Manganese-enhanced magnetic resonance imaging in the acute phase of the pilocarpine-induced model of epilepsy

Imagens contrastadas por manganês na fase aguda da epilepsia induzida por pilocarpina

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ABSTRACT

Magnetic resonance images are useful in the study of experimental models of temporal lobe epilepsy. The manganese-enhanced MRI (MEMRI) technique is of interest since it combines the effects caused by manganese on the increased contrast in activated cell populations, when competing with calcium in synaptic transmission. Thus, the purpose of this study was to investigate the temporal evolution of the contrast related to manganese in the acute phase of temporal lobe epilepsy induced by systemic pilocarpine and compare it to the expression of the c-Fos protein. During this phase, the intensity of the MEMRI signal was analyzed at three different time points (5, 15 or 30 minutes) after the onset of *status epilepticus* (SE). The group that was maintained in *status epilepticus* for 30 minutes showed a decrease in intensity of the signal in CA1 and the dentate gyrus (DG). There were no differences between the control group and the other groups treated with pilocarpine. The expression of the protein, c-Fos, in the same animals showed that even in the short-duration *status epilepticus* (5 minutes), there was already maximal cellular activation in subregions of the hippocampus (DG, CA1 and CA3). Under the experimental conditions tested, our data suggest that the MEMRI signal was not sensitive for the identification of detectable variations of cell activation in the acute phase of the pilocarpine model. Our findings are not consistent with the idea that manganese contrast reflects primarily alterations in cellular activity during SE when other signal-modifying elements can act.

Keywords: Epilepsy/radiography; Hippocampus; Magnetic resonance imaging/methods; Manganese/diagnostic use

RESUMO

As imagens de ressonância magnética são úteis no estudo de modelos experimentais de epilepsia do lobo temporal. A técnica *manganese-enhanced MRI* (MEMRI) é de interesse por combinar os efeitos provocados pelo manganês no aumento do contraste de populações celulares ativadas, ao competir com o cálcio na transmissão sináptica. Assim, o propósito deste estudo foi investigar a evolução temporal do contraste provocado pelo manganês na fase aguda da epilepsia do lobo temporal induzida por pilocarpina sistêmica e compará-las à expressão da proteína c-Fos. Nessa fase, a intensidade do sinal MEMRI foi analisada em três diferentes pontos temporais (5, 15 ou 30 minutos) após o início do *status epilepticus* (SE). O grupo que foi mantido em *status epilepticus* por 30 minutos mostrou diminuição na intensidade de sinal no CA1 e giro denteado (GD). Não houve diferenças entre o Grupo Controle e os outros grupos tratados com pilocarpina. A expressão da proteína c-Fos, nos mesmos animais, mostrou que, mesmo no *status epilepticus* de curta duração (5 minutos) já há ativação celular máxima nas sub-regiões do hipocampo (GD, CA1 e CA3). Nas condições experimentais testadas, nossos dados sugerem que o sinal MEMRI não foi sensível para identificar variações detectáveis da ativação celular na fase aguda do modelo de pilocarpina. Nossos achados não são consistentes com a ideia que o contraste por manganês reflete primariamente alterações na atividade celular durante o SE quando outros elementos modificadores do sinal podem atuar.

Descritores: Epilepsia/radiografia; Hipocampo; Imagem por ressonância magnética/métodos; Manganês/uso diagnóstico

INTRODUCTION

Animal models, such as with pilocarpine and kainic acid [1-3], are widely used for the study of temporal lobe epilepsy (TLE). Compared to histological techniques, magnetic resonance images (MRI) are a very useful form of longitudinal approach in animal models. Despite the

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growing number of scientific publications on technical applications of MRI, including on the use of manganese enhancement (MEMRI) in animal models of TLE[1,2], various anatomical aspects of epileptogenesis still await definition, such as, for example, the sequence of activation of brain areas caused by induced seizures. In addition to morphological and anatomical studies, the MRI also furnishes information according to the sequence used, for example, information on functional and metabolic alterations.

In rodents, the presence of $MnCl_2$ provides an excellent contrast for MRI cerebral anatomy, in which regions such as the hippocampus, pituitary gland, cerebellum, and olfactory bulb are naturally enhanced, despite any stimulus[4]. $MnCl_2$ acts as medium to increase the contrast among the various encephalic structures, by decreasing both the T_1 relaxation time and the T_2 time for adjacent water[4]. In the final phase of action potential, the Mn^{2+} ion competes with the Ca^{2+} ion in the depolarization of the synaptic buttons. In this way, when internalized by the high-activity neurons at the focus of the epileptic discharge, the Mn^{2+} ion serves as a tracer of activated neural routes.

