

Blood-brain barrier breakdown and repair following gliotoxic drug injection in the brainstem of streptozotocin-diabetic rats

Ruptura e reparo da barreira hematoencefálica pós-injeção de droga gliotóxica no tronco encefálico ratos diabéticos

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ABSTRACT

Ethidium bromide (EB) causes local astrocytic disappearance, with glia limitans disruption and blood-brain barrier (BBB) breakdown. The aim of this study was to evaluate the BBB integrity after the injection of 0.1% EB or 0.9% saline solution into the cisterna pontis of Wistar rats submitted or not to the streptozotocin diabetogenic model. Brainstem sections were collected from 24 hours to 31 days post-injection for ultrastructural analysis and glial fibrillary acidic protein immunohistochemical staining. Some animals received colloidal carbon ink by intravenous route at the same periods. In rats injected with EB, results revealed astrocyte disappearance and leakage of carbon particles beginning at 48 hours and persisting for 7 days in non-diabetic rats and for 15 days in the diabetic ones, although, in both groups, several areas remained devoid of astrocytic processes up to 31 days. In rats injected with saline, there was no sign of astrocytic loss or carbon particles leakage.

Key words: blood-brain barrier, central nervous system, diabetes mellitus, ethidium bromide.

RESUMO

O brometo de etídio (BE) determina o desaparecimento local de astrócitos, com ruptura da glia limitans e dano na barreira hematoencefálica (BHE). Este estudo visou avaliar a integridade da BHE após injeção de solução de BE a 0,1% ou de salina a 0,9% na cisterna pontis de ratos Wistar submetidos ou não ao modelo diabetogênico da estreptozotocina. Fragmentos do tronco encefálico foram coletados das 24 horas aos 31 dias pós-injeção para estudo ultraestrutural e marcação imuno-histoquímica para proteína glial fibrilar ácida. Alguns animais receberam carvão coloidal por via intravenosa nos mesmos períodos. Nos grupos injetados com BE, os resultados mostraram desaparecimento astrocitário e extravasamento de partículas de carvão nas lesões a partir das 48 horas, persistindo por até sete dias nos animais não diabéticos e 15 dias nos diabéticos, embora, em ambos os grupos, diversas áreas permanecessem destituídas de astrócitos até 31 dias após. Nos ratos injetados com salina, diabéticos ou não, não houve sinal de perda astrocitária nem de extravasamento vascular de carvão.

Palavras-Chave: barreira hematoencefálica, sistema nervoso central, diabetes mellitus, brometo de etídio.

Ethidium bromide (EB) injection in the central nervous system (CNS) causes local oligodendroglial and astrocytic disappearance, with subsequent primary demyelination, glia limitans disruption, supposed blood-brain barrier (BBB) breakdown and Schwann cell invasion¹⁻³. Hyperglycemia found in diabetes mellitus is known to cause changes in the behaviour of glial cells, such as oligodendrocytes and Schwann cells⁴. As BBB induction and maintenance is closely related to glial cell support, the aim of this study was to evaluate and compare the BBB integrity after the injection of 0.1% EB into the brainstem of diabetic and non-diabetic rats.

METHODS

This experiment was approved by the Ethics Commission of the University Paulista (protocol number 002/09). Adult male Wistar rats were used and some received, after a 12 hours of fasting, a single injection of streptozotocin (50 mg/kg) in 0.01M citrate buffer (pH 4.5) into the tail vein. Ten days after that, blood glucose level was measured and animals with levels of 200 mg/dL or more were considered diabetics. At this time, they were submitted to a local injection of 10 microlitres of 0.1% EB (group I) or 0.9% saline (group II).

