Original Research

Inhibition of matrix metalloproteinases-2 and -9 prevents cognitive impairment induced by pneumococcal meningitis in Wistar rats

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Abstract

Pneumococcal meningitis is a relevant clinical disease characterized by an intense inflammatory reaction into the subarachnoid and ventricular spaces, leading to blood–brain barrier breakdown, hearing loss, and cognitive impairment. Matrix metalloproteinases (MMPs) are capable of degrading components of the basal laminin, thus contributing to BBB damage and neuronal injury. In the present study, we evaluated the effects of MMP-2, MMP-9, and MMP-2/9 inhibitors on BBB integrity, learning, and memory in Wistar rats subjected to pneumococcal meningitis. The animals underwent a magna cistern tap and received either 10 μL sterile saline as a placebo or an equivalent volume of a *Streptococcus pneumoniae* suspension at a concentration of 5×10^9 cfu/mL. The rats were randomized into different groups that received adjuvant treatment with MMP-2, MMP-9 or MMP-2/9 inhibitors. The BBB integrity was evaluated, and the animals were habituated to open-field and object recognition tasks 10 days after meningitis induction. Adjuvant treatments with inhibitors of MMP-2 or MMP-2/9 prevented BBB breakdown in the hippocampus, and treatments with inhibitors of MMP-2, MMP-9 or MMP-2/9 prevented BBB breakdown in the cortex. Ten days after meningitis induction, the animals that received adjuvant treatment with the inhibitor of MMP-2/9 demonstrated that animals habituated to the open-field task faster and enhanced memory during short-term and long-term retention test sessions in the object recognition task. Further investigation is necessary to provide support for MMP inhibitors as an alternative treatment for bacterial meningitis; however, these findings suggest that the meningitis model could be a good research tool for studying the biological mechanisms involved in the behavioral alterations associated with pneumococcal meningitis.

Keywords: Meningitis, *Streptococcus pneumoniae*, metalloproteinases-2 and 9, blood–brain barrier, learning and memory

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Introduction

Pneumococcal meningitis is a relevant clinical problem that is characterized by an intense inflammatory reaction into the subarachnoid and ventricular space, breakdown of the blood–brain barrier (BBB), hearing loss, and neurological sequelae in up to 30% of the survivors.[1–3] Clinical and experimental studies provide evidence that matrix metalloproteinases (MMPs) are implicated in the pathogenesis of various inflammatory diseases of the central nervous system (CNS).[4,5] MMPs comprise a large family of zinc-dependent endopeptidases with substrate affinity for different components of the extracellular matrix,[6] and they are produced by a variety of inflammatory, vascular, and

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resident brain cells.[4] In addition, neurons are the main source of constitutive MMP-9 in the brain, whereas MMP-2 is ubiquitous in most neural cells, including neurons, glial, and endothelial cells.[7,8] During pneumococcal meningitis, the microorganism crosses the BBB through both transcellular traversal and paracellular traversal mechanisms.[9–11] After *Streptococcus pneumoniae* reaches the subarachnoid space, it multiplies rapidly and releases compounds, such as cell wall fragments, lipoteichoic acid, pneumolysin, and peptidoglycan.[2,3,12] Many brain cells can produce cytokines, chemokines, and other pro-inflammatory molecules in response to bacterial stimuli.[13] Consequently, polymorphonuclear leukocytes are attracted from the blood and activated, and large amounts of superoxide anion and nitric oxide are produced.[14–16] Oxidative stress leads to lipid peroxidation, MMP activation, mitochondrial damage, and enhanced secretion of cytokines and chemokines.[14] Tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), and interleukin-6 (IL-6) play roles as early response cytokines. These cytokines trigger, often in synergy, a cascade of inflammatory mediators, including MMPs.[17] MMP-2 and MMP-9 are implicated in leukocyte recruitment and the disruption of the BBB.[18] Furthermore, MMP-2 and MMP-9 are elevated in the cerebrospinal fluid (CSF) of children with meningitis.[19] In the present study, we evaluated the effects of MMP-2, MMP-9, and MMP-2/9 inhibitors on the BBB integrity and on learning and memory in Wistar rats subjected to pneumococcal meningitis.

