Relationship between fluid-attenuated inversion-recovery (FLAIR) signal intensity and inflammatory mediator’s levels in the hippocampus of patients with temporal lobe epilepsy and mesial temporal sclerosis

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ABSTRACT

We investigated a relationship between the FLAIR signal found in mesial temporal sclerosis (MTS) and inflammation. Twenty nine patients were selected through clinical and MRI analysis and submitted to cortico-amygdalo-hippocampectomy to seizure control. Glutamate, TNFα, IL1, nitric oxide (NO) levels and immunostaining against IL1β and CD45 was performed. Control tissues (n=10) were obtained after autopsy of patients without neurological disorders. The glutamate was decreased in the temporal lobe epilepsy (TLE) -MTS group (p<0.001), suggesting increased release of this neurotransmitter. The IL1β and TNFα were increased in the hippocampus (p<0.05) demonstrating an active inflammatory process. A positive linear correlation between FLAIR signal and NO and IL1β levels and a negative linear correlation between FLAIR signal and glutamate concentration was found. Lymphocytes infiltrates were present in hippocampi of TLE patients. These data showed an association between hippocampal signal alteration and increased inflammatory markers in TLE-MTS.

Key words: FLAIR hippocampal signal, cytokines, NO, glutamate and hippocampal sclerosis.

Correlação da intensidade do sinal em FLAIR e os níveis de mediadores inflamatórios no hipocampo de pacientes com epilepsia do lobo temporal e esclerose mesial temporal

RESUMO

Este estudo foi delineado para investigar a presença de relação entre a intensidade de sinal em FLAIR e níveis de citocinas, óxido nítrico (NO) e glutamato no hipocampo de pacientes com epilepsia do lobo temporal refratária, associada com esclerose mesial (TLE-MTS). Vinte e nove pacientes foram selecionados através de análise clínica e de ressonância magnética (RM) que foram submetidos a cortico-amigdalo-hippocampectomia para o controle das crises. Os níveis de glutamato foram avaliados por HPLC, as citocinas TNFα e IL1β por...
ELISA and the levels of NO via NO system. We also assessed the expression of IL1β and CD45 in tissues from patients with epilepsy. Tissue control was obtained from individuals who died without inflammatory and neurological causes (n=10). A decrease in glutamate concentration was observed in patients with TLE-MS (p<0.001) suggesting an increase in the release of neurotransmitter. TNFα and IL1β also exhibited increased levels in the hippocampus of the patients (p<0.05), indicating a process of inflammatory origin. There was a positive linear correlation between the intensity of the signal in FLAIR and the levels of NO and IL1β. In contrast, a negative linear correlation was found between the signal intensity in FLAIR and glutamate levels in the hippocampus, exhibiting a process of inflammatory origin.

Immune system and associated inflammatory processes play an important function in the protection and repair of several tissues, thus constituting an adaptive, beneficial endogenous response. In contrast, immune response activation can also be direct or indirect cause of neuronal dysfunctions. During inflammatory process the communication between cells of the immune system occurs either via direct cell-to-cell contact or via soluble factors called cytokines such as IL1β, IL6 and TNFα. The effects of cytokines on neuronal survival are likely to be mediated by their ability to activate extracellular increase of glutamate by acting on the mechanisms of its release and reuptake, potentiating the function of ionotropic glutamate receptors, enhancing the production of mediators of oxidative stress.

In this study, we investigated the possible involvement of both hemispheres in TLE associated with MTS.
alyzing the intensity of the ipsilateral signal (hippocampal sclerosis) versus the contralateral signal, from each patient. The relationship between the levels of IL1β, TNFα, glutamate and NO with the type of sclerosis using Blumcke criteria was also investigated. Furthermore, immuno-histochemistry against IL1β, TNFα and CD45 in autopsy/control and sclerotic tissues was also performed to localize and identify cellular and humoral components of immune system.