Studies using MRI techniques in the active phase of the kainic acid model resulted in little or no definition of areas with greatest activity, possibly obscured by the cellular lesion that occurs during this phase after prolonged *status epilepticus* – SE (90 minutes)[2]. Similarly, as studies performed in the chronic phase also were not able to correlate the frequency of seizures and MEMRI hyperintensity MEMRI, the authors concluded that the cellular activity resulting from the seizure would not be capable of causing MEMRI hyperintensity[5]. On the other hand, MEMRI hyperintensity was demonstrated in various studies in specific active areas of the brain after several stimuli, such as with sound [6].

Based on these studies, it was concluded that the identification of the brain regions activated in the initial injury is still a significant obstacle to be overcome in the study of animal models of TLE[7] using MRI techniques. Considering that five minutes of continuous epileptic activity is considered sufficient to define SE[1,2], the proposal of the present study was to induce SE and allow it to have a short duration, in the attempt to obtain a better definition of hippocampal areas with greater activity, without interference caused by cell death, which is present in prolonged SE[8]. The expression of the c-Fos protein is an indicator of intense cellular activity, previously used in the identification of activated areas, both in recurring spontaneous epileptic seizures and in reinduction of SE[9]. Thus, the objective of the present study was to identify the existence of parallelism between the expression of this protein and the intensity of the MEMRI signal at different durations of the SE. The data obtained from the analysis of the intensity of MEMRI were compared to the analysis of the expression of the c-Fos protein in the same animals.

METHODS

The animals used in the present study came from the *Centro de Desenvolvimento de Modelos Experimentais* (CEDEME) of the *Universidade Federal de São Paulo* (UNIFESP), and the experimental procedures were approved by the Ethics Committee of UNIFESP (CEP No. 0750/07). Male adult Wistar rats (230 to 280g) were used, maintained in groups of five under a 12/12-hour light/dark cycle.

The dose of $MnCl_2$ used should be sufficient to afford the contrast desired on the MRI, but should be the smallest possible due to its toxicity, besides being calculated for the equipment in use. The dose used in the present study was based on preliminary tests carried out at our laboratory. Doses of 60 to 90mg/kg provide good contrast between structures. We chose the dose of 60mg/kg, since it did not alter the rate of mortality associated with the epileptic status, as opposed to the dose of 90mg/kg.

Initially, the animals received a solution of $MnCl_2.4H_2O$ (Sigma, 60mg/kg, volume of 0.3mL/100g of animal)[10], and 12 hours later, the seizures were induced by intraperitoneal (IP) injection of pilocarpine (Pilo, 300mg/kg, Merck, preceded 30 minutes before by the administration of methylscopolamine (1mg/kg, Sigma, IP) to reduce the peripheral effects of pilocarpine. The animals that displayed SE were divided into three groups, according to the duration of the SE: 5 minutes (Group SE-5; n=5), 15 minutes (Group SE-15; n=6), and 30 minutes (Group SE-30; n=8). The animals that did not present with SE within up to 2 hours after receiving the pilocarpine were excluded from the study. The Control Group (n=8) received 0.9% saline solution instead of other solutions.

The determination of the occurrence of SE observed behaviorally was based on the Racine scale[7]. The onset of SE was defined after 5 minutes of continuous seizure activity. In the different determined time periods previously described, all the experimental groups, including the controls, received a mixture of thionembuthal + diazepam (30 + 10mg/kg; IP). Prior encephalographic recordings showed that the use of this mixture ceases the SE in up to 10 minutes[11]. The same was observed by means of behavioral analysis in the present study.

To decrease the post-SE mortality rate, the animals were placed in a supine position on the surgical table, tracheostomized, intubated (tube with approximately 0.5mm diameter), and connected to a respirator for small animals (model 7025, Ugo Basile) and ventilated with ambient air (21% FiO_2), at a respiratory rate of 70 cycles/min and a volume of 3.5mL/cycle.

The MRIs were acquired using a horizontal superconductor magnet with a 2T field (Oxford Instruments) that operated together with a Bruker® spectrometer. The FLASH (Fast Low-Angle SHot) sequence was used in the acquisition of images weighted by T_1 (TR=200ms, TE=5.8ms, flip angle=90°, 4 means, 40 minutes/animal). The FOV utilized was 40x40x11.2mm³ with a matrix of 192x192x16 points, producing a spatial resolution of 208x208x700mm³.