Materials and methods

Infecting organism

S. pneumoniae (serotype 3) was cultured overnight in 10 mL of Todd Hewitt broth, and then it was diluted in fresh medium and grown to the logarithmic phase. This culture was centrifuged for 10 min at 5000 × *g* and resuspended in sterile saline at a concentration of 5×10^9 cfu/mL. The size of the inoculum was confirmed by quantitative cultures.[20]

Animal model of meningitis

Male Wistar rats (250–300 g body weight) from our breeding colony were used for the experiments. All procedures were approved by the Animal Care and Experimentation Committee of UNESC, Brazil, and were followed in accordance with the National Institute of Health's Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23) revised in 1996. All surgical procedures and bacterial inoculations were performed under anesthesia, consisting of an intraperitoneal (i.p.) administration of ketamine (6.6 mg/kg), xylazine (0.3 mg/kg), and acepromazine (0.16 mg/kg).[21] Rats underwent a cisterna magna tap with a 23-gauge needle. The animals received either 10 μL of sterile saline as a placebo or an equivalent volume of the *S. pneumoniae* suspension. At the time of inoculation, the animals received fluid replacement and were then returned to their cages.[21,22] Meningitis was documented by a quantitative culture of 5 μL of CSF obtained by puncture of the cisterna magna.

Drugs and reagents

MMP-2 selective inhibitor (sc-204092, Santa Cruz Biotechnology) and MMP-9 selective inhibitor (sc-311437, Santa Cruz Biotechnology, USA) were administered intracerebroventricularly (i.c.v.) in a single dose (250 μg/kg) immediately after pneumococcal meningitis induction.[23] The control group received a similar volume of saline injected under the same conditions.

Blood–brain barrier permeability to Evan's blue

To evaluate the BBB integrity, the animals were separated into four groups: sham, meningitis/MMP-2 inhibitor, meningitis/MMP-9 inhibitor, and meningitis/MMP-2/9 inhibitors. Twelve hours after pneumococcal meningitis induction, the BBB integrity was investigated using Evan's blue dye extravasation.[24] The animals were injected with 1 mL of Evan's blue at 1% i.p. 1 h before being killed. The anesthesia consisted of an i.p. administration of ketamine (6.6 mg/kg), xylazine (0.3 mg/kg), and acepromazine (0.16 mg/kg).[21] The chest was subsequently opened, and the brain was transcardially perfused with 200 mL of saline through the left ventricle at a pressure of 100 mmHg until colorless perfusion fluid was obtained from the right atrium. The samples were weighed and placed in a 50% trichloroacetic solution. Following homogenization and centrifugation, the extracted dye was diluted with ethanol (1 : 3), and its fluorescence was determined (excitation at 620 nm and emission at 680 nm) with a luminescence spectrophotometer (Hitachi 650-40, Tokyo, Japan). Calculations were based on the external standard (62.5–500 ng/mL) in the same solvent. The Evan's blue tissue content was quantified from a linear standard line derived from known amounts of the dye, and it was expressed per gram of tissue.[24]

Behavioral tasks

To evaluate behavioral responses, the animals were separated into four groups: sham, meningitis/MMP-2 inhibitor, meningitis/MMP-9 inhibitor, and meningitis/MMP-2/9 inhibitors. Eighteen hours after induction, the animals received ceftriaxone (100 mg/kg body weight given s.c./7 days).[25] Ten days after inoculation, the animals were free from infection. All blood cultures that we performed during this period were negative. The animals recovered their weight and grooming habits, their blood counts returned to control levels, and the reactive protein C values were negative. Thus, we evaluated memory performance through open-field and object recognition tasks.

Open-field test. Behavior was assessed in an open-field apparatus to evaluate both locomotor and exploratory activities. This apparatus is a 40 × 60 cm open-field surrounded by 50 cm high walls made of brown plywood with a front glass wall. Black lines divide the floor of the open-field into nine rectangles. Animals were gently placed on the left rear quadrant and were left to explore the arena for 5 min. The number of crossings (i.e. the number of times that the animals crossed the black lines, which assesses locomotor activity) and rearing movements (i.e. the

exploration behavior observed in rats subjected to a new environment) were measured. The same person, who was blind to group treatment, performed all behavioral testing by manual analyses.[26]

