


ORIGINAL ARTICLE

Short-Term Effects of Sepsis and the Impact of Aging on the Transcriptional Profile of Different Brain Regions

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Abstract—Among the clinical manifestations observed in septic patients, sepsis-associated encephalopathy (SAE) is probably the most obscure and poorly explored. It is well established, however, that SAE is more prevalent in aged individuals and related to a worse outcome. In this context, we decided to investigate the acute effects of sepsis, induced by cecal ligation and puncture (CLP), on the cerebral transcriptional profile of young and old rats. The idea was to highlight important signaling pathways possibly implicated in the early stages of SAE. Global gene expression analysis of three different brain regions (hippocampus, cerebellum, and cortex) indicated a relatively small interference of sepsis at the transcriptional level. Cerebellum tissue was the least affected by sepsis in aged rats. The increased expression of S100a8, Upp1, and Mt2a in all three brain regions of young septic rats indicate that these genes may be involved in the first line of response to sepsis in the younger brain. On the other hand, altered expression of a network of genes involved in sensory perception of smell in the cortex of aged rats, but not in young ones, indicates an earlier disruption of cortex function, possibly more sensitive to the systemic inflammation. The expression of S100a8 at the protein level was confirmed in all brain regions, with clear-up regulation in septic aged cortex. Taken together, our results indicate that the transcriptional response of the central nervous system to early sepsis varies between distinct brain regions and that the cortex is affected earlier in aged animals, in line with early neurological manifestations observed in older patients.

KEY WORDS: Encephalopathy; Inflammation; sepsis; aging; transcriptomics.

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INTRODUCTION

Sepsis can affect people with different health profiles. However, extremes of age and those who already have a chronic health problem or a compromised immune system are at a higher risk of developing the disease [1]. Aged people constitute a group that presents significant vulnerability to sepsis and in the USA, for example, it is estimated that more than 65% of people who develop severe sepsis are 65 years of age or older [2–4]. In addition, in the last 40 years, the incidence of sepsis has increased substantially, which may be partially justified by the increase in the life expectancy of the world population.

Aberrant neurologic signs and symptoms are found in approximately 1/3 of all patients who develop sepsis [5]. In fact, sepsis-associated encephalopathy (SAE) is rare in young individuals, but its frequency is high in the general population. Individuals with SAE may present a broad spectrum of clinical manifestations, ranging from mental confusion to coma [6]. In addition, according to Gotz *et al.* [7], at least 20% of individuals who recover from sepsis have some kind of sequelae, such as physical or cognitive impairment, mood disorders, implicating in a significant decrease in the quality of life. It has been proposed that the initial processes involved in SAE, including the systemic inflammatory stimulus, microglia activation, reduced blood flow, and endothelial injury could lead to permanent cerebral damage [8].

Due to the clear importance of the topic, an increase in the number of scientific articles on the subject has been observed in recent years [9–11]. Consequently, a better characterization of the neurological pathways that are affected in the short and long period following the beginning of the disease is expected [12, 13]. It is now clear that three main processes are involved in the pathophysiology of SAE: diffuse neuroinflammation, ischemic processes, and excitotoxicity [14].

Although new technologies have allowed advances in scientific discoveries related to the neurological pathways affected by sepsis, no study published so far has investigated the transcriptional profile of brain regions of aged animals submitted to septic shock. The aged, in addition to be more affected by sepsis, also presents neurological manifestations more often in the course of the disease than the young. Furthermore, our group and others have been describing that aged people present a characteristic systemic and local inflammatory response, when compared to young individuals [2, 15–17], and we propose that this may also occur in the brain.

The purpose of this study is to investigate the short-term effects of sepsis induced by cecal ligation and perforation (CLP) on the central nervous system (CNS) of young and aged rats using large-scale gene expression analysis of three brain regions: the hippocampus, the cerebellum, and the prefrontal cortex. We have selected these regions due to their importance to the development of SAE [5, 10]. Using this strategy, we concluded that the three brain regions respond differently to sepsis at the transcriptional level and that the effect is significantly distinct between young and aged rats. These results may point to strategies to better comprehend SAE in the elderly.

