Article

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Semi-quantitative analysis of the effects of cyclosporine on remyelination following gliotoxic injection in the brainstem

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ABSTRACT

The use of cyclosporine (CsA) has shown to induce an increase in the density of oligodendrocytes near remyelinating areas following the injection of ethidium bromide (EB), a demyelinating agent, in the rat brainstem. This study was designed in order to evaluate if CsA has the capacity of increasing remyelination. In this context, a comparison between the final balance of myelin repair in CsA treated and non-treated rats was assessed using a semi-quantitative method developed for documenting the extent and nature of remyelination in gliotoxic lesions. Wistar rats were submitted to intracisternal injection of 10 microliters of 0.1% EB. Some were treated during 31 days with CsA (group III - 10 mg/kg/day by 7 days and, thereafter, 3 times a week, with a minimal interval of 48 hours) by intraperitonial route. Others were not treated with CsA (group I). A control group was planned receiving into the cisterna pontis 10 microliters of 0.9% saline solution and following after that the same CsA administration protocol (group II). Results clearly demonstrate that *in vivo* administration of CsA after EB-demyelinating lesions stimulated oligodendrocyte remyelination (mean remyelination scores of 3.72±0.25 for oligodendrocytes and 1.04±0.39 for Schwann cells) compared to non-treated animals (3.13±0.71 and 1.31±0.62, respectively), although the mechanisms by which this positive CsA effect occurs are unclear.

Key words: cyclosporine, central nervous system, ethidium bromide, oligodendroglia, remyelination, Schwann cells, semi-quantitative analysis.

Análise semiquantitativa dos efeitos da ciclosporina na remielinização após injeção gliotóxica no tronco encefálico

RESUMO

O uso de ciclosporina (CsA) mostrou induzir um aumento na densidade de oligodendrócitos próximos a áreas de remielinização após injeção de brometo de etídio (EB), um agente desmielinizante, no tronco encefálico de ratos. Este estudo foi desenvolvido a fim de avaliar se a CsA possui a capacidade de acelerar a remielinização. Neste contexto, foi feita uma comparação entre o balanço final de reparo mielínico em ratos tratados ou não com CsA usando-se um método semiquantitativo desenvolvido para documentação da extensão e natureza da remielinização em lesões gliotóxicas. Ratos Wistar foram submetidos à injeção intracisternal de EB a 0,1%. Alguns foram tratados durante 31 dias com CsA (grupo III - 10 mg/kg/dia por 7 dias e, após, 3 vezes por semana, com um intervalo mínimo de 48 horas entre as aplicações) por via intraperitoneal. Outros não foram tratados com CsA (grupo I). Um grupo controle foi desenvolvido recebendo, na cisterna pontina, 10 microlitros de solução salina e seguindo após o mesmo protocolo de

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administração de CsA (grupo II). Os resultados mostram claramente que a administração *in vivo* de CsA após lesões desmielinizantes induzidas pelo EB estimulou a remielinização por oligodendrócitos (escores médios de remielinização de 3,72±0,25 para oligodendrócitos e 1,04±0,39 para células de Schwann) em comparação aos animais não-tratados (3,13±0,71 e 1,31±0,62, respectivamente), embora os mecanismos pelos quais este efeito positivo da CsA ocorre sejam desconhecidos.
Palavras-chave: análise semiquantitativa, brometo de etídio, células de Schwann, ciclosporina, oligodendróglia, remielinização, sistema nervoso central.

A greater proportion of oligodendroglial cells on the edges of demyelinating lesions was seen in rats treated with cyclosporine (CsA) after the injection of ethidium bromide (EB) in the rat brainstem[1]. This gliotoxic agent is known to induce early astrocyte disappearance and oligodendroglial loss when injected in the central nervous system (CNS) leading to, respectively, blood-brain barrier disruption and primary demyelination. Subsequent remyelination occurs performed by surviving oligodendrocytes and by invasive Schwann cells[1-6].