Object recognition. This task evaluates non-aversive, non-spatial memory. The apparatus and procedures for the object recognition task have been described elsewhere.[27] Briefly, the task was performed in a 40×50 cm open-field surrounded by 50 cm high walls made of plywood with a front glass wall. Black lines divided the floor of the open-field into 12 equal rectangles. All animals were subjected to a habituation session in which they were allowed to freely explore the open-field for 5 min. No objects were placed in the box during the habituation trial. The number of times that the rats crossed the black lines and the number of rearing movements performed in this session were evaluated as locomotor and exploratory activity, respectively. After habituation, training was conducted at different time points by placing individual rats in the field for 5 min, during which two identical objects (objects A1 and A2, both being cubes) were positioned in two adjacent corners, 10 cm from the walls. In a short-term recognition memory test administered 1.5 h after training, the rats explored the open-field for 5 min in the presence of one familiar (A) and one novel (B, a pyramid with a square-shaped base) object. All objects had similar textures (smooth), colors (blue), and sizes (weight 150–200 g), but distinctive shapes. A recognition index calculated for each animal is reported as the ratio TB/(TA + TB). (TA = time spent exploring the familiar object, A; TB = time spent exploring the novel object, B). In the long-term recognition memory test administered 24 h after training, the same rats were allowed to explore the field for 5 min in the presence of the familiar object A and a novel object C (a sphere with a square shaped base). Recognition memory was evaluated in the same way as in the short-term memory test. Exploration was defined as sniffing (exploring the object from a distance of 3–5 cm) or touching the object with the nose and/or forepaws.[28]

Statistics

The results of the BBB integrity are reported as the mean ± standard error of the mean (SEM) of 6–7 animals in each group. Differences among groups were evaluated using analysis of variance (ANOVA) followed by the Student Newman-Keuls *post hoc* test. In the behavioral tasks, 10 animals were used in each group. Data from the habituation to the open-field task were reported as the mean ± SEM and were analyzed with paired Student's *t*-tests and ANOVA, using Tukey's *post hoc* tests. Data from the object recognition task were reported as the median and interquartile ranges, and comparisons among groups were performed using Mann-Whitney U tests. The within-individual groups were analyzed using Wilcoxon's tests. P values < 0.05 were considered statistically significant.

Results

In Figure 1, we evaluated BBB integrity in the hippocampus and cortex at 12 h after pneumococcal meningitis induction in animals administered adjuvant treatment with MMP inhibitors. In the hippocampus, the use of the inhibitor of MMP-2 or MMP-2/9 showed no statistic significant difference with the control group, demonstrated these inhibitors prevented BBB disruption. The meningitis group and the meningitis group that received MMP-9 inhibitor showed statistic significant difference with the control group, demonstrated BBB disruption ($P < 0.05$).

In the cortex, the use of the inhibitors of MMP-2, MMP-9, and MMP-2/9 showed no statistic significant difference with the control group, demonstrated these inhibitors prevented BBB disruption. The meningitis group showed statistic significant difference with the control group, demonstrated BBB disruption ($P < 0.05$).

In the open-field task (Figure 2), we evaluated the influence of the MMP-2, MMP-9, or MMP-2/9 inhibitors on habituation memory 10 days after the induction of pneumococcal meningitis. There were no differences in the number of crossings and rearing movements among groups in the habituation to the open-field training session, demonstrating no difference in motor and exploratory activity among groups. In the test session, there was a significant reduction in both crossings and rearing in the sham group and the meningitis group that received an inhibitor of MMP-2/9 when compared with the training session, demonstrating habituation memory in these groups ($P < 0.05$). However, the meningitis groups that received an inhibitor of only MMP-2 or MMP-9 showed no difference in motor and exploratory activity between training and test sessions, demonstrating habituation memory impairment in these groups.

In Figure 3, the object recognition task, we evaluated the influence of inhibitors of MMP-2, MMP-9, and MMP-2/9 in short- and long-term object recognition retention memory. The animals that received adjuvant treatment with inhibitors of MMP-2 or MMP-9 presented impairment of novel object recognition memory (i.e. they did not spend a significantly higher percentage of time exploring the novel object during the short- and long-term retention memory test sessions in comparison to the training session). However, the meningitis group that received adjuvant treatment with an inhibitor of MMP-2/9 showed evidence of memory during the short- and long-term retention test sessions in comparison to the training trial. These animals spent more time exploring the novel object during the short- and long-term retention memory test sessions in comparison to the training session ($P < 0.05$).