MATERIAL AND METHODS

Rats

We used Wistar rats from the University of Sao Paulo animal facility. All experiments were performed when animals were 8–12 weeks (young rats) or 18 months old (aged rats) and protocols were in accordance with the University of Sao Paulo Faculty of Medicine Animal Facility guidelines and were approved by their ethics committee.

Cecal Ligation and Puncture

We induced peritonitis in rats using the model of CLP as previously described [18]. The CLP model consists of the perforation of the cecum allowing the release of fecal material into the peritoneal cavity eventually leading to systemic infection [9]. Briefly, young (YG) and aged animals (AG) were anesthetized and the cecum was ligated and punctured twice with a 21G needle. As a way of ensuring that the results obtained were mostly due to sepsis, a control group was established. The control group was also divided into young animals (sham YG) and elderly animals (sham AG). In these subgroups, following the same anesthetic procedure adopted in the experimental group, the animals were submitted only to laparotomy with an incision of approximately 2 cm in the median line, enough to exteriorize the cecum and then to return it to the abdominal cavity of the animals. Animals submitted to CLP showed signs of sepsis soon after anesthetic recovery, including lethargy, fever, piloerection, diarrhea, huddling, and malaise.

Brain Samples

Twelve hours after CLP, the animals were taken to an acoustically isolated room where they were decapitated with guillotine. The skullcap of the animals was carefully removed, the brains were removed, and the areas of interest (hippocampus, cerebellum, and prefrontal cortex) were collected and rapidly dissected under a surgical microscope. The collected tissue samples were stored in RNA-later (Thermo Fisher Scientific) at -80°C for gene expression analysis or immersed in liquid nitrogen for analysis *via* ELISA.

In the aged septic animals (AG sepsis) 7 distinct samples of cerebellum, 6 of prefrontal cortex, and 7 of hippocampus were successfully obtained; in young septic animals (YG sepsis), 7 distinct samples of cerebellum, 8 of prefrontal cortex, and 8 of hippocampus were extracted. In

the control group (sham), 7 samples of cerebellum, 6 of prefrontal cortex, and 5 of hippocampus were collected from in the AG sham, and 8 samples of cerebellum, 9 of prefrontal cortex, and 8 of hippocampus were collected in the YG sham. A total of 86 independent microarray experiments were performed.

RNA Extraction and Microarray Experiments

The extraction of the total RNA from the areas of interest of the brains was performed using the Qiasymphony RNA Kit® (Qiagen, Venlo, The Netherlands) and QIASymphony® equipment (Qiagen), following the instructions established by the manufacturer. Overall gene expression patterns were analyzed using DNA microarray assays performed with the SurePrint G3 Rat GE 8x60K Kit (Agilent, California, USA). Labeling using the One-color Low-Input Quick Amp Labeling Kit, hybridization, and washing procedures followed the manufacturer's protocol (Agilent, California, USA). These assays allowed for the analysis of the complete genome expression of the *Rattus norvegicus* species, based on the data compiled in the RefSeq, Ensembl, UniGene, and GenBank databases.

Microarray Data Processing and Analysis

Microarrays were scanned using the SureScan Microarray Scanner (Agilent Technologies) and images were processed using the Feature Extraction Software v12 (Agilent Technologies) for quality control, determination of feature intensities, and for background correction. GeneSpring (Agilent Technologies, USA) was used for data normalization and transformation as well as for subsequent statistical analysis. Principal components analysis (PCA) was used for global gene expression analysis. Moderated *t* test was used for the identification of statistically significant differences in gene expression in septic animals compared to controls. Hierarchical clustering was performed using the hierarchical clustering with support method using differentially expressed genes in order to promote a better understanding of the similarities, as well as the specific differences between the samples of each group studied. For the selection of these genes, we adopted $p \leq 0.01$, adjusted by the Bonferroni test. Differentially expressed genes are represented in terms of fold change (FC). Biological functions enriched among differentially expressed genes were explored using gene ontology (GO) term enrichment analysis and KEGG pathway mapping through DAVID Bioinformatics Resources 6.7 (<http://david.abcc.ncifcrf.gov>), considering a significance threshold of $p \leq 0.01$, adjusted by the Bonferroni test.

Protein Measurements

Protein levels of S100a8 were evaluated in the hippocampus, cerebellum, prefrontal cortex, and plasma using a commercially available kit, as described by the manufacturer (MyBioSource, USA).