Nests of oligodendrocytes similar to those observed in a previous study using CsA following EB injection were seen in attempts of remyelination in multiple sclerosis (MS)[7] and experimental allergic encephalomyelitis (EAE)[8]. Such distribution is not considered a normal aspect of the nervous tissue and may be related to the existence of oligodendroglial progenitor cells (OPCs) and to the action of trophic factors on them[8].

CsA has as its major known effect the capacity of reducing the synthesis and liberation of interleukin-2 (IL-2), a cytokine released from T CD4+ lymphocytes (Th - *helper*)[9] and capable of inhibiting proliferation of OPCs[10,11].

Using a semi-quantitative method developed by Blakemore and Crang[12] and Gilson and Blakemore[13] for documenting the extent and nature of remyelination in gliotoxic lesions, the aim of this study was to investigate if CsA could stimulate oligodendrocyte remyelination after being administered by 31 days in rats submitted to a focal EB injection in the brainstem.

METHOD

This experiment was approved by the Ethics Comission of the University Paulista (protocol number 002/09). Twenty male Wistar rats, 4 to 6 months old, were used. They were divided into 3 groups: I (n=8), constituted of animals injected with EB into the cisterna pontis; II (n=4), including animals injected with saline solution and treated with CsA; and III (n=8), of animals equally injected with EB, but treated with CsA.

The 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 fronto-parietal suture. Injections were performed freehand using a Hamilton syringe, fitted with a 35º angled polished gauge needle into the cisterna pontis, an enlarged subarachnoid space below the ventral surface of the pons.

Ten microliters of 0.1% EB solution were injected into the cisterna pontis of rats from groups I and III and the same volume of 0.9% saline solution was injected in rats from group II.

Rats from groups II and III were daily treated with CsA by intraperitoneal route using 10 mg/kg/day in the first week and, thereafter, 3 times a week, with a minimal interval of 48 hours. A solution containing 10 mg/ml of CsA (Sandimmun®) was obtained by diluting the content for intravenous infusion (50 mg/ml) in sterile 0.9% saline solution.

The animals were anaesthetized and submitted to intracardiac perfusion with 4% glutaraldehyde in 0.1 M Sorensen phosphate buffer (pH 7.4) at 31 days. Thin slices of the brainstem (pons and mesencephalon) were collected and post-fixed in 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 acetate and lead citrate and examined using a Philips EM-201 transmission electron microscope.

Comparison between the final balance of myelin repair in CsA treated and non-treated rats was assessed using the semi-quantitative method developed by Blakemore and Crang[12] and Gilson and Blakemore[13]. Three semithin sections from each animal at 31 days after EB injection from groups I and III were examined for the presence of axons remyelinated by oligodendrocytes and Schwann cells, as well as for demyelinated axons. Remyelination by either Schwann cells or oligodendrocytes was identified using morphological criteria previously described[2-4,6,14,15]. The proportion of each was estimated in a scale ranging from 0 to 5. A lesion in which all axons were remyelinated by Schwann cells would have a Schwann cell (S) score of 5; an oligodendrocyte (O) score of 0 and a demyelination (D) score of 0. If 40% of the demyelinated axons were remyelinated by oligodendrocytes and 40% by Schwann cells with the remaining

axons being demyelinated, then the lesion was assigned a score of O-2, S-2, D-1. To compare remyelination scores from CsA-treated and non-treated rats 31 days after EB injection, a lesion repair profile of O versus S remyelination was made, providing an adequate graphical representation of each group. The mean O and S scores ±2SEM enclose a domain which represent an average of repair. Non-overlapping domains in these graphic representations indicate significantly distinct results.

RESULTS

Observations of semithin sections from groups I, II and III

The examination of semithin sections from rats of group I (EB injection) at 31 days revealed the appearance of lesions of variable extent (from the mesencephalon into the pons), but affecting mostly the ventral surface of the pons, and allowed the establishment of 2 areas with very distinct morphological characteristics. The center of the lesion presented an extended extracellular space, with many foamy phagocytic cells, lymphocytes, demyelinated axons and some myelin debris. At the periphery, macrophages were less conspicuous and thinly remyelinated axons could be seen, some clearly associated with Schwann cells (Fig 1A), others related to oligodendrocytes (Fig 1B).

