induce the apoptosis, so we investigated the AnxA1 effect on apoptosis. The result showed that AnxA1 decreased the apoptosis in brainstem in DAI. All these results indicated that AnxA1 plays an beneficial role in DAI.

Based on these findings, we then wanted to determine the effects of AnxA1 on neuron cell death. Neuronal apoptosis can be resulted from disrupted axon transport. Recent study showed that AnxA1 administration can alleviate neuronal apoptosis in acute ischemic stroke (Xu et al., 2021). In agree with previous data, our results indicated that AnxA1 deficiency increase the neuronal cell death. From these data, we concluded that AnxA1 is a critical molecular in the progression of DAI, alleviates the neuronal dysfunction, and protects the neurological outcomes against the diffused axonal damage.
Oxidative stress is a key mechanism that contributes to axonal deterioration and neuronal death in the development and progression of DAI, which can trigger a cascade of events that exacerbate axonal damage. Increases in the defense mechanisms through the use of exogenous antioxidants may be neuroprotective, particularly if they are given within the neuroprotective time window (Miguel et al., 2021). AnxA1, a protein that has been shown to possess antioxidant properties and can help protect cells from oxidative damage in multiple conditions (Caso et al., 2021), can scavenge ROS and inhibit their harmful effects, thereby reducing oxidative stress. Therefore, we reason that AnxA1 plays its neurological beneficial role via inhibiting the oxidative stress in the early development of DAI. As expected, our research showed that AnxA1 deficiency downregulated crucial pro-oxidative moleculars superoxide and Nox2, and upregulated the synthesis of primary anti-oxidative substance GSH, which implied that AnxA1 may be dependent on suppressing oxidative stress to exhibit its neuroprotective effects in the progression of DAI.

Under pathological state, ROS can activate inflammatory pathways, leading to the release of pro-inflammatory cytokines and chemokines that attract immune cells to the site of injury. These immune cells can further exacerbate oxidative stress and axonal damage by releasing ROS and other toxic molecules. For example, high concentration of TNFα impairs the ability of microglia to eliminate glutamate, and this causes excitatory toxicity and injures neurons (Brás et al., 2020). In addition, AnxA1 exhibits anti-inflammatory function on various conditions, such as AnxA1-/- mice showed a expansion of proinflammatory macrophages in the cardiac ischemic area (Ferraro et al., 2019). In agree with previous data, our results showed AnxA1-/- rat dramatically downregulate the expression of inflammation factors in brainstem of DAI. Combined with above figures, these findings suggested that AnxA1 ameliorates the axonal deterioration and neuron degeneration relying on inhibiting oxidative stress and inflammation in the development of DAI.

In order to confirm our hypothesis, we further investigated the effect of exogenous AnxA1 on oxidative stress and inflammation in the brainstem following DAI. To achieve optimal outcomes following axonal injury by trauma, timely treatment is essential. Our result showed that the high level of pro-oxidative substances superoxide and Nox2 induced by DAI can be inhibited after timely rhAnxA1 treatment. Besides, antioxidants GSH enhanced by AnxA1 treatment. On the other hand, pro-inflammatory cytokines TNF-α and IL-1β in brainstem decreased by timely AnxA1 administration. Importantly, emerging evidences indicated that timely exogenous AnxA1 administration is appreciated for its neuroprotective effects, and detrimental complications of prolonged cerebral hypoxia and ischemia have been attenuated by AnxA1 administration. As expected, our data clearly confirmed that neurological deficit in rat suffered to DAI were ameliorated by the optimal administration of rhAnxA1. All these above findings for the first time suggested that AnxA1 conveys its neuroprotective effect mainly dependent on suppressing the oxidative stress in the murine DAI model.

In conclusion, this current study showed that AnxA1 is significantly increased and activated in the brainstem after DAI. AnxA1 deficiency exacerbates the neurological function, neuronal death, oxidative stress and inflammation. What's more, administration rhAnxA1 suppresses oxidative stress and
inflammation, and improves the neurological outcome. Collectively, we summarized that AnxA1 protects neuronal function in the development of DAI mainly dependent on inhibiting oxidative stress and inflammation, which strongly suggests AnxA1 may be a potent and promising neuroprotective agent against diffuse axonal injury, also in other axonal damage conditions, such as hypoxia/ischemia, infarcts, multiple sclerosis, hypoglycemia.

**Abbreviations**

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<tr>
<th>Acronym</th>
<th>Definition</th>
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<tr>
<td>TBI</td>
<td>Traumatic brain injury</td>
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<td>DAI</td>
<td>Diffuse axonal injury</td>
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<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
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<td>mNSS</td>
<td>modified Neurological Severity Score</td>
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<td>Annexin A1</td>
<td>AnxA1</td>
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<td>rhAnxA1</td>
<td>Recombinant human Annexin A1</td>
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<tr>
<td>I/R</td>
<td>Ischemic/reperfusion</td>
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<td>BBB</td>
<td>Blood brain barrier</td>
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<td>Nox2</td>
<td>NADPH oxidase 2</td>
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**Declarations**

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**Statements & Declarations**

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**Competing Interests**

The authors declare that no competing interest exists.
Author contribution

YH conceived the study and reviewed the manuscript. WZ finished experiments, processed the results, and wrote the manuscript. XL participated experiments and helped analyze the data. SB and YH revised the final version of the manuscript.

