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BRAIN RESEARCH

Research Report

Peripheral thermal injury causes blood–brain barrier dysfunction and matrix metalloproteinase (MMP) expression in rat

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ABSTRACT

Mortality after serious systemic thermal injury may be linked to significant increases in cerebral vascular permeability and edema due to blood–brain barrier (BBB) breakdown. This BBB disruption is thought to be mediated by a family of proteolytic enzymes known as matrix metalloproteinases (MMPs). The gelatinases, MMP-2 and MMP-9, digest the endothelial basal lamina of the BBB, which is essential for maintaining BBB integrity. The current study investigated whether disruption of microvascular integrity in a rat thermal injury model is associated with gelatinase expression and activity. Seventy-two adult Sprague-Dawley rats were anesthetized and submerged horizontally, in the supine position, in 100 °C (37 °C for controls) water for 6 s producing a third-degree burn affecting 60–70% of the total body surface area. Brain edema was detected by calculating water content. Real time PCR, Western blot, and zymography were used to quantify MMP mRNA, protein, and enzyme activity levels. Each group was quantified at 3, 7, 24, and 72 h post thermal injury. Brain water content was significantly increased 7 through 72 h after burn. Expression of brain MMP-9 mRNA was significantly increased as early as 3 h after thermal injury compared to controls, remained at 7 h ($p<0.01$), and returned to control levels by 24 h. MMP-9 protein levels and enzyme activity began to increase at 7 h and reached significant levels between 7 and 24 h after thermal injury. While MMP-9 protein levels continued to increase significantly through 72 h, enzyme activity returned to control level. The increase in MMP-9 expression and activity, associated with increased BBB permeability following thermal injury, indicates that MMP-9 may contribute to observed cerebral edema in peripheral thermal injury.

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1. Introduction

Thermal injury is characterized by increased microvascular permeability, which causes massive fluid volume requirements during resuscitation. Thermal injury to peripheral tissue often causes systemic reactions, such as fever, hyperalgesia, anorexia, and increased permeability of the blood–brain barrier (BBB); and it remains one of the leading causes of childhood death in the United States (Barone et al., 1997a,b, 2000). About 1 million children are injured and 3000 children die each year as a result of burn trauma, according to the Children's Burn Awareness Program, Chicago. Generalized encephalopathy is the most common neurological complication of thermal injury in children, occurring with a 14% incidence. Past research has shown that cerebral complications associated with burn victims are highly correlated with mortality (Asahi et al., 2000, 2001). In addition, there is reason to believe that the high incidence of mortality is linked to a significant increase in cerebral permeability following serious systemic thermal injury, which ultimately leads to cerebral edema (Barone et al., 1997a,b, 2000). The specific mechanisms underlying this increase in cerebral permeability after thermal injury have yet to be illuminated. Thus, research on the effect of peripheral thermal injury on cerebrovascular integrity remains essential.

BBB exists between the systemic circulatory system and the cerebral parenchyma to regulate which substances can enter the brain tissue. The extracellular matrix (ECM) of the BBB forms a basal lamina which surrounds the endothelial cells and provides a physical barrier to obstruct diffusion of most molecules (Mun-Bryce and Rosenberg, 1998; Rosenberg, 2002; Tayebjee et al., 2005). However, when highly toxic proteases, such as metalloproteinases (MMPs), become upregulated due to traumatic conditions, maintenance of the ECM becomes overwhelmed by the degradation of the basal lamina. When this barrier is compromised, previously blocked vascular exudates leak into the surrounding tissues causing detrimental swelling in the cranial cavity, often leading to hemorrhage and death (Hamann et al, 1995).

