Propentofylline reverses delayed remyelination in streptozotocin-induced diabetic rats

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ABSTRACT

Objective: The diabetic state induced by streptozotocin injection is known to impair oligodendroglial remyelination in the rat brainstem following intracisternal injection with the gliotoxic agent ethidium bromide (EB). In such experimental model, propentofylline (PPF) recently showed to improve myelin repair, probably due to its neuroprotective, antiinflammatory and antioxidant effects. The aim of this study was to evaluate the effect of PPF administration in diabetic rats submitted to the EB-demyelinating model.

Materials and methods: Adult male rats, diabetic or not, received a single injection of 10 microlitres of 0.1% EB solution into the cisterna pontis. For induction of diabetes mellitus the streptozotocin-diabetogenic model was used (50 mg/kg, intraperitoneal route – IP). Some diabetic rats were treated with PPF (12.5 mg/kg/day, IP route) during the experimental period. The animals were anesthetized and perfused from 7 to 31 days after EB injection and brainstem sections were collected for analysis of the lesions by light and transmission electron microscopy.

Results: Diabetic rats injected with EB showed larger amounts of myelin-derived membranes in the central areas of the lesions and considerable delay in the remyelinating process played by surviving oligodendrocytes and invading Schwann cells after the 15th day. On the other hand, diabetic rats that received PPF presented lesions similar to those of non-diabetic animals, with rapid remyelination at the edges of the lesion site and fast clearance of myelin debris from the central area.

Conclusion: The administration of PPF apparently reversed the impairment in remyelination induced by the diabetic state.

Keywords: Central nervous system; diabetes mellitus; oligodendrocytes; propentofillyne; remyelination

INTRODUCTION

It is widely described that focal injection of the gliotoxic agent ethidium bromide (EB) in the white matter of the central nervous system (CNS) causes local oligodendrogial and astrocytic death, with consequent primary demyelination, blood-brain barrier disruption and Schwann cell invasion due to the glia limitans breakdown (1-6). Hyperglycemia found in diabetes mellitus is known to cause well described morphological and functional changes in peripheral neurons and Schwann cells (7). Much less is understood about the effects of hyperglycemia on CNS cells, mainly on glia. It is recognized that diabetes exacerbated astrocytic (8,9) and neuronal (10,11) damage induced by ischemia and reperfusion. On the other hand, insulin treatment prevented diabetes-induced alterations in astrocyte glutamate uptake and reverted the decreased GFAP expression in rats at 4 and 8 weeks of diabetes duration (12). Glial modifications were clearly pointed in some studies using streptozotocin-diabetic rats after the injection of EB (13-15), with marked delay on macrophagic scavenging activity of myelin debris, on oligodendrocyte and Schwann cell remyelination (13), on blood-brain barrier repair (14) as well as on glial fibrillary acidic protein (GFAP) expression in reactive astrocytes at the periphery of the injury site (15).

Several in vitro and in vivo studies have shown that propentofylline [PPF, 3-methyl-1-(5’-oxohexyl)-7-propylxanthine], a xanthine derivative, presents profound neuroprotective, antioxidant and anti-inflammatory effects (16,17). Clinically it has shown efficacy in degenerative vascular dementia (18) and as a potential adjuvant treatment to Alzheimer’s disease (19,20), schizophrenia (21) and multiple sclerosis (22). PPF probably depresses activation of microglial cells and astrocytes, which is associated with neuronal damage during inflammation and hypoxia and consequently decreases glial production and release of damaging pro-inflammatory factors (16). In the EB demyelinating...
model, PPF administration after gliotoxic injection significantly increased both oligodendroglial and Schwann cell remyelination at 31 days (23).

Thus, the aim of this investigation was to evaluate if PPF had the capacity of improving activity of myelogenic cells in diabetic rats following EB gliotoxic injury.