After acquisition of images, the animals were perfused by transthoracic route with a buffered 0.9% saline solution and posteriorly, 4% paraformaldehyde at 4ºC. The brains were immediately removed and immersed in 30% sucrose solution for 24 hours, at 4ºC. With the help of a cryostat, the brains were cut into coronal sections with 30mm thickness.

The immunoreactivity for c-Fos was detected using a conventional immunohistochemical technique with avidin-biotin-immunoperoxidase[12].

The relative signals from the MRIs of the regions DG, CA1 and CA3 (Figure 1) were quantified in an image on the coronal plane, approximately at the level -3.6mm caudal to bregma, as per the anatomical atlas of rats[13]. The signals were calculated as the ratio between the intensity of the mean signal in the region

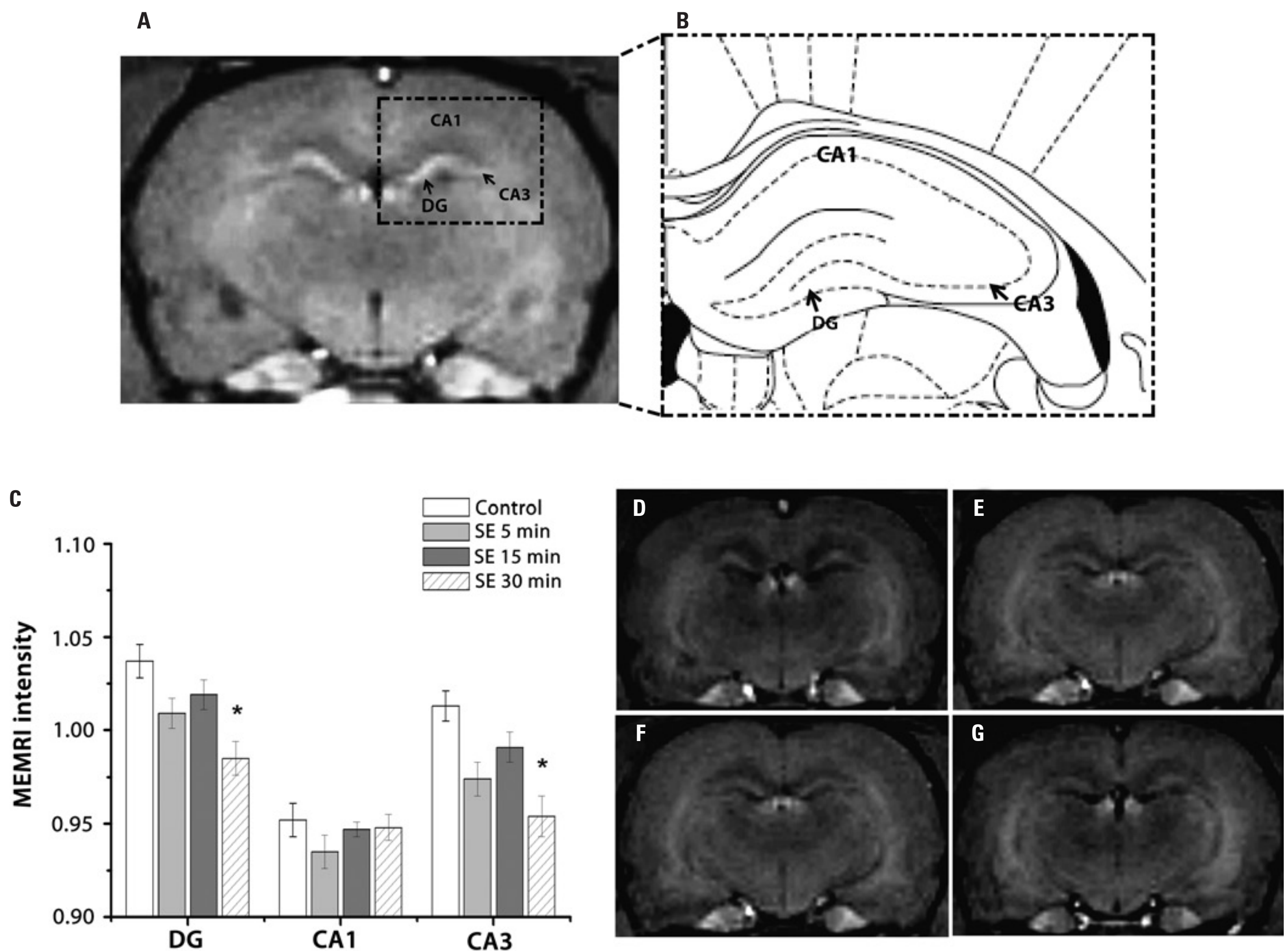

Figure 1. In A, the coronal slice of the magnetic resonance image of a control rat in which the hippocampal sub-regions DG, CA1 and CA3 are identified, and outlined in B[13]. In C, graphic representations of intensities of the MEMRI signal for the groups studied in the DG, in which a difference was detected between the groups Control *versus* SE-30 min (*$p<0.05$); in CA1, in which there was no difference between the groups; and in CA3, in which there was a difference between the groups Control *versus* SE-30 min (*$p<0.05$). The results presented include the analyses carried out in the right and left hippocampi, since no significant difference was detected between them. In D, E, F and G are shown images by T1-weighted magnetic resonance images using manganese chloride as contrast agent for control groups (D), SE 5 (E), SE 15 (F) and SE 30 (G)

of interest (ROI) by the intensity of the mean signal of the adjacent corpus callosum. The increased intensity of the relative signal, when compared to control animals, was determined as MEMRI hyperintensity[14]. A ROI utilized in this study, denominated DG, in reality includes the DG and the proximal portion of the CA3, because it was not possible to separate them for reanalysis.

The images of the histological slices were captured on a high-resolution digital camera (Nikon DXM1200), installed in a Nikon microscope (Eclipse E600FN). The images were acquired with a magnification of 10 X and analyzed using Image J software. The quantitative analysis of the optic densities in regions of the hippocampus was performed in values expressed as levels of grey, having as control the corpus callosum. For each animal, two measurements of each region were made (CA1, CA3 and DG) on both sides of the hippocampus (right and left). Three different levels were used along the rostrocaudal extension of the hippocampus, and illumination was kept stable during the acquisition of images.

