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High serum levels of proinflammatory markers during epileptogenesis. Can omega-3 fatty acid administration reduce this process?

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ABSTRACT

During the epileptogenic process, several events may occur, such as an important activation of the immune system in the central nervous system. The response to seizure activity results in an inflammation in the brain as well as in the periphery. Moreover, CRP and cytokines may be able to interact with numerous ligands in response to cardiac injury caused by sympathetic stimulation in ictal and postictal states. Based on this, we measured the serum levels of C-reactive protein (CRP) and cytokines during acute, silent, and chronic phases of rats submitted to the pilocarpine model of epilepsy. We have also analyzed the effect of a chronic treatment of these rats with omega-3 fatty acid in CRP and cytokine levels, during an epileptic focus generation. C-reactive protein and cytokines such as IL-1β, IL-6, and TNF-α presented high concentration in the blood of rats, even well after the occurrence of SE. We found reduced levels of CRP and all proinflammatory cytokines in the blood of animals with chronic seizures, treated with omega-3, when compared with those treated with vehicle solution. Taken together, our results strongly suggest that the omega-3 is an effective treatment to prevent SUDEP occurrence due to its capability to act as an anti-inflammatory compound, reducing the systemic inflammatory parameters altered by seizures.

1. Introduction

Several lines of evidence have shown the involvement of inflammatory pathway with temporal lobe epilepsy (TLE) [1–3]. Patients with specific forms of epilepsy present with a peripheral inflammatory response linked to the condition; furthermore, an increased cytokine response has also been reported in the literature in these cases [4].

Another event that occurs during long-lasting seizures is dysfunction of the blood–brain barrier (BBB) [5,6], allowing the entrance of proteins and cells from the blood into the brain, increasing local inflammation, and amplifying excitability in a continuous cycle. In this context, lymphocytic infiltrate and high cytokine levels suggest that both humoral and cellular immune systems are exacerbated in these patients [2].

C-reactive protein is an important molecule related to the acute phase of the inflammatory process; it is present in the serum or plasma of many vertebrates [7]. Its main effect is the modulation of several cascades related to immune system activation [8]. It has been linked to inflammatory processes, it has been used clinically as a marker for immune responses to infections, and it has an important biological role in the pathogenesis of cardiovascular disease [9]. Transcriptional regulation of CRP has been extensively studied *in vitro* and *in vivo*. For this regulation, several evidences suggest that IL-6 is the principal inducer of the CRP gene expression, while IL-1, TNF-α, glucocorticoids, and activated complement act synergistically with IL-6 to enhance its effect [10–12]. An increased CRP level in the blood is considered to be an inflammatory marker for brain ischemia, stroke, and vascular events [13].

A few studies provided clinical and experimental evidence suggesting that omega-3 fatty acid supplementation decreases the duration and frequency of seizures [14–16], resulting in neuroprotective effects against seizure-induced brain damage [17]. In patients with refractory seizures, it has been suggested, though not proven, that treatment with omega-3 fatty acid could also reduce seizure-associated cardiac arrhythmias and, in some cases, sudden unexpected death (SUDEP), the most important direct epilepsy-related cause of death [18,19].

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Thus, it becomes necessary to better understand the role played by the immune system in epilepsy; therefore, the main objective of this study was to investigate CRP, IL-1β, TNF-α, IL-6, and IL-10 levels in the blood of rats submitted to the pilocarpine of temporal lobe epilepsy. This model consists of an acute phase, characterized by long-lasting *status epilepticus* (SE) (12 h), followed by a silent period with no seizures (lasting 4 to 44 days) culminating in a chronic phase (period of spontaneous seizures), lasting for the rest of the animal's life [20]. We also analyzed the levels of these immunological markers in the blood of epileptic rats (chronic phase), daily treated with omega-3 fatty acid, after SE onset.

2. Material and methods

2.1. Animals and status epilepticus induction

The animal experiments were performed under UNIFESP Institutional Ethical Committee approval (Hospital São Paulo/Universidade Federal de São Paulo, process n. 313162), and all efforts were made to minimize animal suffering. Wistar adult male rats, weighing 250 g, were housed in groups of three to four per cage and maintained in controlled room temperature, humidity, and light–dark cycle (12:12 h) with chow pellets and tap water available *ad libitum*.

The rats received a single dose of pilocarpine (350 mg/kg, intraperitoneal [i.p.]). To prevent peripheral cholinergic effects, scopolamine methylnitrate was injected subcutaneously at a dose of 1 mg/kg, 30 min before pilocarpine administration.

To stop *status epilepticus* (SE) during the acute phase of the pilocarpine model, diazepam (10 mg/kg, Cristalia-Compaz) was administered subcutaneously 3 h after SE onset. The animals were then allowed to evolve from the acute to the silent and chronic phases of this model as previously reported by us [20]. The occurrence of spontaneous recurrent seizures (SRSs) during the chronic period was video-monitored (24 h per day) for 90 days. Animals were sacrificed, and the blood was collected for CRP and cytokine assay.