Total tissue protein was extracted using the Qproteome Mammalian Protein Prep® (Qiagen), according to the manufacturer's instructions, and the extracted total protein was quantified by spectrophotometry using a Bradford method. Blood plasma and the protein extracted from the brain tissue samples were stored at -80°C until ELISA analysis.

The quantification of protein in AG sepsis was successful for 11 samples of cerebellum, 8 of prefrontal cortex, 11 of hippocampus, and 9 of plasma; in YG sepsis for 8 samples of cerebellum, 8 of prefrontal cortex, 7 of hippocampus, and 9 of plasma; in the AG sham for 10 samples of cerebellum, 8 of prefrontal cortex, 11 of hippocampus, and 9 of plasma; and for YG sham for 8 samples of cerebellum, 9 of prefrontal cortex, 10 of hippocampus, and 9 of plasma. Blood plasma and the protein extracted from the brain tissue samples were stored at -80°C until ELISA analysis.

Statistical analysis was performed using ANOVA followed by Mann-Whitney test for multiple comparison test values. Results are presented as mean \pm standard deviation (SD). The significance level was established as $p < 0.05$.

RESULTS

Global gene expression analysis of cerebral tissue was used to build hypothesis on the effect of sepsis on the CNS and, moreover, to investigate if responses varied at the transcriptional level between young (YG) and aged (AG) animals. The principal components analysis (PCA) indicated that the major source of variability in our data is associated with the brain region (cerebellum, prefrontal cortex, and hippocampus), independently of the experimental conditions (Fig. 1). Additionally, the hippocampal and prefrontal cortex regions were more similar to each other when compared with the cerebellum in terms of global gene expression. We did not analyze any particular cell type but rather bulk tissue from each specific brain region; thus, these results indicate that the molecular particularities of CNS regions, including the diversity of cellular types (i.e., astrocytes, microglia, and a variety of neuron types), surpass the effect of sepsis in this organ.

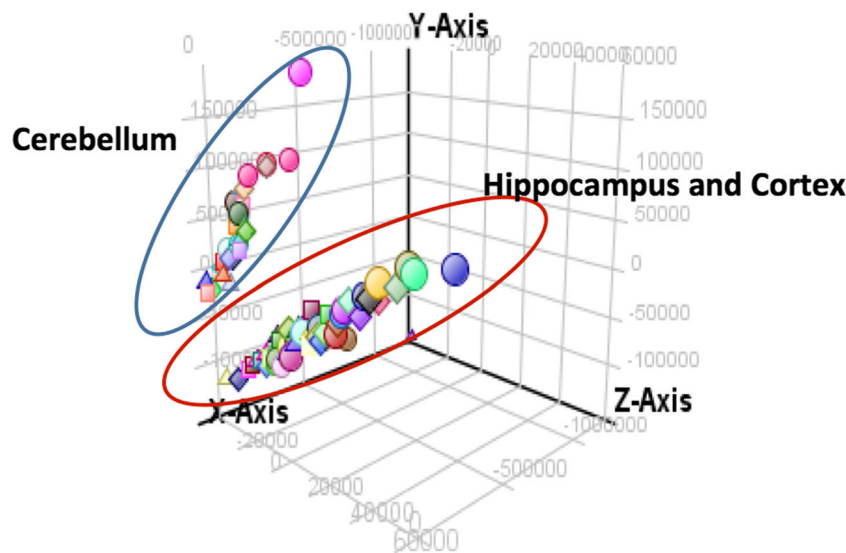


Fig. 1. Principal component analysis depicting global gene expression profiles of the study groups in the hippocampus, prefrontal cortex regions, and cerebellum.

Taking into account this scenario, the subsequent analyses were carried out for each brain region independently. Table 1 summarizes these findings and this restricted list of genes depicts the most consistent markers of the early effect of sepsis in the brain (for a complete list of differentially expressed genes see Supplementary Table 1).