A variety of traumatic stimuli such as physical stretch, arterial pressure, inflammation, ischemia and the local effects of growth factors and cytokines can affect the ECM via activation of matrix MMPs (Lukes et al., 1999). Previous research has demonstrated a correlation between increased MMPs after cerebral ischemic stroke and BBB breakdown leading to cerebral edema (Cunningham et al., 2005). MMPs are divided into five classes including gelatinases (MMP-2 and -9), collagenases (MMP-1, -8 and -13), stromelysins (MMP-3, -10 and -11), membrane-type MMPs (MMP-14 to -17) and others (MMP-7 and -12) (Lo et al., 2003). Increasing evidence has indicated that MMP-2 and -9 are up-regulated after the onset of stroke or other brain damage (Romanic et al., 1998; Rosenberg et al., 1998; Wagner et al., 2003; Pfefferkorn and Rosenberg, 2003; Lee et al., 2005; Wang et al., 2000). Other research has also shown significant elevation of MMP-2 and MMP-9 mRNA during peripheral burn wound healing (Ulrich et al., 2002). However, the effects of MMPs on cerebral integrity after thermal injury remain unknown. In the present study, we address whether BBB dysfunction caused by peripheral thermal injury is associated with MMP overexpression and activity.

2. Results

Brain edema was assessed by the percentage change of water content in the brain after thermal injury at 7, 24, and 72 h (Fig. 1). A one-way ANOVA revealed a significant difference [$F_{(3,25)}=6.72$, $p<0.01$] in the percentage of brain water content between thermally injured and control rats. Duncan's New Multiple Range Test further indicated that the injured rats had higher percentage of water content in the brain compared to the control rats. No difference was detected in brain water content at the three different time points.

Real-time PCR studies demonstrated significant differences in relative mRNA levels of the brain MMP-9 [$F_{(3,20)}=6.35$, $p<0.01$], and MMP-2 [$F_{(3,20)}=4.23$, $p<0.05$] (Fig. 2). A significant increase in MMP-9 mRNA expressions was observed as early as 3 h after thermal injury as indicated by Duncan's New Multiple Range Test. This increase in MMP-9 mRNA level remained at a significant level after 7 h but dropped to control level after 24 h. The same tendency was observed in relative mRNA levels of the brain MMP-2, but the overall increase of MMP-9 mRNA was significantly greater than the increase of MMP-2 mRNA ($p<0.01$).

MMP protein levels in control and thermally injured rats were measured using Western blot. ANOVA analysis revealed significant increase [$F_{(3,17)}=37.19$, $p<0.01$] in brain MMP-9 in the thermally injured groups versus the control group, and an almost significant difference [$F_{(3,12)}=5.34$, $p<0.06$] in MMP-2 protein levels (Fig. 3). MMP-9 protein began to increase at 7 h, reached significantly increased levels between 7 and 24 h, and continued to increase at 72 h after thermal injury (Duncan's

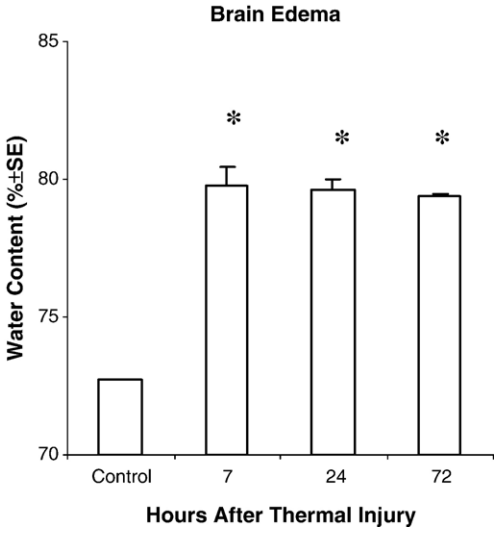

Fig. 1 – Edema quantification in thermally injured rats at 7, 24, and 72 h post thermal injury. Percentage of brain water content was significantly (*$p<0.05$) higher in thermal injury groups at 7 and 24 h following burn, as compared to the control group. Values shown as mean%±SE.

