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Effects of Stress on Hippocampal MMP2 and MMP9 Activity Following Brucella Melitensis Exposure in Rats

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ABSTRACT

Introduction: Matrix Metalloproteinases (MMPs) are inflammatory mediators involved in bacterial infection and other pathological conditions. Inflammation can damage all parts of the brain, particularly sensitive areas such as the hippocampus. Chronic stress can make the brain more susceptible to infection and inflammation. This study aimed to investigate the effects of stress on the activity of MMP2 and MMP9 in the hippocampus of male Wistar rats following the administration of Brucella Melitensis (BM) vaccine.

Methods: The non-stressed group received a Brucella Melitensis vaccine strain via intracebroventicular (i.c.v) and intraperitoneal (i.p) routes. The animals were subjected to heterogeneous sequential stress for nine days and/or received the same volume of Brucella Melitensis vaccine (BMV). The activity of MMP-2 and MMP-9 was measured by Gelatin Zymography.

Results: The results showed that stress increased the activity of MMP9 in both the control group and the BMV, i.p., injected animals. However, stress did not affect the activity of MMP2 in either the control or the BM, i.p., inoculated conditions. Stress also increased the activity of MMP9 following i.c.v. injection of BM, without a concomitant change in the activity of MMP2 in the hippocampus.

Conclusion: The study suggests that vaccination in stressed conditions could activate MMPs, which are essential players in inflammatory processes, in brain of immunized animals. Since the Brucella melitensis vaccine is used for the prophylaxis of brucellosis in small ruminants, these findings have important implications for understanding the effects of stress on the immune response to vaccination and inflammation in the brain.

Keywords: Matrix Metalloproteinases; Brucella melitensis, Inflammation; Hippocampus.

INTRODUCTION

Matrix metalloproteinases (MMPs), a group of Zn-dependent endopeptidases (1, 2) function to remodel the components of extracellular matrix (ECM) including collagens, fibronectin, laminin, and basement membrane proteoglycans (3-5). The emerging role of these proteases has been reported in diverse physiological conditions such as organogenesis, morphogenesis, and pathological states including inflammation and metastasis (6, 7). The functionality of MMP molecules is not limited just to ECM, but they are, also, major regulators of various signaling pathways, resulting from a rich molecular diversity (2). Their encoding genes are located on different chromosomes, and post-translational modifications lead to the production of a variety of enzymes (1). There are more than 25

identified MMPs and based on substrate specificity and also cellular localization of the enzymes, they are divided into four main groups (8), comprising collagenases, gelatinases, stromelysins, and the membrane-type MMPs (MT-MMPs) (8). Gelatinase subfamily of MMPs, proteolytically, cleave denatured collagen (gelatin) type IV and native type IV collagen which are the key components of the basement membrane (9).

MMP2 and MMP9 belong to the gelatinase sub-group of MMPs and, in addition to ECM degradation, they act on cytokines and chemokines (10). For example, MMP9 acts as an activator of IL-8, indicating its role in immune response and inflammation (11). The involvement of MMPs in various cellular processes necessitates a tight control of the enzymes. Controlling mechanisms are applied at the gene expression level, post-transcriptional regulation, cellular and extracellular localization, pro-enzyme activation, and inhibition by specific inhibitors. It has been reported that dysregulation of MMPs is involved in various pathological states such as heart failure (12), pulmonary fibrosis (13), vascular dementia (14), neurodegenerative diseases (3, 15) and depressive disorders (16).

In the past, a classic view was stating that the central nervous system (CNS) is protected against inflammation by the blood-brain barrier (BBB) even in the absence of the lymphatic system (17). However, it has been indicated that various pathological and physiological events lead to arising of inflammation in CNS, majorly by activation of macrophages–microglia and production of immune factors such as cytokines (18). CNS inflammation is generally initiated by various factors such as toxic metabolites, psychological stresses, autoimmunity, aging, and infection (17). The inflammation is important because it exacerbates or initiates other illnesses such as brain injury (19), epilepsy (20), multiple sclerosis, amyotrophic lateral sclerosis (ALS), neurodegenerative diseases (21), and also depression and anxiety (22).