**MATERIALS AND METHODS**

This experiment was approved by the Ethics Commission of the Universidade Paulista (protocol number 023/11). Forty-eight adult (4-6 month old) male Wistar rats were used and 32 animals received, after a period of fasting of 12 hours, a single injection of streptozotocin (50 mg/kg, Sigma) in 0.01M citrate buffer (pH 4.5) by intraperitoneal (IP) route. Ten days after blood glucose level was measured and animals with levels of 300 mg/dL or more were considered diabetic. At this time they were submitted to a local injection of 10 microlitres of 0.1% EB into the cisterna pontis. All rats were anaeasthetized with ketamine and xylazine (5:1; 0.1 ml/100 g) and 2.5% thiopental (40 mg/kg) by IP route and a burr-hole was made on the right side of the skull, 8 mm behind the fronto-parietal 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. Diabetic rats were then distributed into two groups – untreated rats (group I, n = 16) and rats treated with 12.5 mg/kg/day of PPF (Agener União Química, São Paulo, SP, 20 mg/mL solution) by IP route during the experimental period (group II, n = 16). Non-diabetic rats which also received an intracisternal injection of 10 microlitres of EB formed a third group (group III, n = 16).

Body weight and blood glucose levels (Dextrostix, Ames) were recorded at 3 times – at the moment of the streptozotocin injection, at the moment of the EB injection and at the time of euthanasia. The animals were kept under controlled light conditions (12 h light-dark cycle) and water and food were given ad libitum during the experimental period. Four rats were anaeasthetized and were submitted to intracardiac perfusion with 4% glutaraldehyde in 0.1 M Sorensen phosphate buffer (pH 7.4) at each of the following periods - 7, 15, 21 and 31 days post-injection (p.i.). 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.

**RESULTS**

**Clinical observations of diabetic rats**

All rats submitted to the streptozotocin injection presented hyperglycemia (levels from 320 to 730 mg/dL) at the 10th day and at perfusion day. During the experimental period they developed characteristic polyuria, polydipsia and body weight loss. Plasma glucose levels and body weight data are shown in table 1.

**Table 1.** Body weights and plasma glucose levels of the experimental groups

<table>
<thead>
<tr>
<th>Day</th>
<th>Group I (16)</th>
<th>Group II (16)</th>
<th>Group III (16)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>BW (g)</td>
<td>PG (mg/dL)</td>
<td>PG (mg/dL)</td>
</tr>
<tr>
<td>Day -10</td>
<td>268.4 ± 7.8</td>
<td>133.2 ± 8.3</td>
<td>139.7 ± 7.6</td>
</tr>
<tr>
<td>Day 0</td>
<td>254.5 ± 6.5</td>
<td>256.6 ± 7.2</td>
<td>275.3 ± 8.3</td>
</tr>
<tr>
<td>Day 7</td>
<td>382.4 ± 10.1</td>
<td>346.2 ± 8.9</td>
<td>130.3 ± 11.9</td>
</tr>
<tr>
<td>Day 15</td>
<td>251.2 ± 15.9</td>
<td>389.8 ± 4.7</td>
<td>141.7 ± 10.6</td>
</tr>
<tr>
<td>Day 21</td>
<td>239.7 ± 4.2</td>
<td>243.7 ± 15.3</td>
<td>285.3 ± 9.5</td>
</tr>
<tr>
<td>Day 31</td>
<td>490.2 ± 11.4</td>
<td>433.3 ± 8.7</td>
<td>135.7 ± 11.3</td>
</tr>
<tr>
<td></td>
<td>469.7 ± 10.2</td>
<td>432.3 ± 8.1</td>
<td>135.7 ± 15.3</td>
</tr>
</tbody>
</table>

Data are presented as means ± standard deviation (SD) for the number of rats given in parenthesis. Group I – diabetic rats with ethidium bromide (EB) injection; Group II – diabetic rats with EB injection and propentofylline (PPF) treatment; Group III – non-diabetic rats with EB injection. BW: body weight (g); PG: plasma glucose (mg/dL); Day -10: day of streptozotocin injection in groups I and II; Day 0: day of EB administration in groups I, II and III.

**General aspects from EB-induced lesions**

The EB-induced lesions were similar to those previously described in the brainstem of diabetic (13-15) and non-diabetic rats (1-6). In general terms, they were characterized by demyelinated areas in the ventral surface of the pons and mesencephalon, con-
taining in the central region phagocytic cells, variable amounts of myelin-derived membranes in a distended extracellular space as well as naked axons. At the periphery, from 15 to 31 days p.i., it was noted the presence of oligodendrocytes and Schwann cells, the latter occurring in areas of enlarged extracellular space devoid of astrocytic extensions, notably around blood vessels and in subpial areas. Astrocyte processes were invariably seen near the incipient oligodendrogial remyelination and Schwann cells were noted in astrocyte-free areas producing thicker sheaths than those formed by oligodendrocytes at the same period. In the central area a variable proportion of axons persisted without myelin until the 31st day p.i. Few lymphocytes and some infiltrating pial cells were also observed in all periods.

