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The antioxidative, non-psychoactive tricyclic phenothiazine reduces brain damage after experimental traumatic brain injury in mice

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HIGHLIGHTS

- Use of a non-psychoactive tricyclic phenothiazine in acute brain injury.
- Treatment in a post-injury paradigm at 30 min post insult.
- Phenothiazine treatment dose-dependently reduced brain lesion volume.
- No significant differences in markers of cerebral inflammation.

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ABSTRACT

Oxidative stress due to free radical formation is an important mechanism of secondary brain damage following traumatic brain injury (TBI). Phenothiazine has been found to be a strong antioxidant in eukaryotic cells in vitro and in invertebrates in vivo. The present study was designed to determine the neuroprotective potency of unsubstituted phenothiazine in a paradigm of acute brain injury. Thirty minutes after pneumatic, controlled cortical impact (CCI) injury, C57Bl6 mice were randomly assigned to "low dose" (3 mg/kg, LD) or "high dose" (30 mg/kg, HD) s.c. phenothiazine or vehicle treatment. Brain lesion, neurofunctional impairment, body weight, and markers of cerebral inflammation were determined 24 h after the insult. Phenothiazine treatment dose-dependently reduced brain lesion volume (LD: −19.8%; HD: −26.1%) and posttraumatic body weight loss. There were no significant differences in the neurological function score and in markers of cerebral inflammation (Iba-1 positive cells, TNFα expression), whereas iNOS expression was significantly lower compared to vehicle-treated animals. Phenothiazine appears to modify in a post-treatment protocol certain aspects of secondary brain damage in vivo at unusually low concentrations, in particular the cortical contusion volume after TBI. The potential role of the reduced iNOS expression is unclear at present.

1. Introduction

Traumatic brain injury (TBI) is the leading worldwide cause of death and disability in the young population between 25 and 44 years of age [1,2]. The injury mechanisms of TBI are classified into primary and secondary processes. Primary injury is caused by the impact itself, leading to mechanical destruction of brain parenchyma. Due to the nature of TBI, recovery of mechanically destroyed brain tissue is not possible; however, secondary injury is a dynamic process in the surroundings of the primary lesion, which starts immediately after the impact and continues for hours to days after the insult [2–4]. In the past years, oxidative damage has emerged as a potentially important mechanism of secondary brain damage. Oxygen free radicals are accused to impose oxidative stress to lipids, proteins, and nucleic acids – finally enhancing neuronal cell death [5,6]. Already 3 h after TBI, mitochondrial oxidative damage causes mitochondrial dysfunction and loss of mitochondrial calcium buffering capacity [6]. Moreover, oxidative cell membrane damage may cause a cytoplasmic calcium increase, exacerbating

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the excitotoxic wave migrating through the area of secondary brain damage. Different direct oxidants like hydrogen peroxide or peroxynitrite as well as characteristic markers of lipid peroxidation such as F_4-neuroprostanes or 4-hydroxynonenal are present 1 h post insult and peak at different times within the first 72 h after injury [5–7]. Hence, antioxidative strategies could bear the potential to limit secondary brain injury after TBI, even if it is clear that the adverse impact of oxidative stress in acute neurological disease may be even more difficult to counteract than under less severe, chronic conditions [6,8].

Phenothiazine is an organic compound that has been used for decades as lead structure for the development of neuroleptic and antihistaminic drugs such as chlorpromazine or promethazine. Phenothiazine itself has been used in man as an anthelmintic until the introduction of the more potent benzimidazoles [9]. Recent studies on the non-psychoactive phenothiazine core structure have revealed biochemically antioxidative and neuroprotective effects at very low doses in different models of neurodegenerative disease, primarily Parkinson's disease [9–11]. Here, robust efficacy in vivo has been shown in invertebrates [12] and, as published in abstract form, in rats [13]. Moreover, phenothiazine has been shown to be unusually non-toxic and tolerable up to multi-gram doses in humans [14]. In addition, phenothiazine readily crosses the blood–brain barrier [15], making it a potential drug candidate to ameliorate cerebral pathologies.

The present study was designed to determine the dose-dependent influence of phenothiazine on brain lesion volume, functional outcome and cerebral inflammation after experimental brain trauma.

2. Materials and methods

2.1. Animals

Male C57Bl6 mice ($n = 27$, weight 18–20 g) were used for the experiments and cared for in compliance with the Institutional Guidelines of the Johannes Gutenberg University, Mainz. The experiments were performed in accordance with the German Animal Protection Law and following approval by the State Animal Care Authority (Landesuntersuchungsamt Rheinland-Pfalz).