The precise mechanisms of induction of inflammation in the CNS are well studied. For example, during bacterial infection, bacterial components, including DNA, cell wall, or bacterial toxins are recognized by activated microglia cells through the toll-like receptors (TLR), leading to the release of plenty amounts of cytokines and chemokines into the cerebrospinal fluid (CSF) (23, 24). Activation of nuclear factor kappa B (NF- κB), as the indispensable controller of inflammation, in the activated astroglia/microglia cells, flares (25) the activation of MMPs leading to digestion of essential components of BBB and promotes its breakdown, enable extravasation of immune system cells, and thereby contribute to intensifying the inflammation (26).

On the other hand, accumulated data show that stress conditions predispose CNS inflammation (27-29). Stress coping factors are secreted due to unbearable or persistent mental, physical, or emotional pressures (30). Although these hormones are mainly known as anti-inflammatory factors, they will act as inflammation-inducing agents in prolonged exposure to stress (31). The induction of inflammation in the CNS leads to vast biochemical and structural changes in all parts of the system. The hippocampus, as a part of the limbic system, is one of the most sensitive parts of the brain in inflammatory conditions and is known as one of the main centers of glucocorticoid receptors, responsive to stresses (32). Previous studies have indicated that chronic stress has negative effects on neurogenesis and leads to hippocampal atrophy and depression-like behavior (33). Also, it has been reported that cytokines, IL-1 for instance, interfere with hippocampal long-term potentiation (LTP), suggesting the effects of inflammation on learning and memory (34). Accordingly, MMPs production and activity in the hippocampus are influenced by pathological conditions such as epilepsy (35, 36) and hypoxia (37).

Therefore, this study is going to explore the effect of stress on the activity of MMP2 and 9 in the hippocampus of Brucella melitensis (BM) infected brain of male rats.

MATERIALS AND METHODS

Animals

In the current study, adult male Wistar rats weighing approximately 180-220 g were

purchased from Razi Institute, Karaj, Iran. Animals were pair-housed with free access to food and water under a light–dark cycle (12:12 h), the humidity of 40-50%, and temperature (22-25 °C) -controlled conditions. All Experiments were conducted in accordance with the approved guidelines on the care and use of laboratory animals at Damghan University, ethical code IR.DU.REC.1403.009.

Stress model and groups

The chronic heterogeneous sequential stress (HSS) model was created using Espinosa-Oliva et al. introduced model (38). The benefits of this model are the unpredictability of the model. Animals were divided into stressed and non-stressed groups. Non-stressed animals were kept undisturbed in their home cages during the ten days of treatment. The stressed group received 10 µl BM vaccine (BMV; equal to 1×10^6 bacteria; purchased from Razi Institute, Karaj, IRAN) by either Intraperitoneal (i.p) or i.c.v injection, while the non-stressed groups received only saline. This type of bacteria was selected as it is routinely used for vaccination and the results may highlight the vaccination conditions and also the non-dangerous nature of the bacteria for the experimenter and the animal.

A 9-day HSS paradigm was used for animals in the stressed groups. Stressor type and duration are listed in table 1.

Stereotaxic surgery and injection

Animals were anesthetized with ketamine 10% (100 mg/kg, ip) and Xylazine 2% (10 mg/kg, ip). Anesthetized rats were placed in a stereotaxic frame (model 940; David Kopf) to follow image 66 of the brain atlas of Paxinos and Watson (1). Saline or bacteria in 10 µl volumes were i.c.v. injected using the following coordinates: ±3.2 mm medial/lateral, −2.7 mm anterior/posterior, −2.7 mm dorsal/ventral from the bregma. The injections were carried out with a speed of 0.5 µl/min for four min by a 5-µL Hamilton syringe. Injections of either saline or bacteria in the stress group were performed 24 hours after the last stress paradigm and the right hippocampus was extracted another 24 hours following injection.

Table 1. Schedule of stressing agents used during the chronic stress treatment.