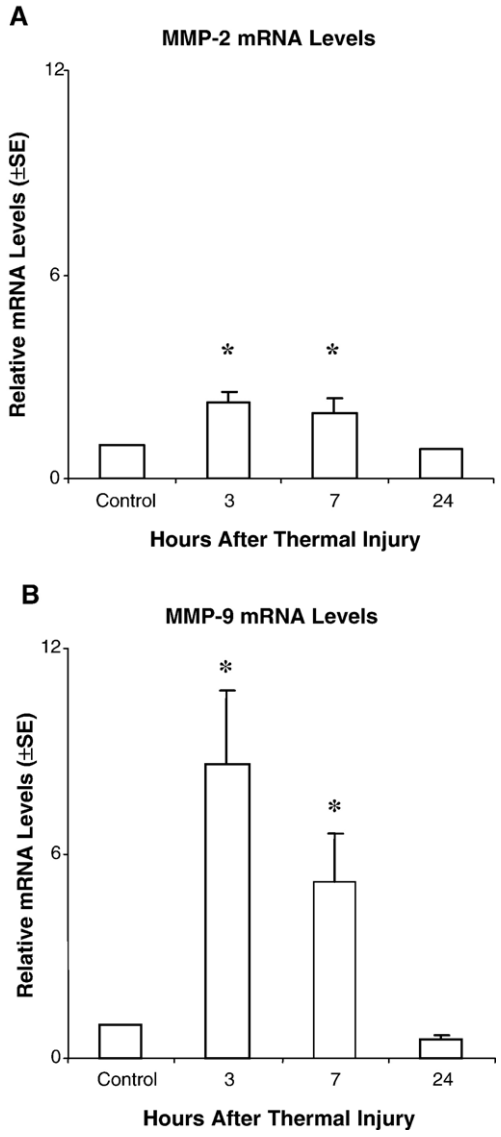

Fig. 2 – Relative mRNA levels of MMP-2 (A) and MMP-9 (B) in thermally injured rats and control rats at 3, 7, and 24 h post thermal injury. (A) Real-time PCR revealed a significant increase (*$p<0.05$) in relative mRNA levels of brain MMP-2 at 3 and 7 h following injury. (B) PCR also demonstrated a significant increase in relative MMP-9 (*$p<0.01$) as early as 3 h after thermal injury, remained significant at 7 h, and returned to control levels after 24 h. The mRNA levels of MMP-9 were significantly ($p<0.01$) higher than those of MMP-2 at both 3 and 7 h after injury.

New Multiple Range Test). Protein expression of MMP-2 revealed a barely significant increase only at 24 h after thermal injury. Overall increase of MMP-9 protein was much greater than MMP-2 following thermal injury ($p<0.01$).

ANOVA analysis of MMP-2 and MMP-9 enzyme activity, quantified by zymography, revealed significant increases in the brain MMP-9 activity [$F_{(3,18)}=9.24$, $p<0.01$], but not in MMP-2 activity in thermally injured rats, as compared to controls (Fig. 4). Duncan's New Multiple Range Test further indicated that the significant increase in the MMP-9 enzyme activity was observed at 7 h and 24 h after thermal injury, without significant difference between the two time points. The MMP-9 activity returned to control level at 72 h after thermal injury. MMP-2 activity was not as increased as MMP-9 activity, with only a slight difference observed at 7 h after thermal injury.

Serum levels for both MMP-2 and MMP-9, measured at 7 h post injury with ELISA, were not increased compared to