**METHOD**

All experiments were performed under approval from the Institutional Ethics Committee of the Universidade Federal de São Paulo (UNIFESP). Surgical specimens from patients with intractable epilepsy were submitted to standard cortico-amygdalo-hippocampectomy at the Hospital São Paulo (UNIPETE/SPDM-UNIFESP-EPM, Brazil). All cases showing neoplasms, vascular malformations, post-traumatic and ischemic lesions on preoperative MRI were excluded. Selected patients (n=29) had detailed anamnesis, video-EEG recordings and MRI studies and after these procedures the epileptogenic zone was delineated. The age of the patients with TLE and HS varied from 19 to 56 years (36.9±9 years) and this group was composed by 72.4% of women and 27.6% of men.

The drugs used by these patients for seizure control include: carbamazepine, phenobarbital, diphenylhydanto- in, clobazam, valproate and topiramate. Control hippocampi were obtained from brains showing no evidence of pathology on the basis of gross and routine histological examination removed from autopsies (less than 6h of postmortem period). The age of the subjects at death varied from 38 to 84 years (67±9 years) and this group was composed by 72.4% of women and 27.6% of men (autopsy/control).

Thus, the levels of IL1β, TNFα, glutamate and NO were measured in the head of hippocampi and correlated with the FLAIR MRI signal, obtained in the head, body and tail of this structure. We also compared IL1β, TNFα, glutamate concentration in the hippocampi of patients with HS with those found in control tissues, in similar hippocampal areas, removed during the autopsy of patients, without neurological disease (autopsy/control). We did not compare NO levels from autopsy/control tissue and those obtained from hippocampi of our patients since autopsy tissue is not an appropriate control. The postmortem levels of NO increases after death due to polyamines production and anoxia and several works have reported arterial synthesis of NO after death.

The autopsies were made by a pathologist from Pathological Anatomic Department Incor, FMUSP, especially trained for this purpose. Thus, the pathologist was able to remove the same structures as performed by the neurosurgeon. Using this procedure, similar hippocampal areas from epileptic patients and autopsied subjects could be compared. All patients and families had signed the consent term authorizing the tissues utilization.

**MRI acquisition**

Patients with clinical diagnostic of TLE and control subjects were underwent imaging with a 1.5-T Gyroscan (Philips Medical System, Eindhoven, The Netherlands) using a standard head coil. The protocol included the following: sagittal T1-weighted (repetition time [TR]=433 milliseconds, echo time [TE]=13 milliseconds, field of view [FOV]=25 cm, 6 mm slice thickness, matrix size=256×512); axial turbo-spin echo FLAIR-weighted (TR=4535 milliseconds, TE=100 milliseconds, FOV=23 cm, 6 mm slice thickness, matrix size=256×512); axial gradient-echo FLAIR-weighted (TR=707 milliseconds, TE=23 milliseconds, flip angle of 15°, FOV=23 cm, 6 mm slice thickness, matrix size=205×256); inversion recovery (IR) T1-weighted (TR=5620 milliseconds, TE=17 milliseconds, inversion time TI=400 milliseconds) and fluid attenuated inversion recovery (FLAIR) (TR=8000 milliseconds, TE=150 milliseconds, TI=2350 milliseconds) with the same section thickness, FOV and matrix (3 mm, 23 cm, 256×512) in the coronal planes perpendicular to the long axis of hippocampus, including the totality of the temporal lobe; fast field echo (FFE) T1-weighted (TR=30 milliseconds), TE=4.6 milliseconds, one acquisition average pulse sequence, flip angle=45°, FOV=23 cm, 1.5 mm slice thickness with no gaps, matrix size=228×512, in the coronal plane. The control group underwent only the coronal FLAIR and FFE T1-weighted sequences.