2.2. Animal groups

2.2.1. C-reactive protein

To study the influence of long-lasting SE or spontaneous seizures (chronic period) on peripheral C-reactive protein, the following groups (n = 8) were analyzed: 5 h, 12 h, and 24 h after SE onset (acute period); 48 h and 5 days after SE induction (silent period), and 90 days after SE onset (chronic phase, period of spontaneous seizures). Saline-treated animals were used as controls for each group (n = 8) as well as rats that received pilocarpine but did not develop SE.

2.2.2. Cytokines

To study the inflammatory profile (IL-1β, TNF-α, IL-6, IL-10) in the blood, the following groups were performed: acute (5 h), silent (5 days), and chronic (90 days after SE onset). These groups were compared with saline-treated rats (n = 6 each group).

2.2.3. Omega-3 treatment

Rats were submitted to SE, which was blocked with diazepam 3 h after onset. After that, animals received vehicle cremophor (0.009%) or fish oil (omega-3, PROEPA, 85 mg/kg/day). These solutions were administered to animals between 11:00 and 12:00 am by gavage. The volume administered was adjusted according to animal weight, which was verified three times a week, for 90 days. Omega-3 fatty acid was formulated as fish oil (EPA 180 mg and DHA 120 mg). The capsule contents were dissolved in cremophor 0.009%, yielding a final concentration of 21.25 mg/ml fish oil, which corresponds to 3.82 mg/ml EPA and 2.55 mg/ml DHA. At the final concentration, fish oil was administered 1 ml per 250 g of animal weight. Animals were killed by decapitation, and serum was collected and stored at −80 °C for CRP and cytokine analyses. Four groups were analyzed: control vehicle (animals which received saline and cremophor), control omega (animals which received saline and omega-3), chronic (animals with epilepsy, 90 days after SE induction), chronic + omega (animals with epilepsy which received omega-3 treatment) (n = 6 for each group).

2.3. Measurement of inflammatory markers

2.3.1. CRP assay

Quantitative assessment of CRP levels was carried out *via* ELISA (C-reactive protein, ELISA kit, Chemicon, Millipore, MA, USA), following the manufacturer's recommendations.

2.3.2. Cytokine analysis

Multiplex immunobead assay technology (MAP Milliplex Rat Cytokine/Chemokine Magnetic Bead Panel Millipore Corp., Billerica, MA, USA; Magpix and analytical test instrument, Luminex Corp., Austin, TX, USA) was performed in serum.

2.4. Statistical analysis

The SPSS statistical package version 22.0 (SPSS, Chicago, IL, USA) and GraphPad version 6.0 were used for statistical evaluation (GraphPad Software, San Diego, CA, USA). Data are expressed as the mean ± standard deviation (SD). Given the sample size and the variable distribution, nonparametric tests were used. Data for three or more independent groups were analyzed by Kruskal–Wallis test. When significant, a multiple comparison *post hoc* test was used (Dunn's test). A two-tailed p-value < 0.05 was chosen as the level of significance. Statistical data are provided in the figures.

3. Results

3.1. Animal behavior

Pilocarpine administration induced the following behavioral changes: akinesia, facial automatisms, and limbic seizures consisting of forelimb clonus with rearing, salivation, and masticatory jaw movements and falling. This type of behavior built up progressively into motor limbic seizures that recurred repeatedly, evolving to long-lasting *SE* as previously reported [21].

3.2. CRP levels

Control rats presented CRP serum levels ranging from 6.8 ± 1.5 to 10.3 ± 3.3 ng/ml. However, after SE induction, the CRP concentration rose drastically mainly after 5 h of SE. During the silent phase, CRP levels were still high within 2 days after SE and remained higher 5 days after SE onset. Animals presenting spontaneous seizures (90 days after SE) showed high CRP concentration, when compared with control levels. The comparison between all groups is presented in Fig. 1A, which shows that CRP elevation is not restricted to seizure period. Its level remained altered in all periods of the epilepsy model induced by pilocarpine.

There were no differences in CRP levels between controls treated with vehicle and controls treated with omega-3 fatty acid. Chronic rats (with spontaneous recurrent seizures) without treatment presented high values, when compared with its proper control groups. In contrast, chronic rats treated with omega-3 for 90 days after SE onset showed decreased levels of this inflammatory marker, when compared with the chronic group. The treatment with omega-3 fatty acid induced a reduction in CRP levels, but the treatment was not enough to bring CRP to normal levels. Treated rats showed CRP levels similar to those values found in animals during the silent period of this epilepsy model (5 days after SE) (Fig. 1B).