Very little effect at the transcriptional level was observed in the cerebellum tissue of AG sepsis *vs* AG sham (none of them with a fold change higher than 2, see Supplementary Table 1) as compared with YG sepsis *vs* YG sham. Although sepsis affected a higher number of genes in the prefrontal cortex and hippocampus of AG sepsis, similarities with YG sepsis were limited to one gene in the case of the cortex (*Bcl3*) and eight genes in the hippocampus (*Cd93*, *Fn1*, *Il1r2*, *Lrg1*, *Slc2a1*, *Tgm2*, *S100a8*, *Upp1*). Noteworthy is that *S100a8* was the mostly differentially expressed gene in YG sepsis when compared with YG sham for every brain region. Other nine genes were deregulated in all three brain regions of YG animals: *Acer2*, *Elt1d1*, *Lcn2*, *Lrg1*, *Mrpl41*, *Mt2A*, *Prex2*, *Tgm2*, and *Upp1*.

Noteworthy is that despite the broad similarity between hippocampus and cortex in global gene expression, only 20 genes were similarly affected in the two brain areas when YG sepsis was compared with YG sham (*Acer2*, *Elt1d1*, *Lcn2*, *Lrg1*, *Mrpl41*, *Mt2A*, *Prex2*, *S100a8*, *Tgm2*, *Upp1*, *Adm*, *Ddit4*, *Fzd9*, *Gjb6*, *Mgst2*, *Pnpla2*, *RGD1359349*, *Sds*, *Timp4*, *Tinagl1*) and 2 genes (*Bcl3* and *Esam*) when considering hippocampus and cortex in

AG sepsis compared with AG sham (Fig. 2a and b). This result indicates that similarities in global gene expression reflected intrinsic characteristics of the brain regions, possibly unrelated with the septic status, and that each of the two regions respond differently to sepsis.

In order to further investigate the mechanisms associated with early sepsis, less stringent statistical filtering criteria to select differentially expressed genes were applied ($p \leq 0.01$ without Bonferroni test adjustment) and the resulting list was used for gene enrichment analysis. The extended set of genes is characterized by Fig. 2c and d. Within the broader set of genes altered in all three brain regions of YG animals following sepsis, response to lipopolysaccharide (LPS) (GO term GO:0032496) appears significantly enriched with eight genes (*Tnfrsf26*, *Adm*, *S100a8*, *Ptges*, *S100a9*, *Slpi*, *Gjb6*, *Mgst2*), three of these genes are also involved in chronic inflammatory response (*S100a8*, *Ptges*, *S100a9*), and *S100a8* and *S100a9* are implicated in neutrophil aggregation and apoptosis. Regarding genes altered only in the cerebellum, cortex, or in the hippocampus regions in early sepsis, no particular enrichment was found but rather alterations in diverse cellular processes (data not shown). This finding indicates that most alterations within brain regions are specific and not directly involved in response to the infection, as previously mentioned in this work.

In AG animals, the overlap of altered genes concerning all three brain regions following sepsis consisted of only seven genes (*Aif1*, *Bcl3*, *Il4ra*, *S100a8*,

Table 1. Differentially Expressed Genes Between Septic Young Animals (YG) vs Sham and Between Septic Aged Animals (AG) vs Sham. This Table Contains Only Annotated Genes Differentially Expressed Between Groups Considering a $p \leq 0.01$ Adjusted by the Bonferroni Test and Presenting a Fold Change (FC) ≥ 2