**MRI quantitative analysis** – Three FLAIR (20% gap, 3 mm thickness) slices including hippocampal head, body and tail on the coronal plane (perpendicular to the hippocampal axis) were taken from both groups. Hippocampal signal was accessed using Leonardo, Syngo MR 2004A (Siemens Medical Solutions, Erlangen, Germany). The signal was also measured using the same procedure in the pons of each patient, from both groups. This procedure was done to “normalize” variations between signals in different MRI as internal standard. Thus, final signal from hippocampal head, body and tail was ever considered as the ratio between its proper signals and those obtained from the pons of the same subject. All hippocampal and pons delimitations were made by the same neuroradiologist.

**Glutamate quantification**

The hippocampi were immediately removed and kept in a cryogenic vial, frozen in liquid hydrogen and stored at −80°C, and then weighted. The high performance liquid chromatography (HPLC) was performed using the
A method described by Cavalheiro \textsuperscript{18} with few modifications as follow: tissue was ultrasonically homogenized in a perchloric acid 0.1 M, 0.02% sodium disulfide and homoserine 10 mg/ml solution (as internal standard). The homogenate was done in a proportion of 15 μL solution for each milligram of wet tissue. The samples were kept at 0°C overnight and then centrifuged at 11,000 g at 4°C for 50 min. The supernatant was filtered and submitted to a reaction with O-phthaldialdehyde (OPA) and then injected into HPLC systems. Amino acid reaction was done dissolving 27 mg of OPA in 1mL of methanol, adding 5 μL of 2-mercaptoethanol and 9 mL of sodium tetraborate 0.1 M (pH 9.3) solution.

Before sample analysis a solution was prepared with 1 mL of stock solution with 2 mL of sodium tetraborate 0.1 M. The pre column derivatization was completed by reacting 100 μL of this solution with 50 μL of amino acid standard solution for exactly 2 minutes before the injection on analytic column.

An isocratic HPLC system coupled to a fluorescence detector, a sample injector of 20 μl for HPLC and a column RP-18 50×4.6 mm were utilized (Chromolith Speed-Rod, Merck, Darmstadt). The mobile phase consisted of sodium phosphate buffer 0.05 M (pH 5.95) with methanol 11.5%. The flow rate was 3.5 ml/minute, detection with excitation of 348 nm and emission of 460 nm in the fluorescence detector were employed. For standard chromatogram amino acid of known concentration were assayed and the retention time of each amino acid was verified for no overlapping after sample delivery.

**Nissl staining and immuno-histochemistry**

Brain tissues removed during surgery or autopsy were rapidly immersed in buffered (pH=7.4) 1% paraformaldehyde, for 24h at 4°C. Collected hippocampi were sliced in 0.5 cm sections through longitudinal axis. All tissues were then dehydrated by an ethanol series, followed by xylol (100%) before inclusion in paraffin. The paraffin included tissue was sliced (3 μm) in microtome (Leica) and kept in silane-covered slides. After paraffin removal (n=3), sections from the hippocampus were submitted to the classical Nissl staining (NS). Cresyl-violet staining was done to assess the specimen orientation and to check the localization and the extent of the lesion, being possible the classification of the sclerosis types as described by Blumcke \textsuperscript{17}.

In summary, five distinct pathological patterns were recognized after histological analysis: no MTS="no hippocampal cell loss , MTS type 1a="classic hippocampal sclerosis , MTS type 1b="severe hippocampal sclerosis , MTS type 2="CA1 sclerosis, and MTS type 3="end folium sclerosis. Adjacent sections were selected for immuno-histochemistry (n=3).