Day of treatment	Agent used	Duration
1	Forced swimming	10 min
2	Restraint	3 h
3	Water deprivation	24 h
4	Restrain at 4 °C	1.5 h
5	Isolation	24 h
6	Food deprivation	24 h
7	Water deprivation	24 h
8	Restrain at 4 °C	2 h
9	Food deprivation	24 h

Tissue homogenization

Anesthetized rats were perfused with ice-cold PBS into the heart by inserting a 25-gauge needle into the left ventricle until the blood became clear. Then, the brain was quickly removed, and the right hippocampus was used for protein extraction and zymographic measurement of gelatinase activity.

Szklarczy et al. introduced protocol was used to extract MMPs from hippocampus tissue with some modifications. Briefly, tissue samples were weighed and then mechanically homogenized by adding 20 µl of 0.25% Triton X-100 buffer (39). The homogenates (750 µl) were centrifuged at 6000 × g for 30 min. Proteins from the supernatant were precipitated in 60% ethanol for 1 min at 4°C and then centrifuged at 15,000 × g for 5 min. The precipitate was solubilized in 200 µl of sample buffer containing 2% SDS for 15 min at 37°C.

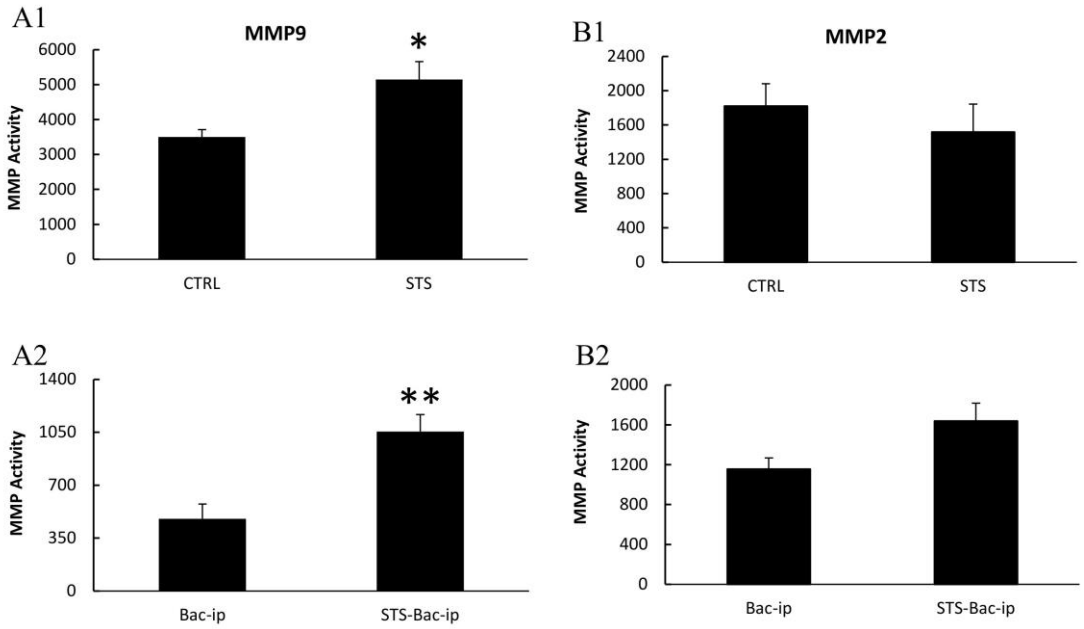

Figure 1. The density of MMP-9 and MMP-2 bands in gelatin zymography of the hippocampus homogenate after IP injection of bacteria. A significant increase was observed in the activity of MMP-9 due to stress in either control, saline-injected animals (A1), or BMV-injected group (A2). MMP2 did not change following the stress. (*P<0.05 and **P<0.01, n=6). CTRL; Control, STS; Stress, Bac.; Bacteria, i.p.; Intraperitoneal

The pellet fraction was resuspended in a buffer containing 50 mM Tris, pH, 7.4, and 0.1 mM CaCl2 in water, heated for 15 min at 60°C, and then centrifuged at 10,000 × g for 30 min at 4°C. This treatment results in releasing ECM-bound MMPs into the solution. Finally, samples were equalized by protein concentration measurements using the Lowry assay (40).