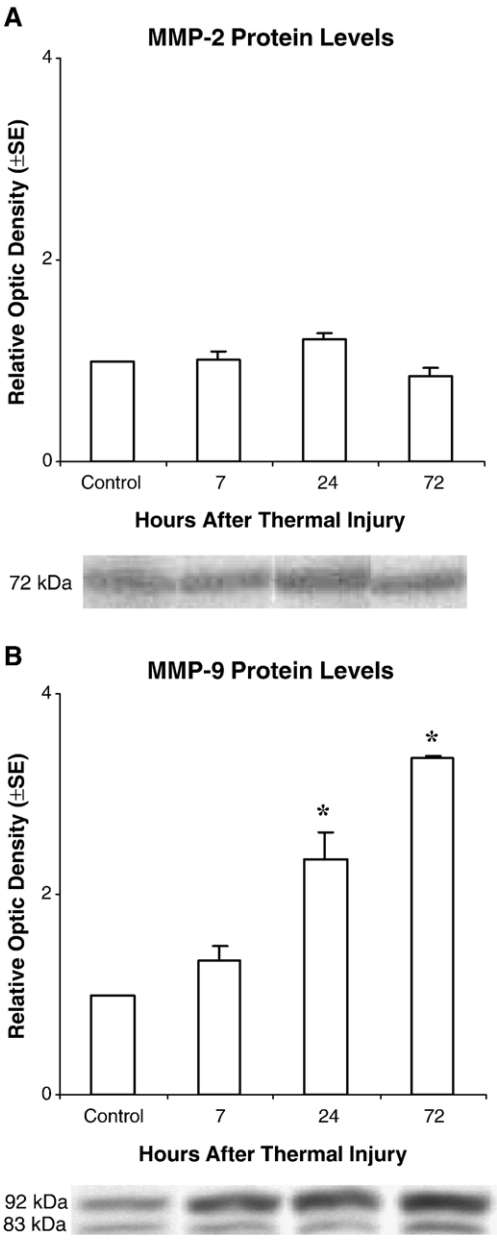

Fig. 3 – Relative MMP-2 (A) and MMP-9 (B) protein levels in thermally injured and control rats as quantified with Western blot. Quantitative Western blot analysis showed protein levels in control and thermal injury groups at 7, 24, and 72 h. Representative immunoblots are presented. Optical density demonstrated a significant increase in MMP-9 (92–83 kDa) at 24 and 72 h post thermal injury compared to controls (*$p<0.01$).

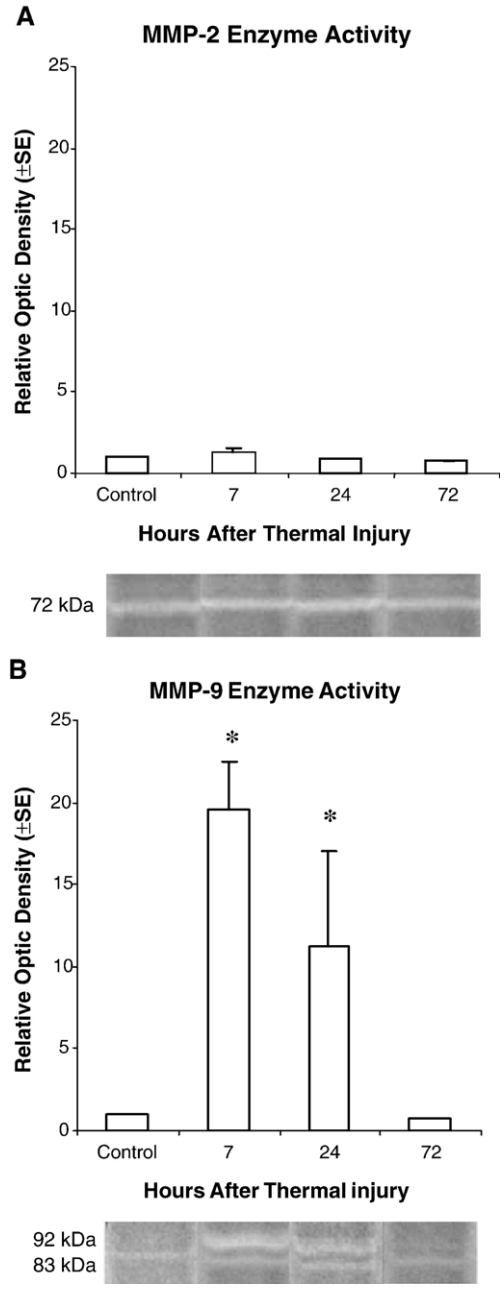

Fig. 4 – Relative MMP-2 (A) and MMP-9 (B) enzyme activity of thermally injured rats at 7, 24, and 72 h as compared to controls. Zymography gelatin of MMP-2 (72 kDa) and MMP-9 (92 kDa, 83 kDa) for control and thermally injured rats revealed MMP-2 expression was slightly higher ($^*p<0.05$) than control at 7 h after thermal injury. MMP-9 was expressed in significantly raised quantities ($^*p<0.05$) at 7 and 24 h after injury, while activity returned to control levels by 72 h. MMP-9 activity level is significantly superior to MMP-2 activity. Representative zymograms are shown.