Paraffin was removed from slices using 100% xylol and endogenous peroxidases were blocked with 3% H<sub>2</sub>O<sub>2</sub> for 15 min. After this procedure, the slices were submitted to 10 min of heating in microwave oven. Tissues were blocked using 5% bovine albumin during 90 min and incubated in humid chamber with primary monoclonal anti-human IL1β, TNFα (1:50) (BD Bioscience, Minneapolis, USA) and CD45 (1:500) (DAKO clone PD7/26) antibodies for 48 hours in the presence of 1% milk. Following three washes in PBS, the sections were incubated with the secondary antibody (anti-mouse (1:500) from Calbiochem, Merck KGaA, Darmstadt, Germany). Immunodetection was performed using the Vectastain ABC Elite Kit (Vector Burlingame, CA, USA) and the complex antigen-antibody was visualized using diaminobenzidine in PBS and H<sub>2</sub>O<sub>2</sub> (1 μL/mL). Tissue sections were mounted on glass slides and the material was examined with a microscope using bright-field illumination.

**Quantification of IL1β and TNFα**

Tissues were homogenized in 0.01M tris-HCl pH 7.6, 0.001M EDTA, nonidet P-40) 10% glycerol, 0.1M NaCl buffer, containing a cocktail of protease inhibitors (Sigma Aldrich Corporation, St Louis, USA). The tissues were homogenized by ultrasound (Virstonic 60, Virts) and stored at –80°C until assay. Total protein was quantified using method described by Lowry \textsuperscript{19}. A standard curve of both cytokines was done to find the liner range of Elisa (BD Bioscience OptEIA\textsuperscript{TM}) method. After this procedure, different amount of cerebral tissues were assayed and 80 pg/ml of protein from all sample homogenates were used due to its inclusion in liner range of the method. All assays were done in duplicate. The assays were done according to method described by manufacturer Kits (Human IL – 1 ELISA Kit BD Opteia\textsuperscript{TM} e Human TNF ELISA Kit BD Opteia\textsuperscript{TM}). The optical density was measure at 450 nm in an Elisa reader equipment μQuant Biotek (USA) and after using the software GEN 5 the concentration of IL1β and TNFα in each sample was determined. The concentrations were expressed in pg/mg protein.

**Nitric oxide quantification**

After tissue removal all hippocampi (control and epileptic) were separated and frozen in liquid nitrogen in less of 20 seconds. The samples were homogenized in Tris-HCl buffer containing 0.2M of KCl being submitted to a centrifugation at 10 000g during 10 min at 4°C. To 1 mL of supernatant were added 2 mL of NaOH 0.5M and 2 mL of ZnSO<sub>4</sub> 10%. This mixture was homogenized during 30s and stayed at room temperature during 15 min. A new centrifugation was done at 10 000g during 10 min and the NO concentration in each sample was quantified using Nitric Oxide Analyzer (NOA\textsuperscript{TM} 280, Sievers Instruments, Inc, Boulder, CO, USA) by chemiluminescence as
This method is highly sensitive and 1 pMol can be detected. The concentrations in all samples were expressed as µMol/mg wet tissue.

**Statistical analysis**

To compare IL1β, TNFα and glutamate concentration between epileptic and control groups Student t test was employed. To analyze the existence of correlation between two different variables the linear correlation of Pearson was employed. For this purpose the software Statistical Package for Social Sciences (SPSS) version 11.0 for Windows was used. A p<0.05 value was accepted as significant.

**RESULTS**

The Table summarizes all clinical data concerning to patients submitted to cortico-amygdalo-hippocampectomy at the Hospital São Paulo (UNIPETE/SPDM) including sex, age at surgery, presence of initial event, duration of epilepsy, brain side involved, presence of hyperintense signal in temporal pole structures, Engel’s scale after surgery and type of sclerosis, according to Blumcke classification17.

In this context, the Engel’s distribution between patients was 55.2% as IA, 10.3% as IB, 24.1% as IIIA, 3.4% as IVA, 3.6% as IIIB and 3.4 as IVC showing that a great majority of patients presented improvement in their quality of life after surgery, due to minor seizure episodes.