Consent to publish

All authors reviewed and approved the final manuscript.

References


**Figures**

**Figure 1**

*Endogenous annexin A1 molecule was activated in rat brainstem after DAI.* (A) DAI resulted in significantly increased mRNA level of annexin A1 in rat brainstem tissue compared to control group. (B) Protein level of annexin A1 was dramatically upregulated in brainstem of DAI rats compared to control.
rats. (C) Quantification revealed an increase in the protein expression levels of annexin A1 in the brainstem of DAI rats compared with control group rats based on images in B. The relative protein expression level was normalized to β-actin. The values are represented as mean ± SEM. * \(P<0.05\), DAI rats vs control group rats, \(n = 5\)

Figure 2

**Figure 2**

Annexin A1 deficiency exacerbated neurological outcomes of rats after DAI. (A) Modified neurologic severity score (mNSS) was detected for rats at different time points (1 day, 3 days and 7 days) after DAI. Compared to wild type DAI rats, annexin A1 deficiency rats have higher mNSS score, especially at 3 days and 7 days after DAI. (B) Quantitation of brain water content at different time points (1 day, 3 days and 7 days) after DAI. Compared to wild type DAI rats, brain water content of \(AnxA1^{+/+}\) DAI rats is much higher than the \(AnxA1^{-/-}\) rats subjected to DAI, especially at 3 days and 7 days after DAI. Pre: before the DAI. The values are represented as mean ± SEM. * \(P<0.05\), \(AnxA1^{+/+}\) DAI group vs \(AnxA1^{-/-}\) DAI group, \(n = 5\) for each group.
Annexin A1 deficiency significantly increased the apoptotic index in the injured brainstem of rats. TUNEL staining was detected in injured brainstem of AnxA1+/+ rats and AnxA1−/− rats at 1 day, 3 days and 7 days after DAI respectively. There were more apoptotic index in injured brainstem of AnxA1−/− rats compared with wild type DAI rats. The values are represented as mean ± SEM. * P < 0.05, AnxA1+/+ DAI group vs AnxA1−/− DAI group, n = 5 for each group. Scale bar: 50 μm
Annexin A1 deficiency increased DAI-stimulated oxidative stress in brainstem of rat. (A) Superoxide level in brainstem of rats subjected to DAI significantly increased in annexin A1 deficiency rats compared to wild-type rats. (B) Annexin A1 deficiency remarkably downregulated glutathione (GSH) level in rat brainstem at 3 days after DAI. (C) mRNA level of annexin A1 was not detected in annexin A1-deficient DAI rat compared with wild-type DAI rat. mRNA level of pro-oxidative factors Nox2 increased in annexin A1-deficient DAI rats compared with wild-type DAI rats. All assays were performed at 3 days after DAI. The values are represented as mean ± SEM. * $P < 0.05$ vs wild-type DAI group, n = 5 for each group.
Annexin A1 deficiency promoted inflammatory molecules expression in brainstem of DAI rats. (A) mRNA level of pro-inflammatory molecules TNF-α, IL-1β and ICAM-1 increased in annexin A1-deficient DAI rats compared with wild-type DAI rats. (B-D) Western blotting results indicated that protein level of TNF-α, IL-1β and ICAM-1 were also upregulated in annexin A1-deficient DAI rats compared with wild-type DAI rats. Quantification based on Western blotting images confirmed an upregulation in the protein expression levels of TNF-α, IL-1β and ICAM-1 in the brainstem of the AnxA1⁻/⁻ rats subjected to DAI compared with AnxA1⁺/⁺ rats following DAI. The relative protein expression level was normalized to β-actin. All assays were performed at 3 days after DAI. Values are represented as mean ± SEM. * P < 0.05 vs wild-type DAI group, n = 5 for each group.
Figure 6

Recombinant annexin A1 downregulated oxidative stress and inflammatory factors in rat brainstem after DAI. (A) Recombinant human Annexin A1 (rhAnxA1) treatment suppressed superoxide level in rat brainstem after DAI compared to vehicle group treated with same concentration IgG. (B) rhAnxA1 upregulated GSH level in rat brainstem after DAI compared to vehicle group treated with same concentration IgG. (C) Real time PCR results indicated that mRNA level of oxidative factor Nox2 and inflammatory factors TNF-α and IL-1β increased in brainstem of DAI rats administrated with rhAnxA1 compared with vehicle group treated with same concentration IgG. rhAnxA1 treatment was conduct at 30 mins after DAI modeling. All assays were performed at 3 days after DAI. Values are represented as mean ±SEM. * P < 0.05 vs vehicle group, n = 5 for each group.
Figure 7

Recombinant annexin A1 protected neurological function for rats against DAI. mNSS was quantified for rats at different time points (1 day, 3 days and 7 days) after DAI. Recombinant annexin A1 was injected through tail vein at 30 mins after DAI modeling. Compared to vehicle group DAI rats, rats treated with recombinant annexin A1 showed lower mNSS score, especially at 3 days and 7 days after DAI. All assays were performed at 3 days after DAI. Values are represented as mean ±SEM. * P < 0.05 vs vehicle group, n = 5 for each group