Semithin sections from rats belonging to group III (EB injection and CsA treatment) at the same period revealed lesions similar to those seen in group I and also presenting macrophages, lymphocytes, few naked axons and some myelin derived membranes in the extracellular space of the central area. As previously noted in a former study from 2008 using the immunosuppressive agent CsA[1] the most prominent finding at the peripheral site was the high density of round cells identified as oligodendroglial cells (Fig 2A), as well as many thinly remyelinated axons (Fig 2B). Schwann cell remyelinated axons were also observed, mainly at perivascular and subpial sites. No mortality was recorded in this group due to CsA treatment using the drug administration scheme previously mentioned.

Examination of ultrathin sections from EB injected groups (I and III) confirmed the ultrastructural descriptions outlined by Bondan et al.[1].

No rat from group II (saline solution injection and CsA treatment) presented any sign of lesion or tissue disorganization in the brainstem at 31 days post-injection.

Score comparison between groups I and III at 31 days

Comparison between group I (rats non-treated with CsA) and III (rats treated with CsA) was made by observing semithin sections at 31 days after EB injection,

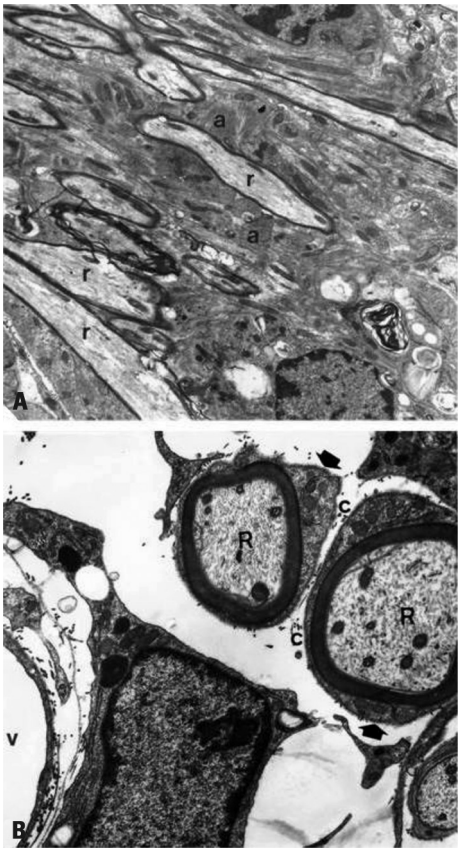

Fig 1. Group I - Electronmicrographs [A] Oligodendrocyte remyelinated axons (r) next to astrocyte processes (a). 6.064×. [B] Schwann cell remyelinated axons (R) near a blood vessel (v). Note the presence of Schwann cell cytoplasm (arrow) surrounding the axon, as well as the presence of collagen fibers (c). 12.672×.

with documentation of the remyelination balance and construction of the corresponding diagrams, according to the semi-quantitative method developed by Blakemore and Crang[12] and Gilson and Blakemore[13]. Scores are represented in Table and the respective diagrams in Fig 3.

DISCUSSION

In the present study the observations obtained from semithin and ultrathin sections in groups I and III confimed those described in a previous study using CsA following EB injection in the rat brainstem[1]. Again it was clearly noted that rats injected with EB and treated with this immunosuppressive drug had a higher density of oligodendrocytes on the edges of the lesions, in relation to the immunocompetent animals from group I and those treated with cyclophosphamide[4] or dexamethasone[5] from previous investigations.

With the semi-quantitative analysis (by comparing the domains of the mean scores for groups I and III and their limits – ±2SEM – at 31 days), it was evident that CsA treatment in the present study caused an increased oligodendroglial remyelination[14,15].