Zymographic measurement of gelatinase activity:

The proteolytic activity of MMP-2 and MMP-9 was measured by gelatin substrate zymography according to previous descriptions by Zhang et al. (41). Briefly, six ng of each hippocampal lysate were incubated and subjected to electrophoresis, in SDS-PAGE 10.5% gels containing 0.1% gelatin under, nonreducing conditions. Gels were washed twice for 30 min in 2.5% Triton X-100 to remove SDS and incubated overnight in 50 mM Tris, pH 7.5, 10 mM CaCl2, 1 µM ZnCl2, 1% Triton X-100 and 0.2% Brij35 at 37°C. Gels were then stained with 0.1% Coomassie blue G-250 for 3 hours and de-stained with a solution containing 5% acetic acid and 25% methanol until clear bands of gelatinolytic appeared on a dark background. Zymograms were imaged by UVI doc, and the density of bands was measured using Image-j software.

Statistical analysis:

Statistical analysis was performed using IBM SPSS Statistics version 23. To compare two independent groups, Student's t-test was applied. Results are presented as Mean ± Standard Error of the Mean (SEM). Statistical significance was set at $P < 0.05$.

RESULTS

Stress enhances the i.p. BMV-induced changes in the activity of the MMP

The i.p. injection of the bacteria mimics the natural condition of encountering bacteria as a vaccine. The statistical analysis of the effect of BMV inoculation and stress application revealed significant differences. Independent T-test analysis showed an increase in the band intensity of MMP9 due to stress (STS; Independent T-test, P<0.05, n=6) and BMV in the stressed condition (STS- Bac-i.p; Independent T-test, P<0.01, n=6). Neither the stress, nor the same amount of BMV injection (10 µl; 1×10^6 bacteria) in stress