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solution into the cisterna pontis. All rats were anaesthetized with ketamine and xylazine (5:1; 0.1 mL/100g) and a burr hole was made on the right side of the skull, 8 mm rostral to the frontoparietal suture. Injections were performed freehand using a Hamilton Syringe, fitted with a 35° angled polished 26-gauge needle into the cisterna pontis, an enlarged subarachnoid space below the ventral surface on the pons. Non-diabetic rats also received 10 microlitres of 0.1% EB solution (group III) or 0.9% saline solution (group IV). Body weight and blood glucose levels (Dextrostix, Ames) were recorded at 3 times – at the moment of the streptozotocin injection, 10 days after and at the time of euthanasia. Water and food were given *ad libitum* during the experimental period. At different times after EB or saline injection, the rats were anaesthetized and submitted to intracardiac perfusion with 4% glutaraldehyde in 0.1 M Sorensen phosphate buffer (pH 7.4) at each of the following periods - 24, 48, 72 hours, and 7, 11, 15, 21 and 31 days after intracisternal injection. Thin slices of the brainstem (pons and mesencephalon) were collected and post-fixed in 0.1% osmium tetroxide, dehydrated with graded acetones and embedded in Araldite 502 resin, following transitional stages in acetone. Thick sections were stained with 0.25% alkaline toluidine blue. Selected areas were trimmed and thin sections were stained with 2% uranyl and lead acetate and viewed in a JEM -1200 EX2 JEOL transmission electron microscope.

From each group, some animals received colloidal carbon ink by intravenous route at the same periods previously mentioned, 20 minutes before being submitted to euthanasia and perfusion with 10% buffered formalin solution (pH 7.4). Immunohistochemical staining to glial fibrillary acidic protein (GFAP) (GFAP - rabbit anti-cow GFA, code number ZO334, Dako) was performed as an astrocyte marker according to Sanchez et al.⁵

RESULTS

In rats from groups II and IV, there was no sign of astrocyte loss and no leakage of ink from blood vessels in the injection site. In groups III (Fig 1A) and I (Fig 1B), astrocyte disappearance began at 48 hours and some areas were still devoid of astrocytic processes 31 days after. In the EB-induced lesion, leakage of carbon particles was seen from 48 hours to 15 days in group I (diabetic) and from 48 hours to 7 days in group III (non-diabetic). Tight junctions did not show any detectable ultrastructural change due to the lack of perivascular astrocytes in groups injected with EB. Diabetic rats from group I presented delayed macrophagic activity (Fig 2B) and lesser remyelination (Fig 3B) in comparison to non-diabetic rats from group III (Fig 2A and 3A). Although oligodendrocytes were the major remyelinating cells in the brainstem, Schwann cells invaded EB-induced lesions, firstly appearing at 11 days in non-diabetic rats and by 15 days in diabetic rats. Results indicate that short-term streptozotocin-induced diabetes hindered BBB reconstruction and both oligodendrocyte and Schwann cell remyelination in comparison to non-diabetic rats after local EB injection (Fig 4A and 4B).

DISCUSSION

A previous study⁶ had already demonstrated that normal Wistar rats presented leakage of carbon particles from 48 hours to 7 days after injection of this gliotoxic drug, as well as astrocyte disappearance for up to 31 days. The present results in the non-diabetic group corroborate those findings. In streptozotocin-diabetic rats, however, the most important difference noted was that ink leakage persisted until the 15th day, suggesting that the diabetic state somehow delayed the functional repair of the barrier.

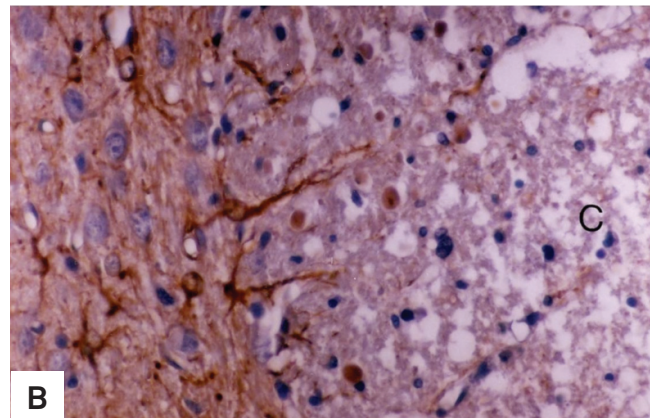
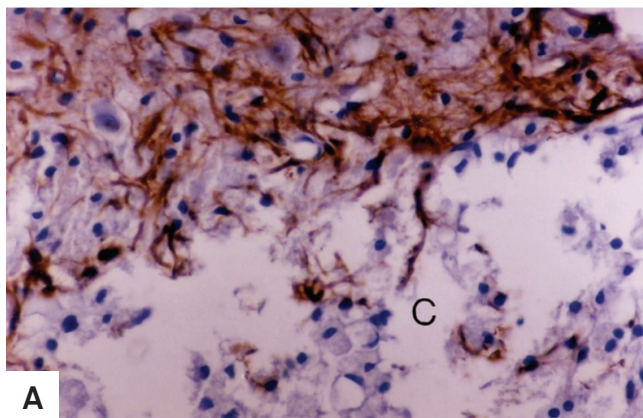