2.2. Experimental protocol

All mice were randomly assigned to 3 groups ($n = 9$ each): low dose (LD, 3 mg/kg phenothiazine), high dose (HD, 30 mg/kg phenothiazine), and vehicle (Veh). Mice were subjected to brain trauma and received either phenothiazine or vehicle subcutaneously 30 min after insult. Histological brain damage and microglia activation were determined 24 h after insult. Inflammatory marker gene expression (iNOS, TNFα) was quantified by real-time polymerase chain reaction (PCR) in pericontusional brain tissue.

2.3. Traumatic brain injury

Experimental TBI was performed as previously published in detail [16]. Anesthesia was induced in a bell jar filled with 5 vol% sevoflurane in 100% O_2 for 2 min and maintained by sevoflurane via facemask (2.3 vol% sevoflurane in 40% O_2/60% N_2). Anesthetized mice were fixed in a stereotactic frame and a rectal temperature probe was placed to maintain body temperature at 37 °C with a feedback-controlled heating pad. A cranial window (4 mm × 4 mm) was drilled above the right parietal cortex between the sagittal, lamboid, and coronal sutures. The injury was induced with a pneumatic controlled cortical impact (CCI) device (L. Kopacz, Mainz, Germany) perpendicular to the surface of the brain (diameter of the impactor tip 3 mm, impact velocity 8 m/s, impact duration 150 ms, penetrating depth 1 mm). The craniotomy window was closed with the initially removed bone flap using conventional tissue glue and the skin wounds were closed. After surgery the mouse was transferred into a cage and placed in a neonatal incubator for 2 h with controlled air temperature (33 °C) and ambient humidity (35%).

2.4. Drug preparation

The half-maximum effective concentrations (EC50 values) of phenothiazine as neuroprotective antioxidant in vitro have been determined to be 0.02–0.04 μM in different cellular paradigms [9]. To attain a more than half-maximum effective concentration in vivo, and to leave sufficient allowance for potentially rapid compound degradation and imperfect drug mobilization and brain tissue penetration, a starting dose yielding in theory a 300-fold EC50 concentration was chosen, i.e. 3 mg/kg as single bolus injection. As phenothiazine cannot be dissolved and administered directly in aqueous media, it was first dissolved in diethylether at 166 mM concentration and then rapidly injected (1:9 vol:vol) into a 20% oil-in-water emulsion used clinically for parenteral nutrition (Lipovenös MCT20, Braun, Melsungen, Germany), which produced a solubilized phenothiazine preparation stable for at least 24 h. The final solution contained 10% diethylether in MCT20; the vehicle solution was prepared correspondingly.

2.5. Assessment of functional outcome

Before and 24 h after CCI, body weights were determined and animals were rated by an investigator blinded toward group allocation, using the 10-point neurologic severity score (NSS). This test has been established for mouse TBI and evaluates motor function, alertness, balancing, and general behavior [17].

2.6. Histological evaluation

The brains were carefully removed from isoflurane-anesthetized animals, immediately frozen in powdered dry ice and stored at −20 °C. Brains were cut in the coronal plane with a cryostat (HM 560 Cryo-Star, Thermo Fisher Scientific, Walldorf, Germany). The first slide was taken at bregma +3.14 mm according to the Mouse Brain Library atlas (http://www.mbl.org). Subsequently, 10 μm thick sections were collected every 500 μm, placed on Superfrost Plus slides (Thermo Fisher Scientific) and stained with cresyl violet. The area of both hemispheres and the region of contused brain tissue, defined as the region lacking cresyl violet staining, were analyzed using a computerized image system (Optimas 6.51, Optimas Corporation, Bothell, USA) by an investigator blinded to the experimental groups. Contusion volume was calculated by multiplying contusion areas obtained from 16 consecutive sections with a 500 μm distance between histological sections based on following formula: $0.5\ \text{mm} \times (A_1 + A_2 + \ldots + A_n)$ [16].

2.7. Ionized calcium binding adaptor molecule-1 (Iba-1) staining

For immunohistochemical staining of activated microglia, cryosections were fixed in 4% paraformaldehyde, rinsed with PBST (phosphate-buffered saline (PBS) with 0.3% Triton X-100; Sigma, St. Louis, USA), and incubated in blocking solution consisting of PBS with 5% normal goat serum (DAKO, Glostrup, Denmark). Antibodies were dissolved in PBST. At room temperature, sections were incubated overnight with rabbit anti-Iba-1 antibody (1:1500; WAKO Pure Chemical Industries, Osaka, Japan). After rinsing, biotinylated anti-rabbit IgG (H+L) (Vector Laboratories Inc., Burlingame, USA) was applied as secondary antibody. A peroxidase ABC-Complex (Vector Laboratories Inc., Burlingame, USA) was applied after rinsing. The non-bound complex was washed out, and