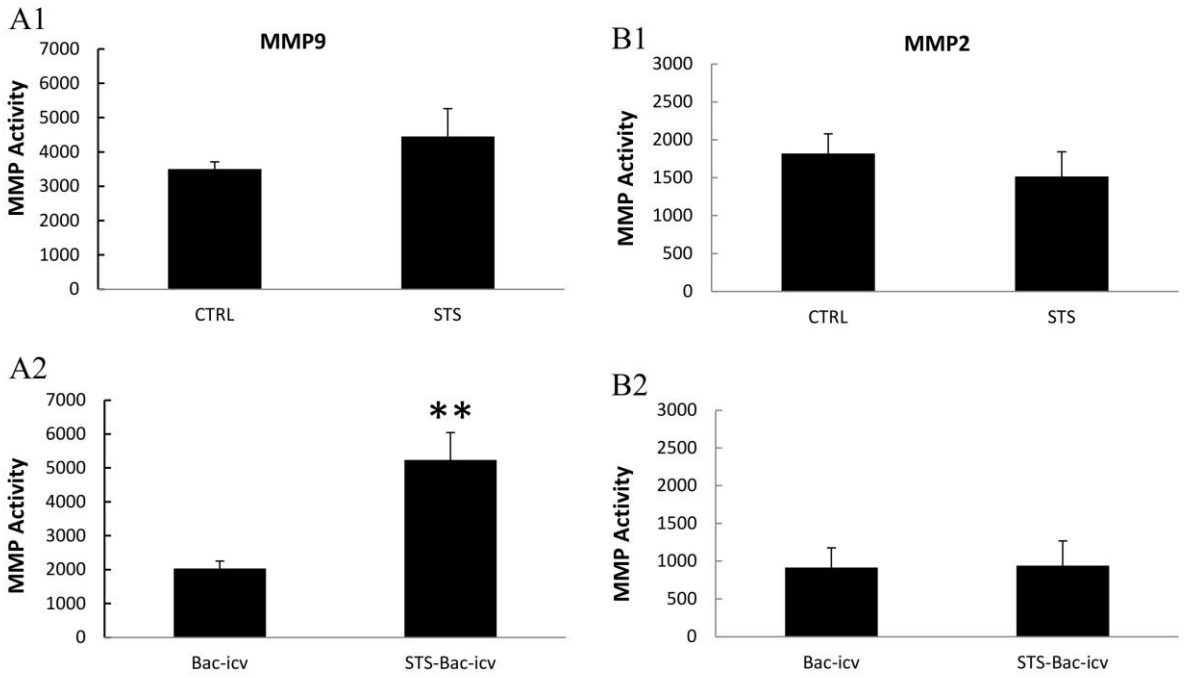

Figure 2. The density of MMP-9 and MMP-2 bands in gelatin zymography of the hippocampus homogenate after i.c.v injection of bacteria. A significant increase was demonstrated in the activity of MMP-9 due to stress in the BMV-injected group (A2). MMP9 activity was not altered in the control condition, while MMP2 activity did not change following either control or stress. (**P<0.01, n=6). CTRL; Control, STS; Stress, Bac.; Bacteria, i.c.v.; Intracerebroventricular

conditions demonstrated any difference of MMP2 concerning their control (p>0.05). The results highlight the importance of stress condition in the infection outcome (Figure 1).

Stress augments the i.c.v BMV-induced changes in the activity of the MMP

Stress may facilitate the entrance of bacteria into the brain; crossing the blood-brain barrier (BBB). Therefore, this experiment was designed to study the influence of i.c.v perfused bacteria on MMPs in stressed and naive conditions. Comparing the stressed and control animals, showed no significant difference, while the band intensity for MMP9 developed a higher significant intensity following BMV perfusion (STS- Bac-i.c.v; Independent T-test, P<0.01, n=6). In contrast, MMP2 did not change significantly, either in stressed or the BMV i.c.v injection in stressed conditions (p>0.05). The data confirms that stress may be a negative intervention in the bacterial complications in the brain (Figure 2).

DISCUSSION

This study investigated the influence of stress conditions on MMP activity following bacterial inoculation. The results demonstrated that stress enhanced the brain MMP9 activity following either saline or bacterial i.p. injection. Furthermore, stress intensified the MMP9 activity in bacterial i.c.v infusions.

Stress, as a pathogenic factor, induces the development of inflammation, which may lead to stress-induced vulnerability (42, 43). The proteolytic MMP enzymes also play a variety of functions in the development of inflammation, which in turn may be destined to pathologic conditions (7, 36, 44, 45). Our data showed an increase in MMP9 activity due to stress, which was not replicated in MMP2 activity. Although the bacterial injection, in this study, revealed a very light MMP activity, stress increased the activity to a higher level. This effect of stress in the condition of healthy BBB and i.p. injection, once more, discuss the likely breakdown of the BBB in stressful conditions (44, 46).

Neuroinflammation-induced release of enzymes and oxidative agents perpetuate the brain and vessel injury by activating the MMPs, they try to remodel the vessels without success and even worsen the situation at the level of BBB (47). Our results showed no difference in MMP9 activity of control animals experiencing stress, which indicates the likely effect of direct BMV, i.c.v, import into the brain tissue and BBB breakdown through surgery. On the other hand, stress augmented the brain MMP9 activity due to i.c.v bacterial injection in the stressed conditions. It is known that lipopolysaccharides increase the expression of MMP9 in cell lines (48) and brain tissue microvascular system (49). It is worth to noting that the bacteria used in this study were a vaccine form of weakened Brucella Milletensis without the behavior of live and active bacteria, which can be considered to function like LPS in the development of inflammation. Nevertheless, the BMV was differently functioning in the naïve and stressed condition.

The data demonstrated the importance of conditions in which the animal receives a vaccine or exposure to an infection and indicates that stress will lead to the dominance of bacterial influence on the fate of inflammation.

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ABBREVIATIONS

CTRL; Control, CSF; Cerebro-spinal Fluid, CRT; Corticosterone, HPA; Hypothalamic Pituitary Axis, Central Nervous System; CNS, CRH; Corticotropin-Releasing Hormone, PVN; Paraventricular Nucleus, ICV; IntraCerebroVentricular, GR; Glucocorticoid Receptor.

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