Discussion

In the present study, we demonstrated the influence of MMP-2, MMP-9, and MMP-2/9 inhibitors on BBB integrity and on learning and memory in an animal model of pneumococcal meningitis. In previous studies, cytokines were produced mainly in the first 6–24 h[29] and oxidative stress was formed at 24 and 48 h after meningitis

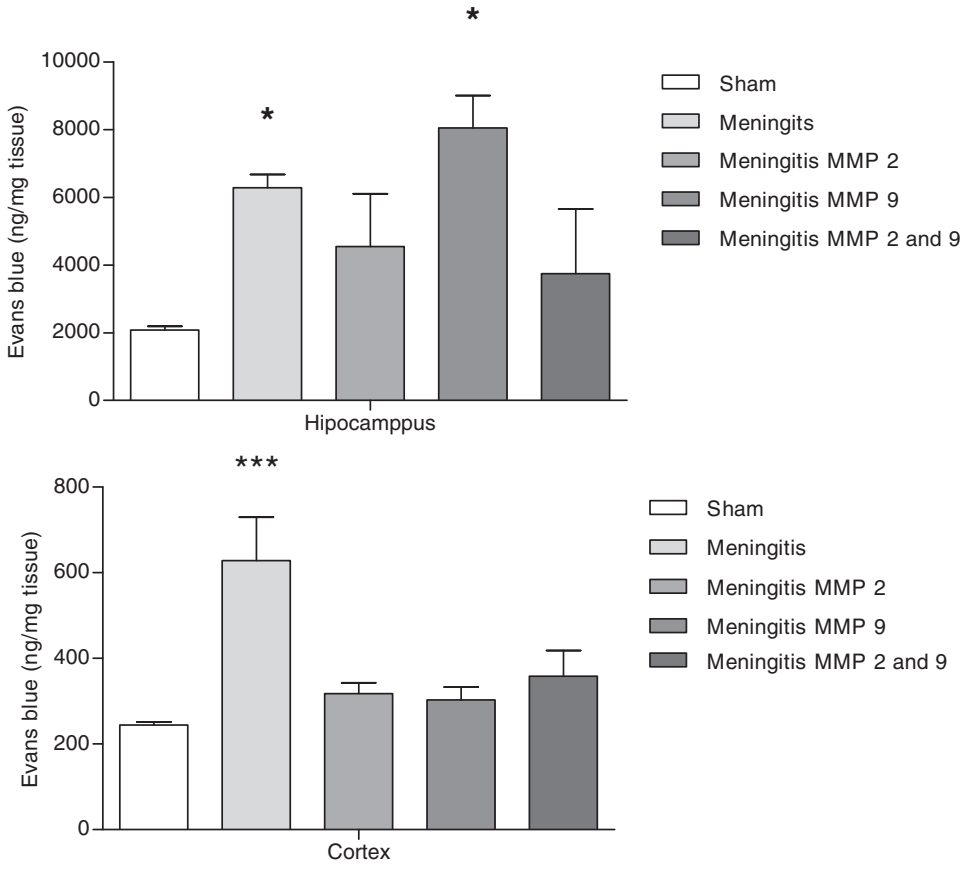

Figure 1 The integrity of the blood–brain barrier (BBB) was investigated using Evan's blue dye extravasation in hippocampus and cortex after induction of *Streptococcus pneumoniae* meningitis. The animals were separated in sham, meningitis, meningitis/inhibitor matrix metalloproteinases (MMP)-2, meningitis/inhibitor MMP-9 and meningitis/inhibitor MMP-2/9. The results of the BBB integrity are shown by the mean ± standard error of the mean (SEM) of 6–7 animals in each group. Differences among groups were evaluated by using analysis of variance (ANOVA) followed by the Student Newman-Keuls *post hoc* test. In all comparisons, $P < 0.05$ indicated statistical significance