controls ($p>0.05$) (Fig. 5). However, zymography on serum MMP enzyme activity at 3, 7, 12, and 24 h post thermal injury revealed that MMP-9 activity remained low at 3 h, increased at 7 h ($p<0.01$), but quickly returned to control levels between 7 and 12 h and remained low through 24 h. (ANOVA: main effect on time [$F_{(4,10)}=26.97$, $p<0.01$]). ANOVA analysis and a Duncan's New Multiple Range Test of serum MMP-2 enzyme activity indicated that serum MMP-2 activity was slightly increased at 7 h following thermal injury compared to measurements at 3, 12, and 24 h (ANOVA: main effect on time $F_{(4,10)}=5.12$, $p<0.05$).

3. Discussion

MMPs are directly involved in tissue remodeling during development and homeostasis, but are also produced by endothelial cells, microglia and astrocytes in response to pathological conditions such as atherosclerosis, arthritis, cancer, and neurodegeneration (Cunningham et al., 2005). MMPs degrade proteins and polysaccharides that compose the

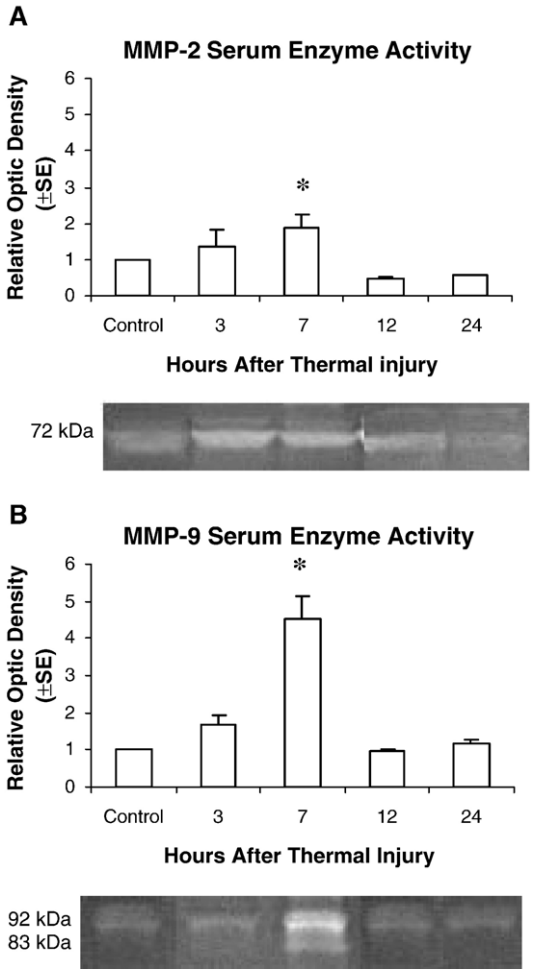

Fig. 5 – Relative MMP-2 (A) and MMP-9 (B) serum enzyme activity of thermally injured rats at 3, 7, 12, and 24 h as compared to controls. Zymography gelatin of serum MMP-2 (72 kDa) and MMP-9 (92 kDa, 83 kDa) for control and thermally injured rats revealed that expression of MMP-2 was slightly higher ($^*p<0.05$) than controls at 7 h. Serum MMP-9 expression was significantly increased ($^*p<0.05$) for a short time 7 h after thermal injury and returned to control levels between 7 and 12 h. Relative zymograms are shown.

neurovascular matrix, including basement membrane components (i.e. type IV collagen, heparin sulphate proteoglycan, laminin and fibronectin). Recently, studies have emphasized the role of MMPs in regulating the integrity of the BBB following injury (Romanic et al., 1998; Lo et al., 2003). Our previous study showed, using Evans blue analysis, that BBB function is significantly diminished by 7 h following thermal injury (Berger et al., in press). The present study confirms the results of previous work and further demonstrates a temporal association between BBB breakdown and cerebral expression of MMP at the level of transcription (mRNA), translation (protein–Western blot), and enzyme activity (zymography) in thermally injured rats.