CR YG sepsis vs CR YG sham	FC	CX AG sepsis vs CX AG sham	FC	CX YG sepsis vs CX YG sham	FC	HP AG sepsis vs HP AG sham	FC	HP YG sepsis vs HP YG sham	FC
S100a9	5.62	Ildr1	3.77	Lcn2	4.25	Tm7sf4	3.36	Lcn2	4.07
Lcn2	4.42	Bcl3	3.61	Upp1	2.94	Epcam	3.00	Acer2	3.70
Cdkn1a	3.98	Olr1407	3.56	Pla1a	2.92	Bcl3	2.91	Mosc1	3.61
Upp1	3.82	Vom2r4	3.25	Bcl3	2.76	Olr903	2.60	Angpt2	3.25
Mt1a	3.61	Has1	3.10	Tgm2	2.73	Slc8a3	2.49	Lrg1	3.20
Zbtb16	3.55	Gpx2	3.06	Adm	2.64	S100a8	2.48	Hmgcs2	3.10
Mt2A	3.45	Slc38a11	3.02	Mgst2	2.63	Fn1	2.35	Lyve1	3.05
Mrap	3.30	Il4ra	3.01	Map3k6	2.60	Cyp2f4	2.29	Cdkn1a	2.99
Pla1a	2.82	Il20ra	2.93	Sds	2.40	Upp1	2.26	Mgst2	2.89
Lrg1	2.78	Olr1519	2.91	Acer2	2.30	Il1r2	2.13	Upp1	2.81
Tgm2	2.65	Fetub	2.86	Mt1a	2.22	Olr1525	2.08	Adm	2.81
Sult1a1	2.57	Raet11	2.63	Lrg1	2.10	Gpr4	2.06	Adamts1	2.80
Fibin	2.55	Nek2	2.56	Olr1055	2.10	Car5a	2.01	Afp	2.79
Gpatch4	2.32	Tubb6	2.55	Mt2A	2.04	Cd200r1	-2.27	Tgm2	2.75
Pdlim1	2.27	Cox6c1	2.46	Slc40a1	-2.00	Sp9	-2.59	Sds	2.74
Kremen1	2.27	Masp1	2.42	Alox12	-2.23	Runx2	-2.75	Fmo3	2.64
Pnpla7	2.21	Pla2g4b	2.25					Il1r2	2.62
Mobp	2.21	Dpep1	2.20					Sult1a1	2.61
Map3k6	2.17	Gpx2	2.19					Emp1	2.51
Acer2	2.17	Tmem211	2.01					Napsa	2.49
Cebpd	2.13	Tbx4	-2.04					Pou2f3	2.48
Il1rn	2.12	Tmem195	-2.11					Omd	2.46
Htr3a	2.04	Grif1	-2.18					Pnpla2	2.42
Col4a1	2.00	Klhl4	-2.23					Cp	2.42
Opn3	-2.03	Pleg2	-2.24					Fn1	2.41
Adra1b	-2.11	Tmem129	-2.42					Olr1421	2.41
Cdcp1	-2.23	Phf13	-2.45					Pex11a	2.32
G0 s2	-2.38	Sulf1	-2.77					Mrap	2.31
Tnc	-2.44	Ctsc	-3.21					Serpinb1a	2.23
Sh2b2	-2.49							Il1b	2.22
Slc16a4	-2.71							Heph	2.20
								Pla2g2c	2.17
								Ret	2.16
								Tinagl1	2.11
								Mt2A	2.11
								Cyp4v3	2.10
								Tmem140	2.05
								Cd93	2.02
								Mcpt114	2.02
								Pdlim1	2.00
								Prom1	-2.14
								Slc38a5	-2.17
								Rep15	-2.51
								Olr1501	-2.53
								Slc16a4	-2.92
								Sult1c2a	-3.20