Fig 1. EB-induced lesions in groups III (A, non-diabetic) and I (B, diabetic). Note the absence of glial fibrillary acid protein (GFAP) - positive cells in the central areas (C) of both groups at 31 days post-injection. Increased astrocyte immunoreactivity to GFAP is seen at the lesion boundaries. Greater amounts of cellular debris are found in the center of the lesions of the diabetic group. GFAP immuno-histochemical staining - Obj. 100x.

The BBB, a physical and metabolic barrier, is an important and complex structure designed to maintain a constant neuronal extracellular environment by restraining the penetration of a wide range of hydrophilic molecules, proteins and cellular elements into the CNS^{7,8}. It is composed of endothelial cells lining CNS microvessels that are interconnected by tight junctions and are surrounded by pericytes and astroglial processes. Besides the capillary endothelium and adjacent glia, this anatomical barrier may also include components of the extracellular matrix. The BBB involves several

mechanisms by which free exchange of non-lipid soluble molecules between blood and cerebrospinal fluid (CSF) space is restricted, such as active (energy-requiring) processes (e.g., amino-acid transporters) and passive mechanisms (e.g., endothelial tight junctions) that regulate CNS homeostasis, because blood contains very high levels of potential neurotoxic substances and neural exposure to them would render ineffective neuronal excitability⁹. Intravenous injection of an exogenous contrast agent (that is normally excluded from the CNS space, but can enter with damage to the integrity of the

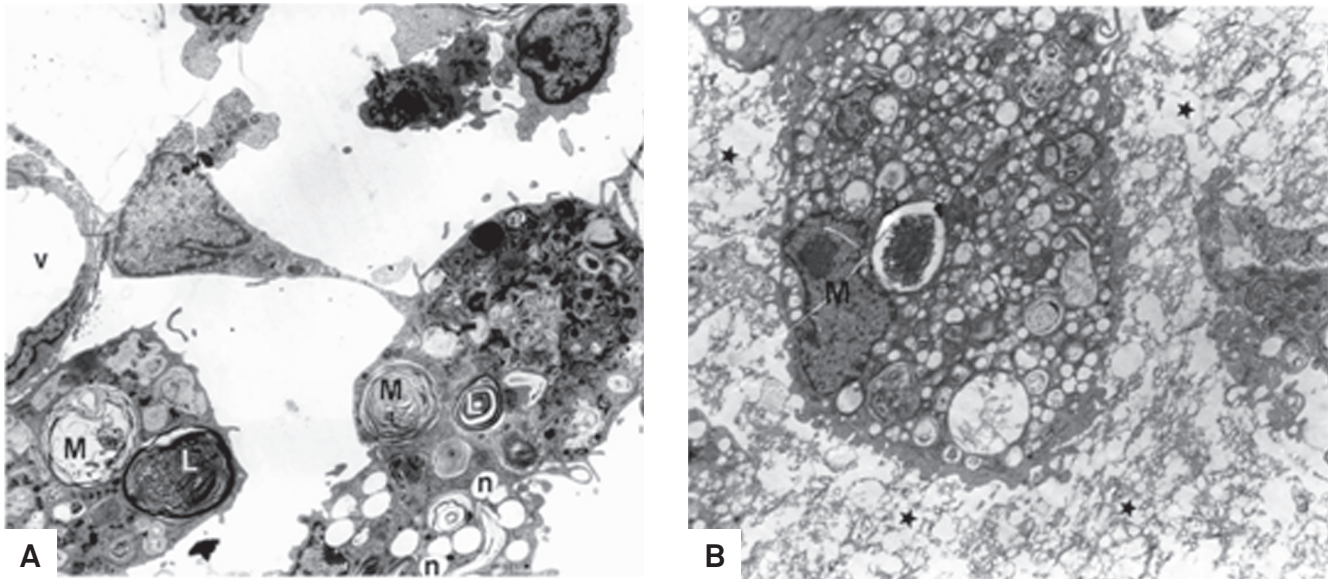


Fig 2. Central areas of ethidium bromide-induced lesions at 21 days in non-diabetic (A, group III) and diabetic (B, group I) rats containing macrophages (M) in phagocytic activity. (A) Myelin can be seen in different stages of breakdown, from the visualization of lamellae (L) to neutral fat droplets (n). v: blood vessel. Electron micrograph - 4.309x. (B) Diabetic rats show huge amounts of myelin-derived membranes in the extracellular space (asterisk) suggesting delayed phagocytic activity. Electron micrograph - 3.968x.