In the central nervous system, the early appearance of MMP-2 or -9 is associated with an alteration of BBB permeability through destruction of the endothelial basal lamina of the BBB which often results in vasogenic edema (Gasche et al., 1999; Fujimura et al., 1999; Rosenberg et al., 1998; Heo et al., 1999; Yong et al., 2001; Lo et al., 2002). In addition, pharmacologic inhibition of MMPs can ameliorate brain-injury-associated edema (Rosenberg et al., 1998; Romanic et al., 1998). It was reported that MMP-9-deficient knockout mice had reduced BBB disruption and edema after transient focal cerebral ischemia (Asahi et al., 2000, 2001) and traumatic brain injury (Wang et al., 2000). The present study demonstrates for the first time that BBB dysfunction, as indicated by the severe edema as early as 7 h after thermal injury, is also associated with cerebral temporal overexpression of MMPs, especially MMP-9.

In addition, while brain MMP-9 mRNA is greatly increased by 3 h post thermal injury followed by rising MMP-9 protein levels and enzyme activity through 72 h, serum MMP-9 protein and enzyme activity levels remain low with only a short increase in activity at 7 h. The finding that serum protein levels and enzyme activity of MMP-2 and MMP-9 were not increased compared to controls suggests that early cerebral expression of MMP mRNA up to 7 h after injury has not led to systemic protein expression of MMPs. These results further suggest that other traumatic mediators, such as cytokines, may act as triggers to induce cerebral MMP expressions and associated BBB dysfunction. Interestingly, our preliminary study has demonstrated a significant increase of tumor necrosis factor-alpha (TNF-α) and interleukin-1 beta (IL-1β) as well as intercellular adhesion molecule-1 (ICAM-1) in brain after peripheral thermal injury (Reyes et al., 2006).

An association between increased expression of MMPs and neuroinflammation has been shown (Leib et al., 2001). MMP expression can result in tissue injury and inflammation (Lukashev and Werb, 1998), facilitating leukocyte infiltration (Romanic et al., 1998). In addition, *in vitro* activation of brain microvascular endothelium with proinflammatory cytokines, such as TNF-α and IL-1β, has been shown to result in a selective up-regulation of MMP-9 expression (Harkness et al., 2000). Also, the addition of dexamethasone, a chemotherapy drug, partially inhibited the cytokine-induced up-regulation of MMP-9.

We also found that while MMP-9 protein levels remain increased at 72 h following thermal injury, MMP-9 enzymatic activity has returned to control levels at this time. Although the pathway of activation and inhibition of MMPs is complex, the inverse relationship between MMP-9 protein and activity levels could be explained by the presence of endogenous MMP inhibitors known as tissue inhibitors of metalloproteinases (TIMPs) (Cunningham et al., 2005; Dzwonek et al., 2004). TIMPs inhibit MMP activity through high-affinity, non-covalent binding. Therefore, as MMP-9 levels and activity rise following thermal injury, it is possible that TIMPs bind to MMP-9 and inhibit its activity.

Our studies demonstrate a temporal correlation between disruption of BBB integrity and MMP expression. Moreover, our data indicate that MMP-9 (vs. MMP-2) dominates in the response to thermal injury, suggesting that this gelatinase plays a key role in the skin-burn-induced BBB disruption. Previous research examining levels of MMP-2 and MMP-9 in stroke induced rats found an increase in MMP-2 between 1 and 3 h after ischemia and a more marked increase in MMP-2 at 5 to 21 days following ischemic injury (Lukes et al., 1999). They suggest that early rise of MMP-2 may be involved in MMP-9 activation, while later, higher levels may be involved in the repair processes of angiogenesis and scar formation. These findings may explain our results that show only a very small increase in MMP-2 between 3 and 72 h following injury.