The results obtained are expressed in values of mean ± standard error and were submitted to one-way variance analyses (ANOVA) followed by post-hoc Tukey/Kramer tests, assuming $p<0.05$ as level of significance.

RESULTS

The MEMRI signal in the DG of the animals of Groups SE-5 (1.009±0.008) and SE-15 (1.019±0.008) presented no MEMRI hyperintensity when compared to the control group (1.037±0.009). In Group SE-30, a reduction of intensity of the MEMRI signal (0.985±0.008; $p<0.05$) was noted relative to the control group (Figure 1C).

In CA1 of animals SE-5 (0.935±0.009), SE-15 (0.947±0.004) and SE-30 (0.948±0.007), there was no statistical difference in the MEMRI signal relative to the animals from the control group (0.952±0.009), (Figure 1C).

The CA3 of animals SE-5 (0.974±0.009) and SE-15 (0.991±0.008) showed no differences as to intensity of the MEMRI signal relative to the animals in the control group (1.013±0.008). In CA3 of the animals of Group SE-30 (0.954±0.011), a significant reduction was observed ($p<0.05$) of the MEMRI signal when compared to the control group (Figure 1C). Figure 1 (D-G) illustrates the MRI representative of the animals of the same groups.

Figure 2 demonstrates that in the DG region the expression of c-Fos was intense and differed statistically ($p<0.05$) between groups SE-5 (37.74±2.80), SE-15 (41.38±2.29) and SE-30 (40.57±2.04) when compared to the control (10.39±0.44). There were no significant differences between the groups treated with pilocarpine and maintained for increasing times of SE. The same result was observed for the CA1 region of groups SE-5 (26.95±1.93), SE-15 (30.95±1.35), SE-30 (26.62±1.59) and control (11.22±0.34); and also in the CA3 region for groups SE-5 (20.65±1.76), SE-15 (23.66±1.24), SE-30 (20.11±1.34) and control (9.04±0.30).

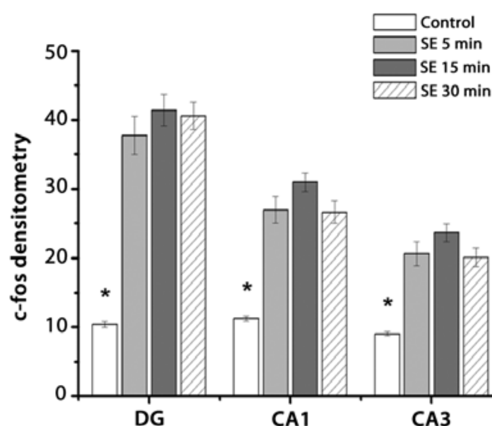

Figure 2. Graphic representations of the results obtained as to densitometry of the expression of c-Fos for the groups studied in the hippocampal sub-regions DG, CA1 and CA3 studied. Differences were detected between all the groups treated with pilocarpine and the control group (*$p<0.05$)

DISCUSSION

Using the model of pilocarpine in the acute phase, in the present study the animals were submitted to different durations of SE (5, 15 or 30 minutes). By means of the MEMRI technique, the objective was to analyze the activated hippocampal areas during the short duration SE and compare the result to the expression of the c-Fos protein in the same areas.