It is known that CsA permeates into target cells and binds to cyclophilins, a family of peptidyl-prolyl isomerases. Its immunossupressive effect is due to the binding to cyclophilin A and the drug-receptor complex then inhibits the dephosphorylase activity of calcineurin, preventing nuclear factor of activated T cell (NFAT) dephosphorylation and thereby inactivating the transcription factor NFAT[16]. As NFAT dephosphorylation is necessary for the transcription of a number of cytokine genes, including IL-2, IL-4, IFN-δ, TNF-α, activation of various T cells, macrophages and B cells is inhibited[17,18]. Notably the suppression of IL-2 expression by CsA is thought to play an important role in T-cell-mediated immunological processes[17].

IL-2 may be present in the CNS in pathological states following disruption of the blood-brain barrier (BBB) and infiltration of activated T cells in this site, more precisely of those producing the referred cytokine such as Th1 cells and T cytotoxic cells[10,19].

In the CNS it is recognized that IL-2 may modulate the function and genic expression of oligodendrocytes and their progenitors[10,11,19]. Human recombinant IL-2 appears to influence proliferation and differentiation of rat oligodendrocytes *in vitro*, increasing as far as in 3 times their numbers in cultures containing IL-2 and stimulating their maturation, as shown by the augmented expression of myelin basic protein (MBP) and of MBP mRNA[10,18]. On the other hand, IL-2 seems to inhibit the proliferation of oligodendrocyte progenitor cells (OPCs)[10,11,19], suggesting that IL-2 has variable biological effects on oligodendrocytes depending on their stage of differentiation.

Lymphocytes were found in the demyelinating lesions from both EB-injected groups, sometimes contacting myelin debris in the extracellular space as well as activated macrophages containing phagocytosed myelin, in a relationship suggestive of antigenic recognition[1].

A possible explanation for the high density of oligodendrocytes in rats submitted to CsA treatment could be the inhibition of IL-2 secretion induced by the drug. This requires that lymphocytes in the EB-induced lesions had the capacity of secreting the cytokine (such as the Th1 subpopulation) and were activated by antigenic stimulation. Since the referred cytokine inhibits *in vitro* proliferation of rat OPCs[10,11,19], it is possible that depletion of IL-2 in the lesion site facilitates division and migration of such cells. Paradoxically, mature oligodendrocytes are capable of accelerating their capacity of myelination or remyelination in the presence of IL-2[10,19]. This would be beneficial in demyelinating diseases, although it is not observed in immune-mediated conditions, such as MS and EAE, characterized by abundant lymphocytic infiltrates, specially of Th1 cells[19]. It is important to notice

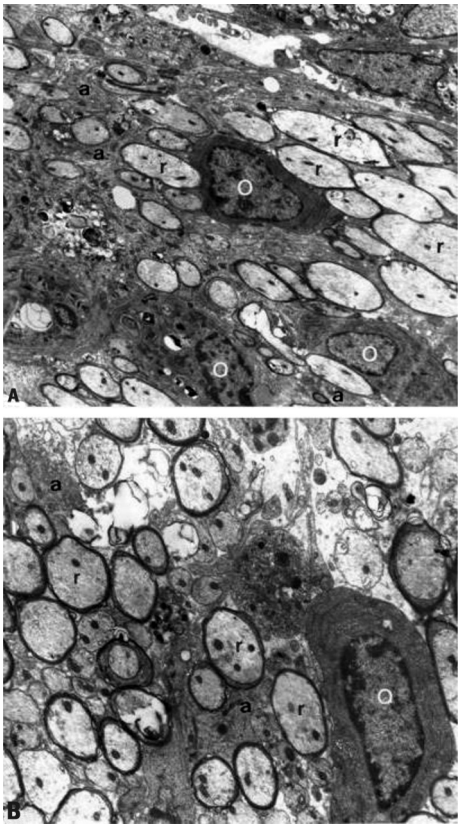

Fig 2. Group III - Electronmicrographs [A] Oligodendrocyte (O) presenting extensive rough endoplasmic reticulum next to remyelinating axons (r) and astrocyte processes (a). 4.836×. [B] Amplification of an oligodendroglial remyelinated area. (a) hypertrophic processes; (O) oligodendrocytes; (r) remyelinated axons. 10.460×.