Further studies examining the effect of an MMP inhibitor on edema and BBB damage following thermal injury may directly link MMP-9 with degradation of the BBB. Studies including Evans blue analysis at later time points may more specifically indicate the temporal destruction of the BBB related to the temporal activity of MMP-9. Understanding the mechanisms of the MMP pathway underlying BBB dysfunction may lead to the development of a therapeutic intervention for burn victims with cerebral complications that are highly correlated with mortality.

4. Experimental procedures

A total of 72 adult male (260 to 280 g) Sprague-Dawley rats (Charles River, Wilmington, MA) were used. Throughout the experiment, animals were housed in the same care facility with food and water available ad libitum during a 12-h light/dark cycle. Animal care was carried out in accordance with guidelines approved by the NIH and the University of Texas Health Science Center Animal Investigation Committee. Animals were divided into control and thermal injury groups. Groups were created for analysis of brain edema and MMPs. MMP analysis included quantification of mRNA levels, protein levels, and enzyme activity. Each group of animals was tested at 7, 24, and 72 h post thermal injury (mRNA levels were measured at 3, 7, and 24 h). All groups contained six rats for each time point with an additional six for sham procedure.

In thermal injury groups, animals were anesthetized with a face mask using 2% halothane in 30% O_2 and then submerged horizontally, in the supine position, in 100 °C water for 6 s producing a third-degree burn affecting 60–70% of the animals body surface area (Jimenez et al., 1994; Barone et al., 1997a,b; Barone et al., 2000). A heating pad and heating lamp were used to help maintain the animal's body temperature at 37.5 °C. The control group underwent a sham procedure in which they were submerged in 37 °C water. Animals were anesthetized or given analgesic relief throughout the entire procedure. Blood

was drawn from each animal for ELISA analysis to determine serum levels of MMPs in order to study molecular changes in serum and rule out the possibility that the increased MMP level in brain was derived from blood.

Edema was measured directly. Brains were harvested after thermal injury or sham procedure. Tissue samples were immediately weighed to obtain wet weight (WW). The tissue then was dried in an oven at 70 °C for 72 h and weighed again to obtain the dry weight (DW). The formula, [(WW−DW)/WW]×100%, was used to calculate the water content and expressed as a percentage of wet weight (Masada et al., 2001; Kawai et al., 2001).

mRNA levels of MMP-2 and MMP-9 were measured using an RNA STAT-60 kit (Tel-Test Co., tel-test.com) to extract the total RNA from each brain sample according to the manufacturer's instructions. Next, the sample was purified using the RNeasy kit (RNeasy Mini Kit, Qiagen). Random primers from Promega were used to create First-strand DNA synthesis using SuperScript RNase H Reverse Transcriptase kit (Invitrogen). The cDNA was then amplified using an ABI Prism 7900HT sequencing detection system for real-time PCR with SYBR Green PCR Master Mix from Applied Biosystems. The MMP-2 and MMP-9 gene-specific rat primers were designed from previous studies (Kelly et al., 2003; Chavey et al., 2003; Ding et al., 2004) (Table 1). For internal PCR control, the rat ribosomal protein L32 (rpL32) was used for each sample. The relative mRNA levels of gene expression were determined using the threshold cycle (C_T) and arithmetic formulas. Subtracting the C_T of the housekeeping gene from the C_T of target gene yields the ΔC_T in each group (control and experimental groups), which was entered into the equation $2^{-\Delta CT}$ and calculated for the exponential amplification of PCR. The mean amount of gene from control group was arbitrarily assigned to 1 to serve as reference. The expression of the target gene from experimental groups therefore represents the fold-difference expression relative to the reference gene.