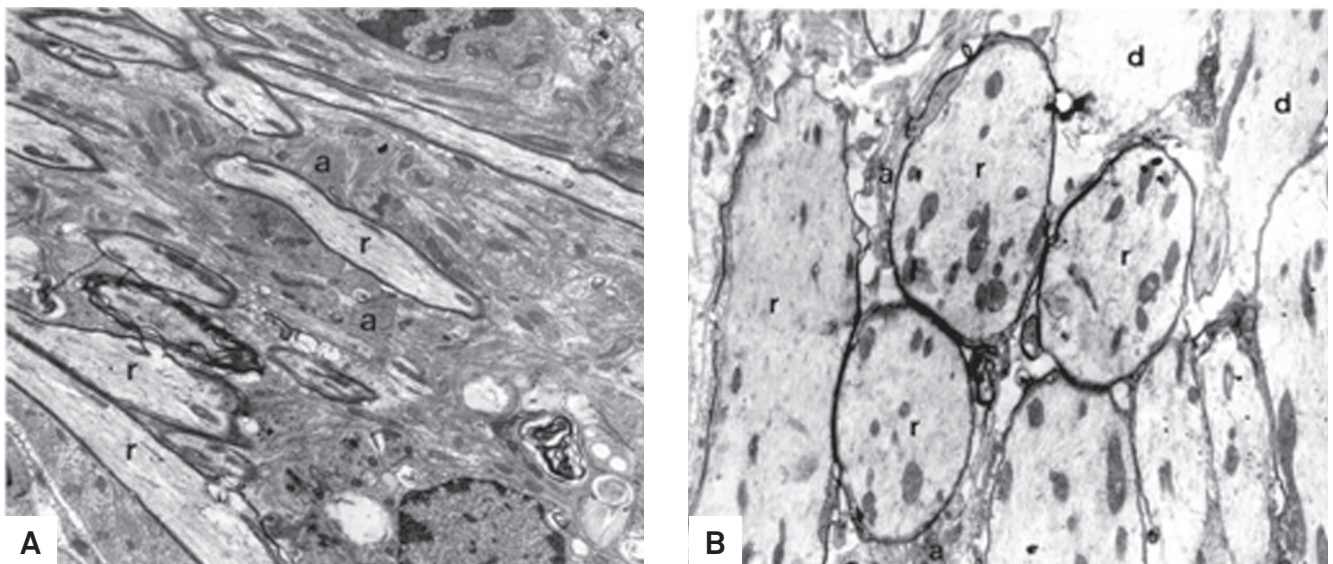


Fig 3. Oligodendrocyte-remyelinated areas in non-diabetic (A, group III) and diabetic (B, group I) rats at peripheral sites after ethidium bromide injection. (A) Axons in initial remyelination (r) among hypertrophic astrocytes (a) at 21 days. Electron micrograph - 6.064x. (B) Fewer axons (r) show thinner myelin sheaths even at 31 days. Many axons remain demyelinated (d). Electron micrograph - 7.152x.