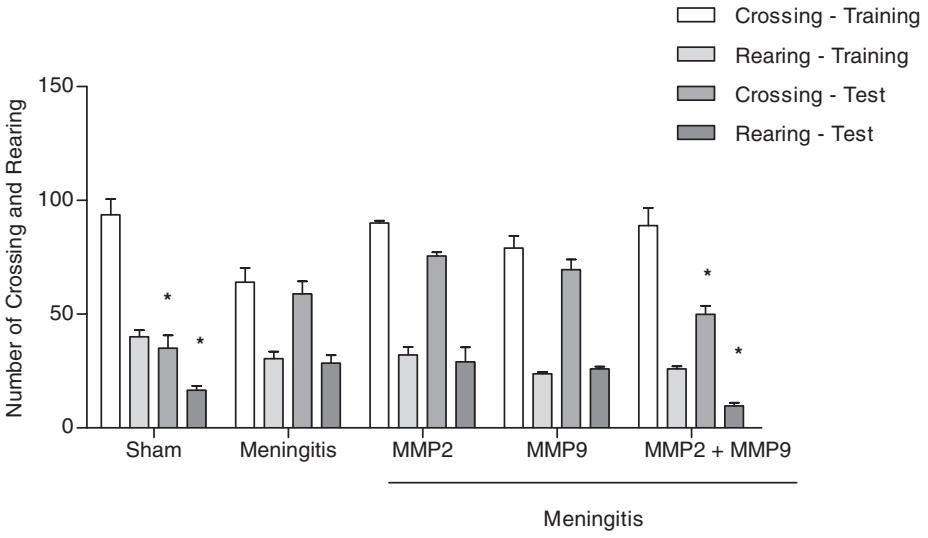

Figure 2 Open field test 10 days after induction of meningitis by *Streptococcus pneumoniae*. The animals were separated in four groups sham (control group), meningitis, meningitis/inhibitor matrix metalloproteinases (MMP)-2, meningitis/inhibitor MMP-9, and meningitis/inhibitor MMP-2/9. Data are reported as mean ± SEM in training and test sessions, $n = 10$ per group, and were analyzed by the paired Student's *t*-test and ANOVA *post hoc* Tukey. In all comparisons, $P < 0.05$ indicated statistical significance

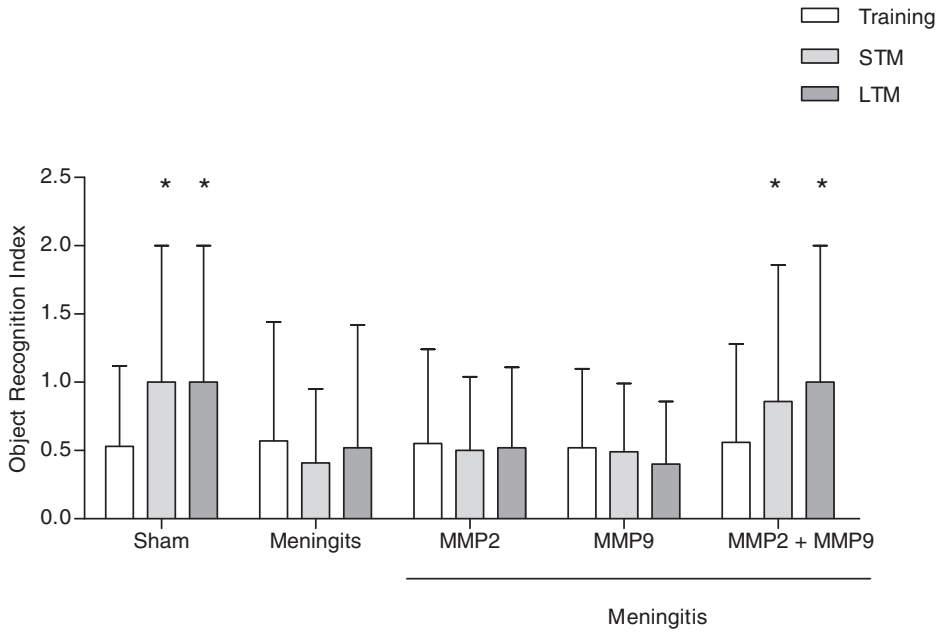

Figure 3 Object recognition task 10 days after induction of meningitis by *Streptococcus pneumoniae*. The animals were separated in four groups sham (control group), meningitis, meningitis/inhibitor matrix metalloproteinases (MMP)-2, meningitis/inhibitor MMP-9 and meningitis/inhibitor MMP-2/9. Data are reported as median and interquartile ranges, and comparisons among groups were performed using Mann-Whitney U tests, $n = 10$ per group. The within-individual groups were analyzed by Wilcoxon's tests. In all comparisons, $P < 0.05$ indicated statistical significance