To investigate protein synthesis of MMP-2 and MMP-9, Western blot analysis was used to quantitatively detect protein expression patterns (Ding et al., 2002; Lindsey et al., 2005). A sample containing one hemisphere was homogenized in lysis buffer including protease inhibitors on ice. Equal volumes (10 μl) of tissue extracts normalized by protein concentration were mixed with SDS sample buffer without reducing agent and without boiling the sample. The samples were separated by electrophoresis through 10% polyacrylamide gel and then transferred to a nitrocellulose membrane. Primary polyclonal anti-rat MMP-9 antibody (M 5427, 1:2000, Sigma) and monoclonal anti-rat MMP-2 antibody (AB808, 1:500, Chemicon) was incubated with the membrane for 1 h at 25 °C. After 3 wash cycles, the membrane was then incubated with secondary antibody conjugated to horseradish peroxidase (Sigma) for 30 min. Finally, the targeted antigens was visualized by using the standard chemical luminescence methods (ECL, Amersham Parmacia Biotech) (Romanic et al., 1998; Lo et al., 2003; Li et al., 2005; Lindsey et al., 2005). To quantify the relative levels of MMP protein expression, images were analyzed using an image analysis program (ChemiGeniusQ System), and the intensity of MMP expression from different groups were statistically compared with a significance level of $p<0.05$.

MMP-2 and MMP-9, known as gelatinase A and B, have intrinsic gelatinase activity. This property allows the zymographic analysis by electrophoresis in polyacrylamide gels containing gelatin. Zymography has been used for qualitative separation of the distinct MMP forms and for quantitative assessment of gelatinase activity (Kleiner and Stetler, 1994; Zhang and Gottschall, 1997). Based on methods described previously (Romanic et al., 1998; Wielockx et al., 2001; Sironi et al., 2003; Asahi et al., 2001; Wagner et al., 2003; Rosenberg et al., 2001), gelatin zymography was used to determine the activity levels of MMP-2 and MMP-9. Phenylmethylsulphonylfluoride (PMSF) was added to protein samples to block endogenous serine protease activity. The protein samples were then subjected to electrophoresis through 10% polyacrylamide gel containing 1 mg/l gelatin (Sigma) at 4 °C. After electrophoresis, the gel was incubated in a 2.5% Triton X-100 solution at 25 °C for 1 h, and then incubated in a 0.05 mol/l Tris–HCl buffer, pH 8.0, containing 10 mmol/l $CaCl_2$ at 37 °C overnight. Duplicate samples loaded onto another gel were incubated in the buffer containing 10 mmol/l EDTA as a control for the nature of the gelatinolytic activity. The gels were fixed with 40% methanol and 7% acetic acid, stained with 0.25% Coomassie blue R-250, and then destained with 10% methanol and 7% acetic acid. Enzyme activity attributed to MMP-2 and MMP-9 were visualized based on the molecular weight in the gelatin-containing zymograms as clear bands against a blue background. As a positive control, purified preparations of latent and active recombinant MMP-2 (72 kDa) and MMP-9 (latent 92 kDa, active 83 kDa) were run in parallel. To quantify the relative levels of MMP expression, the zymogram images were analyzed using an image analysis program (ChemiGeniusQ System). The intensity of MMP expression from different animal groups was statistically compared with a significance level of $p<0.05$.

Additional enzyme-linked immunosorbent assay (ELISA) studies were performed on these animals to determine levels of MMP-2 and MMP-9 in serum. Serum samples from 6 thermal injury and 6 control rats were tested by using an ELISA kit (R&D Systems) at 7 h post injury and sham procedure. Further zymography experiments were carried out to determine serum MMP enzyme activity. Serum activity of MMP-2 and MMP-9 were measured at 3, 7, 12, and 24 h post thermal injury.

Table 1 – Sequence of primers

Gene	Primer/Probe	Sequence
MMP-2	Primer (forward):	5′-ATC TGG TGT CTC CCT TAC CGP-3′
	Primer (reverse):	5′-GTG CAG TGA TGT CCG ACA AC-3′
MMP-9	Primer (forward):	5′-AAA TGT GGG TGT ACA CAG GC-3′
	Primer (reverse):	5′-TTC ACC CGG TTG TGG AAA CT-3′
rpL32	Primer (forward):	5′-TGTCCTCTAAGAACCGAAAAGCC-3′
	Primer (reverse):	5′-CGTTGGGATTGGTGACTCTGA-3′

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