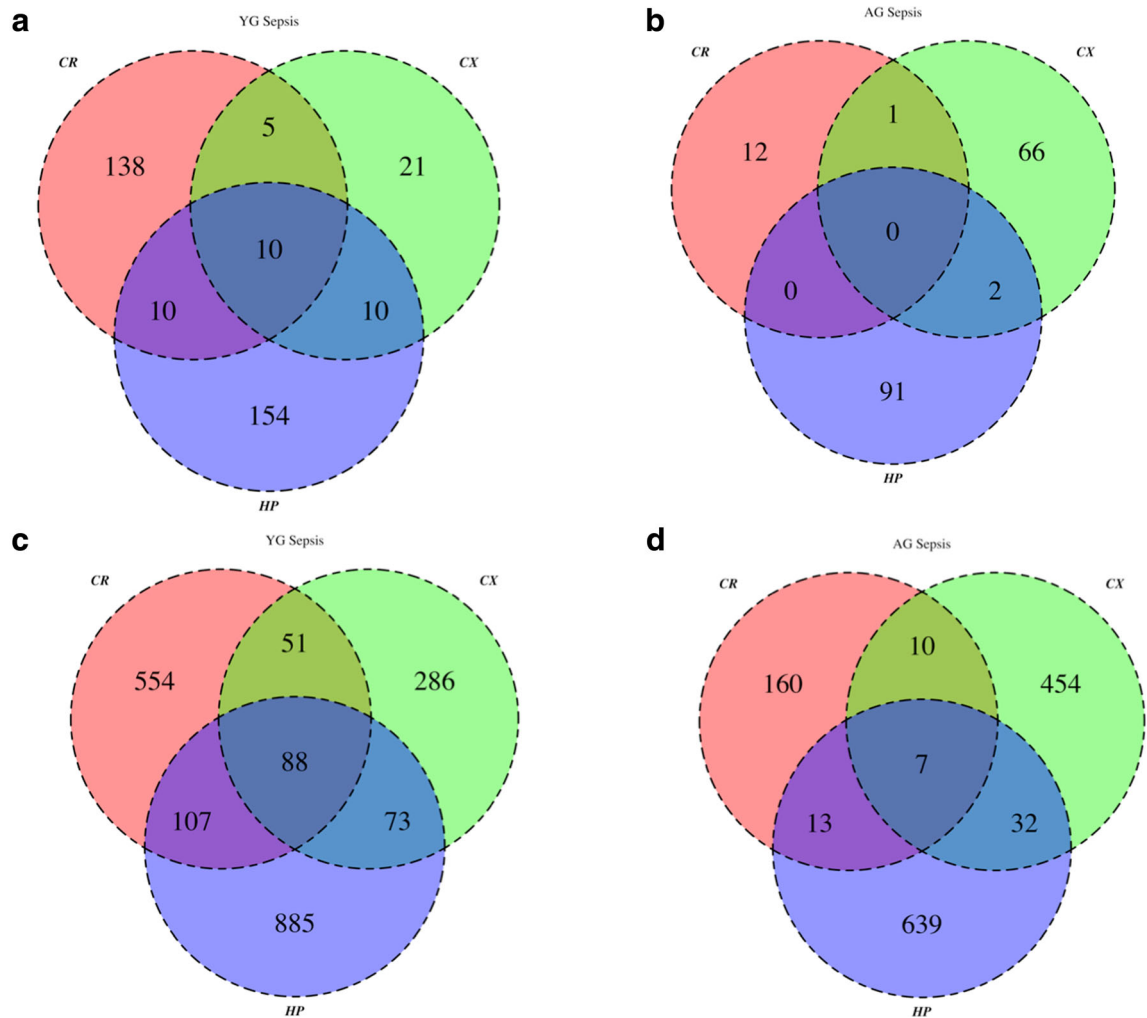


Fig. 2. Venn diagrams illustrating the differentially expressed genes in each evaluated brain region. (a) Comparison of differentially expressed genes between the three brain regions of young animals ($p \leq 0.01$ adjusted by the Bonferroni test); (b) comparison of differentially expressed genes between the three brain regions of aged animals ($p \leq 0.01$ adjusted by the Bonferroni test); (c) comparison of differentially expressed genes between the three brain regions of young animals ($p \leq 0.01$). YG: young sepsis vs young sham. AG: aged sepsis vs aged sham. CR: cerebellum. HP: hippocampus. CX: prefrontal cortex.

S100a9, Smad7, Upp1) (Fig. 2D), two of which in common with YG animals: S100a8 and S100a9. Interestingly, the transcriptional profile of genes involved in sensory perception of smell was thoroughly altered in the cortex of AG animals (41 genes from GO:0050911). This alteration could be an indication of early brain dysfunction, not observed in YG animals, associated with the systemic inflammatory state following sepsis.

Since the only overlap at the gene expression level when we considered all brain regions of AG sepsis vs AG sham and YG sepsis vs YG sham were differences for S100a8 and S100a9 expression, the protein levels of

S100a8 were measured in brain regions using the ELISA technique. The protein was detected in all brain regions, but despite the fact that S100a8 was the mostly differentially expressed gene in YG sepsis compared with YG sham, this result was not confirmed at the protein level. However, S100a8 protein levels were significantly higher in the prefrontal cortex of AG sepsis when compared with AG sham, YG sham, and YG sepsis (Fig. 3). S100A8 has been previously detected in plasma and evaluated as an inflammation marker [19]. We tried to detect S100a8 in plasma samples but the protein was not detectable.