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M: male; F: female; Age: at surgery intervention; Initial event: presence of febrile seizures or status epilepticus; Duration: duration of epilepsy in years; Side: side with major involvement; Engel scale: Engel classification after surgery and sclerosis types: according to (8) classification; IA: type IA; IB: type IB; AT: atypical hippocampal sclerosis.
After Nissl staining, the types of hippocampal sclerosis were identified as shown in Fig 1, panel A, B, C and D. The Fig 1A and B shows the stained tissue from patients presenting HS type 1B. Fig 1 C shows an atypical HS (AT) and in the Fig 1D we can visualize an unclassified HS due to damage of the tissue during surgery.

The quantification of IL1β by ELISA method in sclerotic and autopsy/control hippocampi showed values of 117.6±15.4 and 92.3±28.8 pg/mg protein (p=0.023) respectively. The levels of TNFα obtained in both groups were 28.9±14.5 (sclerotic hippocampi) and 17.8±16 pg/mg protein (autopsy/control tissue) (p=0.049), showing increased concentration of both cytokines in the hippocampi of patients with TLE. These findings could be visualized and confirmed, in part, through the immunohistochemistry analysis of IL1β. The immunohistochemistry done against IL1β in autopsy/control and sclerotic hippocampus is shown in Fig 2. The panels A and B show

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**Fig 1.** Hippocampal sclerosis. Nissl staining of hippocampus of patients with TLE and HS

[A] Patient number 5 with HS Type 1B (scale bar 1000 μm). [B] Patient number 13, HS Type 1B (scale bar 1000 μm; Cresil). [C] Patient number 12, atypical HS; [D] HS without classification.

**Fig 2.** Immunohistochemistry (IL1β). IL1β immunohistochemistry of patients 5 and 9 and autopsy/control tissue. [A] Patient number 9, CA2-CA3 regions (scale bar 100 μm). [B] Patient number 5, CA2-CA3 regions (scale bar 100 μm; [C] Autopsy/control tissue CA2-CA3 regions (scale bar 100 μm; IL1-β).
CA2 and CA3 regions from the hippocampus of two different patients. Panel C shows an autopsy/control tissue submitted to the same staining procedure. As can be visualized, there is an increased expression of IL1β in HS, when compared with the same regions of autopsy/control tissue. Immuno-histochemistry done with TNFα did not show difference between autopsy/control and sclerotic hippocampus (data not shown).

The amino acids levels in sclerotic and autopsy/control tissues demonstrated decreased concentration of glutamate in patients (4.76±1.17 ng/mg wet tissue), when compared with autopsy/control tissues (6.31±0.83 ng/mg wet tissue, p=0.00051).

A presence of perivascular lymphocytes infiltrate could also be identified in Fig 3, when the tissues were submitted to immunostaining against CD45 antibody. This infiltrate could not be visualized in autopsy/control tissues.

A positive correlation was found between the level of IL1β and the signal presented in the tail of hippocampus as shown in Fig 4 (top panel) (p=0.033, Pearson coefficient=0.436). In contrast, a negative correlation was found between glutamate levels and FLAIR MRI signal intensity in hippocampal tail (p=0.025, Pearson coefficient=0.457) as shown in Fig 4 (lower panel).

No relationship between the levels of TNFα and FLAIR MRI signal intensity in head, body and tail of the hippocampus of patients with TLE was found, showing no association between these variables (data not shown). In addition, a positive correlation was found analyzing NO concentration and the FLAIR MRI signal intensity in the head (Pearson coefficient=0.498, p=0.013); body (Pearson coefficient=0.505, p=0.012) and tail of the hippocampus of patients with epilepsy (Pearson coefficient=0.532, p=0.007) (Fig 5).

**DISCUSSION**

The present work showed changes in the concentration of different inflammatory mediators such as IL1β and TNFα in the hippocampus of patients with MTS. In addition, a decreased glutamate concentration was also found, when compared with autopsy/control tissues. The mea-
Measurement of cytokines NO and glutamate was done in the head of the hippocampus since Bernasconi et al. described this region as that responsible for the major signal alteration on MRI. We also found a linear relationship between cytokines and NO levels with the signal on FLAIR MRI in head, body and tail of the hippocampus.