Comparison between EB-induced lesions from groups I, II and III

By 7 days p.i., the examination of semithin and ultrathin sections from diabetic rats from group I revealed the appearance of a larger demyelinated area filled with huge amounts of myelin-derived membranes around naked axons and foamy macrophages when compared with rats from groups II (diabetic rats treated with PPF) and III (non-diabetic rats) at the same period. The quantity of myelin debris was remarkably greater in group I and such difference appeared to increase from 15 to 31 days p.i. (Figure 1A and B) in comparison with the other groups (Figure 1C and D). No astrocytic processes were seen in the central area of the lesions in all groups, but in groups II and III astrocytic processes appeared to be more frequently found at the edges of the injury site after 15 days. At peripheral locations, by day 15 p.i. cells with morphological resemblance to oligodendrocytes were seen over the edges of the lesions, some of them already forming thin myelin sheaths. Schwann cells appeared associated with one or multiple demyelinated axons or already forming thin myelin lamellae around single axons in astrocyte-free areas. Remyelination was a relatively rare finding in diabetic rats from group I even at 31 days p.i. (Figure 2A and B) in relation to groups II and III (Figure 2C and D). Axons with signs of degeneration (Figure 3A and B) persisted until day 31 p.i. in animals from all groups, although a much larger number of degenerating fibers was observed in diabetic rats from group I than in diabetic rats treated with PPF or non-diabetic rats. Pial cell infiltration was noted from 15 to 31 days p.i. in all groups. Differences in remyelination between diabetic rats from group I and diabetic rats treated with PPF or non-diabetic rats from group III clearly appeared from day 15 p.i. as the last two groups presented a greater proportion of oligodendrocyte remyelinated axons when compared to the diabetic ones without PPF. Schwann cell remyelination in diabetic rats was also increased with PPF treatment (Figure 4A and B).

DISCUSSION

Diabetic rats presented lesions similar to those observed in earlier investigations (13-15,24) involving EB injection in streptozotocin-induced diabetes and comparable to those observed in cyclophosphamide immunsuppressed rats (5), in which large amounts of myelin debris and demyelinated axons were found as well as delayed remyelination by both oligodendrocytes and Schwann cells. The persistence of myelin-derived membranes in an expanded extracellular space suggested that somehow phagocytic activity was impaired (13). Diabetes has also shown to decrease GFAP expression in reactive astrocytes around EB-induced lesions (15). The mechanisms by which the diabetic state affect CNS glial cells, such as oligodendrocytes, and astrocytes, remain unclear. On the other hand, it is known that hyperglycemia causes intracellular glucose accumulation and increases polyol pathway activity by aldose reductase in cells that do not need insulin for glucose transmembrane transport, such as Schwann cells, thus resulting in increased levels of sorbitol and fructose, a fact that may cause osmotic swelling and even cell death (25). Hyperglycemia may also increase oxidative stress and reactive oxidative species (ROS) generation, initiating apoptotic signaling pathways (26). It is suggested that the impaired remyelination found in diabetic rats following EB injection may be caused by a lack of trophic factors for proliferation and differentiation of myelinogenic cells, such as surviving oligodendrocytes; their progenitors, the OPCs (oligodendrocyte progenitor cells); and/or invading Schwann cells. Insulin and IGF-1, for example, which are recognized as stimulating factors for Schwann cells (27), are decreased in experimental diabetes (28). IGF-1 and 2 also stimulate OPC proliferation (29) and receptors for IGF-1 are expressed in astrocytes and oligodendrocytes over the edges of demyelinating lesions (30).
Figure 1. Macrophages (m) in intense phagocytic activity in a central area of the lesion at 15 days after EB injection. Note in (A) and (B) from diabetic rats (group I) the presence of demyelinated axons (d) and the larger quantity of myelin-derived membranes (asterisk) in the extracellular space. (A) Bar = 2 µm; (B) Bar = 1 µm. (C) Group II (diabetic rats treated with PPF); Bar = 3 µm. (D) Group III (non-diabetic rats); Bar = 4 µm.