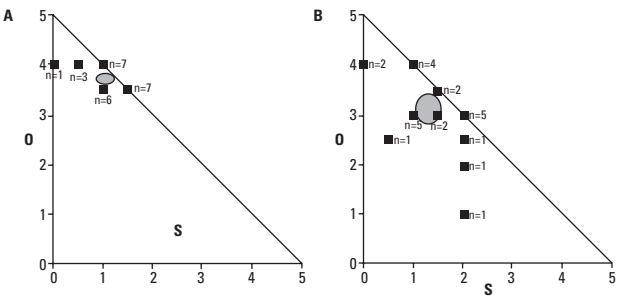

Fig 3. Diagrams of oligodendrocyte (O) versus Schwann cell (S) remyelination scores in CsA-treated [A] and non-treated [B] rats 31 days post-lesioning. Non-overlapping repair domains indicate significantly different results. The majority of axons are remyelinated by oligodendrocytes. n: number of score observations.

that those conclusions connecting IL-2 and oligodendroglial cells were obtained from *in vitro* studies, where a simpler scenery is found. The *in vivo* environment is a much more complex and unpredictable milieu due to the intricate interactions between the different cell types involved and their secreted factors.

Table. Remyelination scores by 31 days after EB injection in groups I (rats non-treated with CsA) and III (rats treated with CsA), according to the semi-quantitative method developed by Blakemore and Crang[12] and by Gilson and Blakemore[13] for semithin sections.

	Group I (non-treated with CsA)			Group III (treated with CsA)		
Animal	O	S	D	O	S	D
1	4.0	1.0	0.0	4.0	0.5	0.5
1	3.5	1.5	0.0	3.5	1.0	0.5
1	3.0	2.0	0.0	3.5	1.5	0.0
2	3.0	1.0	1.0	3.5	1.5	0.0
2	3.0	1.0	1.0	4.0	1.0	0.0
2	3.0	2.0	0.0	4.0	1.0	0.0
3	2.0	2.0	1.0	3.5	1.0	0.5
3	4.0	1.0	0.0	4.0	1.0	0.0
3	3.0	2.0	0.0	4.0	0.5	0.5
4	3.0	1.0	1.0	4.0	0.0	1.0
4	1.0	2.0	2.0	4.0	1.0	0.0
4	4.0	0.0	1.0	3.5	1.5	0.0
5	3.0	1.0	1.0	4.0	0.5	0.5
5	3.0	1.5	0.5	4.0	1.0	0.0
5	3.5	1.5	0.0	3.5	1.0	0.5
6	3.0	2.0	0.0	3.5	1.0	0.5
6	3.0	1.0	1.0	3.5	1.5	0.0
6	2.5	2.0	0.5	4.0	1.0	0.0
7	4.0	1.0	0.0	3.5	1.0	0.5
7	4.0	0.0	1.0	3.5	1.5	0.0
7	3.0	1.5	0.5	3.5	1.5	0.0
8	3.0	2.0	0.0	3.5	1.0	0.5
8	4.0	1.0	0.0	3.5	1.5	0.0
8	2.5	0.5	1.0	4.0	1.0	0.0
Mean	**3.13**	**1.31**	**0.52**	**3.72**	**1.04**	**0.23**
SD	**0.71**	**0.62**	**0.56**	**0.25**	**0.39**	**0.29**
SEM	**0.14**	**0.12**	**0.11**	**0.05**	**0.08**	**0.06**

O: axons remyelinated by oligodendrocytes; S: axons remyelinated by Schwann cells; D: demyelinated axons; SD: standard-deviation; SEM: mean standard error.

Astrocyte disappearance and direct mechanical damage due to intracisternal injection of the gliotoxin are identified as factors capable of disturbing the blood-brain barrier (BBB) and thus allowing lymphocyte infiltration. Blood vessels devoided of nearby astrocytic prolongations were still seen at 31 days after EB injection, suggesting the lack of a completely developed BBB, as it is known that astrocyte presence appears to be essential to the induction of BBB tight junctions[20].