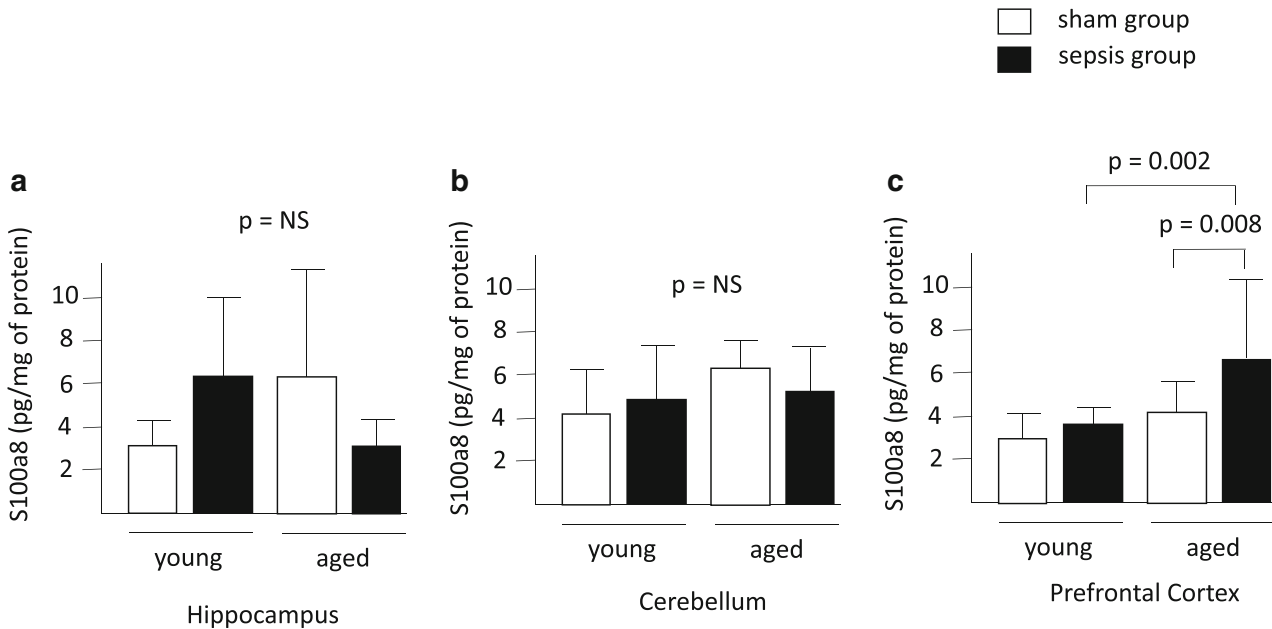


Fig. 3. Protein levels of S100a8 in different brain regions of healthy and septic rats of different age (young and aged). Statistical analysis was performed using the ANOVA followed by the Mann-Whitney test for multiple comparison test values. Results are presented as mean \pm standard deviation (SD). The significance level was established as $p < 0.05$.

DISCUSSION

Systemic inflammation and endothelial activation are characteristics that define sepsis. These factors can promote changes in the blood-brain barrier (BBB), inducing leukocytes infiltration in the CNS [20]. Cells and mediators that are part of the systemic inflammatory process, in contact with the nervous system, can alter the cellular metabolism, leading to oxidative stress and mitochondrial dysfunction, which cause further pathological abnormalities, ranging from neurotransmission to cell death by apoptosis [21]. At 12 h following CLP, we were unable to detect a characteristic gene expression pattern of acute inflammation in the CNS of aged and young rats. Among processes related with neuroinflammation, we looked for the activation of microglia, increased secretion of chemokines and cytokines, or altered expression that could suggest a compromised blood-brain barrier allowing the infiltration of peripheral immune cells. A limitation in our experimental design is that we did not include in the analysis a group of truly naïve animals as controls for the surgical stress. In our experience, however, the surgical procedures associated with CLP model induce limited inflammatory response and it would be unlikely that the observed gene expression alterations in the

brain occurred due to surgical stress rather than to bacterial infection and sepsis.