induction.[30] We verified BBB disruption at 12 h after pneumococcal meningitis induction in the hippocampus and cortex, whereas cytokine-induced neutrophil chemoattractant 1 (CINC-1) levels were increased at 6 h in the arterial plasma and at 3 h in the jugular plasma.[21] We verified that inhibitors of MMP-2 and MMP-2/9 prevented BBB disruption in the hippocampus and that all three inhibitors prevented BBB disruption in the cortex. The host immune response, the production of cytokines and chemokines, and leukocyte migration represent the first line of defense in response to bacterial infection.[31] Leukocytes can produce nitric oxide, superoxide anion radicals, and hydrogen peroxide that can lead to the formation of peroxynitrite,[14] which is a strong oxidant that exerts cytotoxic effects on endothelial cells,[32] increases the BBB permeability,[33] and provokes lipid peroxidation, mitochondrial damage and MMPs activation.[14] The MMPs break down the extracellular matrix, thus increasing BBB permeability, degrading myelin proteins and enhancing the flow of leukocytes from blood into the cerebral spinal fluid,[4,34,35] Furthermore, MMPs appear to be involved in the pathophysiology of pneumococcal meningitis.[14] The resulting release of cytokines and the production of oxidative stress can transform MMPs into the biologically active form. The cytokines induce MMPs via proto-oncogenes of the c-*fos* and c-*fun* family that bind to various gene promoter elements, such as the activator protein-1.[4] Lipoteichoic acid also induces MMP-9 expression and can be activated by Toll-like receptor 2 (TLR2)-activated c-Src-dependent transactivation of the platelet-derived growth factor (PDGFR) pathway.[36] Furthermore, MMP-2 and MMP-9 have also been shown to induce BBB breakdown through their substrate affinity for a variety of components of the extracellular matrix,[37] and they also facilitate leukocyte extravasation in experimental bacterial meningitis.[38] Patients with bacterial meningitis present high concentrations of MMP-9[39,40] and MMP-8 in the CSF; indeed, high concentrations of MMP-9 are correlated with risk factors for the development of postmeningitis neurological sequelae.[41]

Animals subjected to pneumococcal meningitis show memory and learning deficits and anxiety-like and depressive-like behavior.[42,43] Therefore, following this reasoning, we evaluated learning and memory using adjuvant treatment with inhibitors of MMPs. The animals that received an inhibitor of MMP-2/9 presented normal habituation memory and retention of short- and long-term memory. In contrast, treatment with either MMP-2 or MMP-9 alone did not prevent cognitive impairment. Inhibitors of MMPs reduce cortical damage in experimental pneumococcal meningitis,[44] and this cortical damage is associated with changes in parenchymal MMP-9/TIMP-1 ratio and increased collagen type IV degradation.[45] Thus, the prevention of the cognitive damage was associated with the use of the MMP-2/9 inhibitors in rats after the induction of pneumococcal meningitis. Although the hippocampus is not exposed to microorganisms or infiltrating leukocytes directly, it is surrounded by an interstitial fluid, which is contiguous with the cerebral spinal fluid, permitting the secreted bacterial toxins and immune system mediators to diffuse into the parenchyma.[2]

Conclusion

Despite significant progress in treatment options, pneumococcal meningitis remains one of the most important infectious diseases of the CNS with high mortality and

morbidity. Although there are some limitations to this study, this experimental animal model contributes to our understanding of this complex pathophysiological disease and demonstrates the involvement of MMPs.

Author contributions: All authors participated in the design and interpretation of the studies, analysis of the data and review of the manuscript; JSG, CMM, LRS, SGE, FV and CMC conducted the experiments, FDP and JQ supplied critical reagents (or animals) (Wistar rats), and TB wrote the manuscript.

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