Prior studies showed that, 12 hours after its injection, $MnCl_2$ had permeated the hippocampus of the rodents[1,2]. Nevertheless, it was noted that the animals that remained for 5 or 15 minutes in SE showed no alteration in intensity of the MEMRI signal when compared to the control group. Even more interesting, the animals that remained for more time in SE (30 minutes), had a decrease in the MEMRI signal relative to the controls. These results corroborate previous results in which it was not possible to access the activated areas during the acute phase of the kainic acid model after the animal had remained in SE for 90 minutes[7]. In order to explain the reduced MEMRI signal, the authors proposed that this was due to massive cell loss caused by prolonged injury, which could be avoided by the animal's remaining

in SE for a short period of time. In a previous study [15], it was demonstrated that 5 minutes of continuous epileptic activity in the pilocarpine model are sufficient to cause cell loss only in the hilus of the hippocampus, when evaluated 2 hours after the end of the seizure. Other cerebral areas showed mild lesion when the animals were perfused 24 hours after the end of the SE that lasted 5 minutes. Still in the same study, it was demonstrated that it is necessary that SE last more than 30 minutes to promote neuronal damage to the pyramidal cells and to the stratum oriens of CA1 and CA3. At this point, it is important to highlight that a SE that lasts less than 30 minutes is not sufficient to provoke a chronic epileptic condition[16-19].

The increased T_1 associated with the formation of edema during the 30-minute SE may be considered an event capable of decreasing the contrast provided by Mn^{2+} and should be better investigated. In more magnetic fields, the intensity of the signal provoked by the accumulation of manganese is more evident. However, the dose used in this study in a 2T magnetic field is sufficient for the study of the hippocampal region in animal models of epilepsy.

The immune reaction to the c-Fos protein expressed by cells in activity, in the same animals in which the MRIs were acquired, allowed the evaluation of the hypothesis of non-activation of specific cell populations. The expression of the c-Fos protein is a reliable marker of cellular activation that is expressed rapidly and transiently after stimuli unknown to the animal. The expression of this protein appears in various specific cerebral regions after the induction of several types of seizures[17].

The present result demonstrates intense marking in the hippocampal regions of the DG, CA1 and CA3 for all the groups treated with pilocarpine, regardless of the duration of the SE, which indicates activation of the cells in all hippocampal areas. In the model of epilepsy induced by lithium-pilocarpine in rats 10 days after birth, the expression of c-Fos was also verifiable in various regions of the brain, among them, the CA1 and the DG, 2 hours after SE induction[17].

In analyzing spontaneous short-duration seizures, it was previously demonstrated[20] that there is no expression of the c-Fos protein. Possibly, such a result is due to the fact that the recurring seizure is not a new stimulus, i.e., not previously experienced by these cells. Accordingly, Peng and Houser[21] showed c-Fos labeling in the DG after spontaneous seizures in Guiney pigs. It was not discussed by the authors, but there is a possibility that the labeling of these cells is related to the cells that divided after the SE. On the other hand, no studies are known in which the short-duration SE is used for the evaluation of the expression of the c-Fos gene. In this way, the present study complements the results obtained with the use of the MEMRI technique, showing that short-duration SE truly activates the hippocampal areas of the DG, CA1 and CA3.

The similarity in the levels of c-Fos protein labeling among the groups studied herein favors the idea that 5 minutes of continuous epileptic activity, induced by pilocarpine, are sufficient to promote generalized cellular activation in the hippocampus. Since all these cells, or the majority of them, have already been activated, greater durations of the SE will not result in greater labeling. In this way, such results indicate that the reduction of the MEMRI signal in this experimental protocol cannot be related to the quantity of cells activated.

CONCLUSION

The results show that short-duration SE does not alter the intensity of the MEMRI signal in the acute phase of the pilocarpine model. The histological analysis of the expression of the c-Fos protein indicates that in the times studied, there was cellular activation in the regions of the DG, CA1 and CA3. The elements involved in MEMRI that seem to be altered and therefore do not allow the formation of enhancement after continuous induced epileptic activity, should be studied further and are targets of future investigations.

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