Unsupervised global gene expression analysis highlighted the similarity of gene expression patterns among samples collected from the same brain region, despite age or septic status. Clearly, 12 h following CLP was not sufficient to cause significant changes in brain cells function. In fact, response to infection, characterized by the activation of a few genes associated with response to LPS was detected only in YG animals. On the other hand, the altered regulation of a set of genes related with the perception of smell in AG animals, but not in YG ones, could be an indication of the earlier onset of brain function disruption in the aged animals. Although we did not verify behavioral parameters that could have confirmed SAE following CLP, this result illustrates the higher susceptibility of the aged brain to infection. Actually, the prevalence of SAE in the general population admitted to intensive care units is only 1–2%, while among aged individuals, this incidence increases considerably to rates around 70–87% [22], a fact that is currently not understood.

Three genes were consistently upregulated in all three brain regions of YG septic rats when compared with YG sham: S100a8, Upp1, and Mt2a. The important increase in expression of Upp1 in YG sepsis when

compared with YG sham is worth noting. Uridine phosphorylases (UPases) are key enzymes in the homeostatic regulation of intracellular uridine concentrations. Intracellular uridine, a pyrimidine nucleoside, under the action of UPases, provides a substrate for maintaining adequate ATP cellular levels [23]. It is known that an increase in UPase levels is important for the survival of nerve cells after ischemia performed in *in vitro* and *in vivo* models [24]. Since cerebral ischemia frequently occurs during sepsis [25], the results of our study suggest that the nerve damage due to the ischemic process in sepsis may better prevented in YG animals due to the increased levels of UPP1. Mt2a has been associated with astrocyte activation following CNS injury [26]. Metallothioneins such as Mt2a are expressed by reactive astrocytes and their interaction with injured neurons has been reported to lead to improved neuroregeneration. Although this possible function remains to be confirmed in our model, the fact that increased gene expression was found mostly in YG animals following systemic infection, suggests a prompt response of astrocytes in the younger brain, when compared with aged animals.

S100a8, also known as calgranulin A or myeloid-related protein (MRP) 8, is part of a family of proteins involved in enzymatic regulation and Ca^{2+} homeostasis, in addition to interacting with the cytoskeleton [27]. S100A8/A9 proteins are localized in the cytoplasm or nucleus of myeloid cells, and they significantly upregulated in response to cell damage or inflammation, often in association with infection; they may promote systemic inflammation through activation of TLR4 [28]. Studies have also suggested an important role of this protein in the defense against oxidative stress [29]. A recent publication put in evidence that in young mice submitted to the CLP model showed significant changes in the S100a8 gene expression levels in the brain of septic animals, when compared to the animals of the control group [12]. The study evaluated only young animals 14 days following CLP, our findings indicate that this protein may be important in even earlier molecular events following sepsis since we detected not only differential gene expression levels but also the protein being expressed in every brain region tested.

S100a8/a9 were the only two genes that showed consistent deregulation between sepsis and sham when all brain regions were taken together and for both aged and young animals. S100a8 protein was detected in all brain regions tested, but a significant change was seen only between prefrontal cortex of AG sepsis

compared with the other AG sham, YG sham, and YG sepsis. This result is not consistent with the gene expression results and can be due distinct dynamics of RNA and protein levels in the brain tissue. Noteworthy, contrary to other reports in the literature [30], the S100a8 protein concentrations that we found were near the limit of detection for most ELISA assays. S100a8 detection is dependent not only on the degree of calcium chelation in the lysis buffer but also on whether or not the ELISA antibodies bind to sites that are exposed both in monomeric and heterodimeric forms of the protein. Therefore, we cannot guarantee that the detection of the protein was not impaired by the conditions of the assay.

Considering the important role of this protein in inflammation, this result for the aged could have biological implications. The role of these molecules in sepsis has been recently reviewed and it is currently known that patients with sepsis display elevated circulating levels of S100A8/9.

CONCLUSION

This work shows effects of early stages of sepsis to the CNS of aged and young rats. A limited amount of genes was differentially expressed following 12 h of CLP both in the aged and young brain. Differences between the aged and the young brain suggest early onset of brain function disruption in aged animals and a more specific response to infection in the young brain. A deeper comprehension of the early effects of sepsis in the brain will help to identify early biomarkers and implement drug regimens tailored to specific patient populations, such as the aged.

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COMPLIANCE WITH ETHICAL STANDARDS

Conflict of Interest. The authors have no financial or ethical conflicts of interest.

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