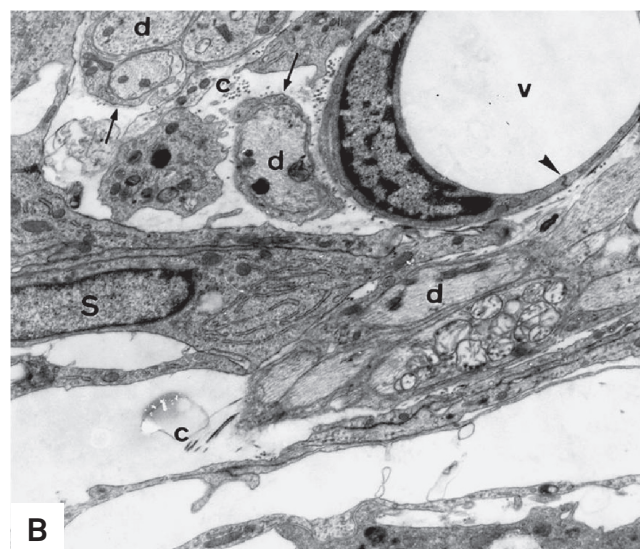
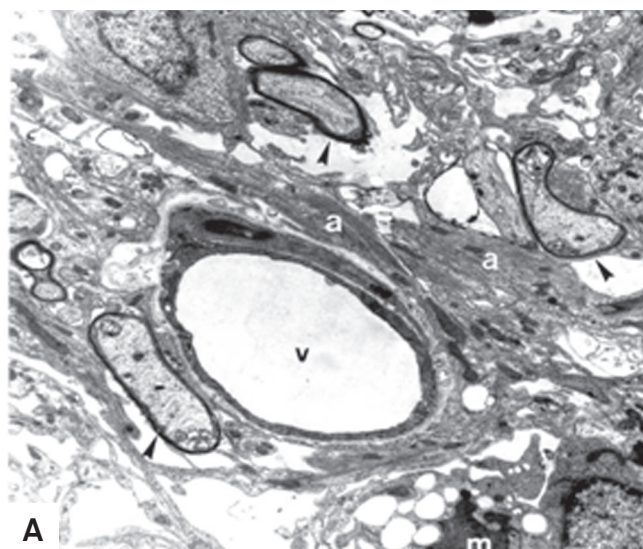


Fig 4. (A) Oligodendrocyte-remyelinated axons (arrowheads) near to a blood vessel (v) at 15 days after ethidium bromide injection. Note the presence of surrounding astrocyte processes (a). m: macrophage. Group III (non-diabetic) - Electron micrograph. 7.077x. (B) Demyelinated axons (d) at 31 days after EB injection in a perivascular area. Observe the presence of a tight junction (arrowhead) and Schwann cell (S) cytoplasm around the naked axons (arrows). v: blood vessel; c: collagen fibers. Group I (diabetic) - Electron micrograph. 10.864x.

BBB) allows magnetic resonance imaging (MRI) to visualize the breakdown of the BBB in patients with neurological disorders⁹, that can be even seen by light microscopy observation in experimental conditions using animal models⁶.

BBB breakdown is present in several common CNS disorders, such as stroke, trauma, brain tumors, multiple sclerosis, HIV-1 dementia, Alzheimer's and Parkinson's disease¹⁰. On the other hand, it is known that prolonged BBB disruption may lead to neurological deficits, as delayed cortical dysfunction and epileptiform activity involving glutamatergic and GABAergic neurotransmission⁸.

Diabetes mellitus is a metabolic disorder associated with structural and functional alterations of various organ systems. Although the peripheral neuropathy along with small and large blood vessel angiopathy can explain most of the diabetes-related organ failures, tissue injury is attributed mainly to chronic hyperglycemia¹¹. In contrast to the high prevalence of renal and retinal disease in diabetic patients, the chronic diabetic complications of the CNS are hardly noticeable and the deleterious effects of persistent hyperglycemia on brain metabolism and cognitive function are often unrecognized. This glucotoxicity is not tissue specific, even

though the severity of organ dysfunction in diabetes is highly variable¹¹. Oxidative stress plays an important role in tissue damage caused by both insulin-induced hypoglycemia and streptozotocin-induced diabetes, which may be the result of deterioration in glucose homeostasis caused by these metabolic changes.

During severe energy deprivation following hypoglycemia and diabetes, mitochondrial free radicals scavenger system is down regulated leading to reactive oxygen species generation and activation of processes conducting to DNA damage and neural cell death¹². Neuroinflammation may expose endothelium to proinflammatory cytokines, such as IFN-gamma, TNF-alpha and IL-1beta, which in turn disorganize cell-cell junctions, affect leukocyte endothelial adhesion and migration, increase expression of class II major histocompatibility complex molecules and decrease the brain solute barrier¹³.

Exact mechanisms for breakdown of the BBB in pathologic conditions, including EB gliotoxic injection, are not completely understood and may involve direct effects of these cytokines on endothelial regulation of BBB components, as well as indirect cytokine-dependent leukocyte mediated injury.

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