These data suggest the presence of an inflammatory process in the hippocampus, associated with increased release of glutamate, which is indicative of a persistent excitotoxicity. Our data are supported by previous report, which demonstrated increased extracellular glutamate levels (increased release), using microdialysis probes in humans with TLE. A deficiency in glutamine synthesis in astrocytes, found by several authors, can also explain the high levels of glutamate in extracellular space, suggesting increased release of this amino acid. Working with synaptosomes from hippocampus of patients with TLE and MTS, was found increased basal release of glutamate and GABA, data that could support our findings. Thus, the decreased concentration found in sclerotic hippocampus could be associated to increased release of this neurotransmitter, as already reported by several authors.

Cytokines, a diverse group of polypeptides that are generally associated with inflammation, immune activation, cell differentiation or death include interleukins, interferons, tumor necrosis factors, chemokines and growth factors. Cytokines can exert direct action on neurons, indirect action via glial cell, direct action on brain vasculature and on physiological parameters such as regional blood flow and temperature. IL1β and TNFα act on distinct cell-surface receptors but share some common signaling mechanisms related to neurodegeneration. IL1β can induces cell death, as well as cyclooxygenase 2 (COX2) and oxide nitric synthase overexpression (iNOS). In addition, seizures stimulate increase in the levels of IL1α, IL1β, IL6 and TNFα in the brain. The mechanism by which IL1 affect seizure activity is not known, but Allan showed that the association between administration of AMPA receptor agonist and IL1 produce widespread cortical cell death. This fact is not observed when AMPA or IL1 are administered alone. This fact indeed support the hypothesis that suggest the participation of increased release of glutamate associated with the increased levels of cytokines, in the process of cell death, observed in the hippocampus of patients with TLE and MTS. Glutamate during excitotoxic phenomenon stimulates AMPA and NMDA receptors producing excessive depolarization of postsynaptic membrane, which results in an osmotic imbalance when countered by an influx of Na+, Cl− and water.

Glial cells can also secrete IL1β and TNFα, which in turn, can act on these cells in an autocrine manner. IL1β and TNFα induce the proliferation of astrocytes, a process known as astrogliosis, which is also found in hippocampal sclerosis.

The NO level from sclerotic hippocampus was correlated with FLAIR hyperintense signal in the head, body and tail of this structure, supporting the hypothesis of participation of NO production in the hippocampal sclerosis. Neuronal NOS is localized in the hypothalamic supraoptic and paraventricular nuclei, both of which are mainly involved in the neurosecretory activity of this
area. NO is able to interact with many intracellular targets to trigger an array of signal transduction pathways, resulting in stimulatory or inhibitory output signals. However, it becomes noxious if it is produced in excess recently. The term “nitrosative stress” has been used to indicate the cellular damage that is elicited by excess of NO and reactive nitrogen species. In addition, NO has been shown to stimulate the release of noradrenaline and glutamate in the hippocampus. This data can support our hypothesis concerning to increased release of this neurotransmitter since we found low levels of glutamate in sclerotic tissues, when compared with autopsy/control tissues. Taken together, these data support the idea that the hyperintense signal on FLAIR MRI could be the result of increased expression of cytokines and NO associated with increased excitotoxicity in TLE.

The correlation between ipsilateral versus contralateral signals showed that although the hyperintense signal was major in the sclerotic side the contralateral hippocampus is frequently involved since it also presented increased FLAIR signal.

As lymphocytes were also visualized in epileptic tissue we can suppose that both humoral and cellular immune systems are activated in TLE associated with HS. This data were previously reported by other authors, who demonstrate the presence of cytokines, complement activation as well and NO release in the blood after an ictal event.

Taken together, these data show the participation of markers of inflammatory process in the physiopathology and in FLAIR signal related to temporal lobe epilepsy, associated to hippocampal sclerosis.

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