Figure 2. (A,B) Demyelinated axons (d) persisted among macrophages (m) and myelin-derived membranes (asterisks) at 31 days in diabetic rats from group I despite the presence of oligodendrocytes (O). (A) Bar = 3 µm; (B) Bar = 2 µm. (C,D) Thinly remyelinated axons by oligodendrocytes (O) at 31 days. (C) Group II (diabetic rats treated with PPF); Bar = 2 µm. (D) Group III (non-diabetic rats); Bar = 1 µm.
As previously observed in non-diabetic rats (23), PPF administration seemed to increase both oligodendrocyte and Schwann cell remyelination in the rat brainstem of diabetic animals following gliotoxic injury, apparently reversing the deleterious effects of diabetes on remyelination. Known mechanisms of PPF include inhibition of cyclic AMP (cAMP) and cyclic GMP phosphodiesterases (PDE) and action as a reuptake inhibitor for the purine nucleoside and neurotransmitter adenosine by blocking the activity of membrane nucleoside transporters (ENTs). This leads to increased intracellular cAMP levels and greater extracellular concentrations of adenosine (16), stimulating adenosinergic neurotransmission and adenosine 2 (A2) receptor-mediated cAMP synthesis (31,32).

In the CNS, PPF acts as a glial modulator, with direct actions on microglia, decreasing microglial proliferation and expression of inflammatory cytokines in vitro, such as tumor necrosis factor-α (TNF-α) and interleukin 1β (IL-1β) (33,34). Regulation of cytokine production by leukocytes includes the adenylate cyclase – cAMP – protein kinase pathway, which also affects the activity of a great number of other cell types (35).
Intracellular levels of the second messenger cAMP can be increased by adenylate cyclase activation or by cAMP-degrading phosphodiesterases (PDE) inhibition. Elevation of cAMP in leukocytes mainly down-regulates inflammatory and immune responses. Inhibition of secretion of the T helper cell type 1 (Th1) – derived cytokines IL-2, IL-12 and IFN-δ and of TNF-α synthesis has been described for a number of PDE inhibitors (PDEIs), including PPF, and for activators of adenylate cyclase, such as prostaglandins (35). Yoshikawa and cols. (34) reported that PPF, a type III-IV specific PDEI, although decreasing in a dose-dependent manner the production of the inflammatory cytokines TNF-α, IL-1 and IL-6 by LPS-activated microglial cells in vitro, increased up to three times the production of IL-10, an inhibitory cytokine, which is recognized to impair cytokine production of Th1 lymphocytes. Besides IL-10 also inhibits the activation of macrophages and microglia induced by IFN-δ (34).

It has been hypothesized that inflammatory mediators could create a detrimental environment to myelin and myelinogenic cells with potentially damaging molecules to the neural tissue. Products secreted by macrophages, lymphocytes and astrocytes during the inflammatory reaction to EB may exacerbate the direct damaging effects induced by the gliotoxin. In such context the anti-inflammatory and antioxidant effects performed by PPF may be supportive to myelin repair even in diabetic rats, whose remyelinating capacity is undoubtedly compromised.

A Ca++ – dependent and excessive activation of glial cells is involved in neuroinflammation. In cultured microglial cells, several days’ treatment with adenosine agonists or PPF increased apoptosis in activated microglial cells and strongly inhibited the secretion of pro-inflammatory substances and the formation of ROS, as well as their transformation into macrophages after injury (31,32). Probably by affecting Ca++- and cAMP-dependent molecular signaling pathways, PPF stimulates the production of trophic factors in astrocytes, apparently avoiding a harmful and secondary astrocytic activation caused by previous microglial up-regulation.

Diabetic rats treated with PPF presented lesions that resembled those observed with cyclosporine treatment following streptozotocin-induced diabetes (24), with a higher density of oligodendrocytes over the edges of the lesions and increased remyelination. The precise mechanisms by which the beneficial effects of PPF occur in diabetic rats remain obscure, although it has been believed that drugs that elevate extracellular adenosine and/or block the degradation of cyclic nucleotides, like PPF, may explain the improvement of remyelination.

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REFERENCES