Even during inflammation, the total number of lymphocytes in the CNS is comparatively small in relation to other body sites. So the amount of cells capable of reacting to a given antigen is proportionally reduced and lymphocytic response is restricted to a relatively small number of clones (oligoclonal response)[21].

In the present investigation, there was no evidence that CsA treatment was detrimental to macrophagic activity, because, on the contrary to that observed with the use of cyclophosphamide[4] or dexamethasone[5], there was no significant difference in the amounts of myelin-derived membranes between groups I and III (treated or not with CsA).

Nests of oligodendrocytes similar to those observed in our study were found in attempts of remyelination in MS[7] and EAE[8]. Such distribution is not a normal feature of the nervous tissue and may reflect the existence of OPCs responding to trophic factors on them[8].

In the EB model, it was described a cell population with membrane immunoreactivity to ganglioside D3 (GD3) from 6 to 12 days after the gliotoxic injection,

being considered as probable OPCs that came out in response to the demyelinating process[22].

Oligodendrocytes originate from migratory and mitotic precursors, then progenitors, and mature progressively into postmitotic myelin-producing cells[23,24]. These OPCs derive from neuroepithelial cells of the ventricular zones, at very early stages during embryonic life, and migrate long distances away from these zones, populating the developing neuropil to form white matter throughout the brain[23,24].

The subventricular zone (SVZ) is a germinal matrix of the forebrain that first appears during the later third of murine embryonic development. It enlarges during the peak of gliogenesis, between P5 and P20, and then shrinks but persists into adulthood[23,24]. Although most cells give rise to homogeneous progeny, some SVZ cells originate both oligodendrocytes and astrocytes, and a rare cell will develop into both neurons and glia[25].

During remyelination local adult OPCs must switch from an essentially quiescent state to a regenerative phenotype and this transition seems to be triggered by factors derived from activated microglial cells and astrocytes, leading to OPC proliferation and recruitment to demyelinated areas where differentiation of OPCs to remyelinating oligodendrocytes takes place[24]. Adult OPCs have received numerous names such as synantocytes and polydendrocytes, showing proliferative activity *in vivo* and remarkable plasticity *in vitro*[26].

When submitted to CsA *in vitro*, neural stem cells, collectively referred as neural precursors cells (NPCs) and isolated from the forebrain subependyma of adult male CDI mice, showed increased numbers and larger colonies, without any alteration in the differentiation profile of these colonies, indicating that CsA did not promote selective survival of a particular neural lineage. Consistent with *in vitro* observations, *in vivo* administration of CsA to adult animals increased the numbers of NPCs within neurogenic niches lining the lateral ventricles[27].

On the other hand hipoccampal-derived precursors have shown *in vitro* decreased proliferation, diminished numbers of neurons and increased numbers of astrocytes within CsA-treated colonies[28]. These different observations probably reflect the use of temporally (embryonic vs adult) and regionally distinct (hippocampal vs subependymal) precursors pools and emphasize the differences between starting populations of cells on those studies.

Our study clearly demonstrates that *in vivo* administration of CsA after EB-demyelinating lesions stimulated oligodendrocyte remyelination (mean remyelination scores of 3.72±0.25 for oligodendrocytes and 1.04±0.39 for Schwann cells) compared to non-treated animals (3.13±0.71 and 1.31±0.62, respectively), although the mechanisms by which this positive CsA effect occurs are unclear.

As already stated[1], CsA could produce a direct or indirect effect on persisting niches of OPCs in the mature CNS environment by affecting the final balance between proliferative/antiproliferative factors related to oligodendroglial populations in the lesion site, maybe facilitating the first ones and by doing this resulting in greater numbers of oligodendrocytes. Although CsA in this study changed the dynamics of the nervous tissue repair after EB injection, it is not possible to assume that these alterations were directly due to the suppression of lymphocyte activity.

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