Changes in neuromedin S and its receptor after traumatic brain injury in cycling rats

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Abstract

Animal studies indicate that gonadal steroids have prominent neuroprotective effects in several models of experimental traumatic brain injury (TBI). Neuromedin U (NMU) and neuromedin S (NMS) are regulatory peptides involved in inflammatory and stress responses, and modulation of the gonadotropic axis. Since steroid hormone levels change during the estrous cycle, we sought to determine whether variations in ovarian hormones would affect blood-brain barrier (BBB) permeability and brain levels of NMS, NMU, and neuromedin S   receptor 2 in experimental TBI. Two groups (proestrus and non-proestrus) of female rats underwent diffuse TBI. At 24 hrs after TBI, results showed a significantly decrease in BBB permeability in traumatic-proestrus animals (TBI-P) in comparison to traumatic non-proestrus (TBI-NP) rats. Western blot analyzes demonstrated an enhanced expression of prepro-NMS in TBI-P compared with that in the TBI-NP group. Likewise, TBI-P rats exhibited significantly higher NMUR2 gene expression compared with those of TBI-NP, whereas no significant difference in brain NMU content was seen between sham and traumatic animals. Our findings indicate that diffuse TBI induces an increase in prepro-NMS and neuromedin S receptor 2 expression in traumatic-proestrus rats which may mediate the anti-edematous effects of gonadal hormones in proestrus rats following trauma.

INTRODUCTION

It is well documented that female sex steroid hormones provide neuroprotection in various models of central nervous system (CNS) injury such as diffuse traumatic brain injury (TBI). However, the underlying mechanisms have not been totally clarified. There are evidences that circulating estradiol and progesterone at the time of injury are important for neuroprotection. High circulating estradiol levels in cycling female rats are associated with less brain damage after cerebral ischemia.[1,2] A converse correlation between serum levels of progesterone and the extent of edema after TBI has been reported.[3] Administration of estradiol and progesterone, exclusively or in combination, results in a rising concentration of sex steroid hormones and the decline of brain edema after TBI.[4-6] It has shown that rodent estrous cycle influences the outcome of brain injury.[7]

The normal estrous cycle in a female rat contains two main phases: the proestrus phase and the non-proestrus phases including estrus, diestrus and metestrus.[8] Since plasma levels of female sex hormones are significantly elevated throughout the proestrus stage of the estrous cycle[9,10], it appears that differences in gonadal hormone concentrations between proestrus and non-proestrus phases affect TBI outcomes. Recent study in our laboratory showed that the brain edema and intracranial pressure in injured proestrus rats is less than that in the non-proestrus animals.[11] It has demonstrated that vulnerability in the proestrus stage is lower than the other phases of the reproductive cycle[2]; however, the underlying mechanisms are not well understood.

The regulatory peptides, neuromedin U (NMU) and structurally related neuropeptide neuromedin S (NMS), are implicated in the regulation of numerous biological processes such as autonomic, endocrine and behavioral functions. They share the same C-terminal amino-acid sequence and bind to the same G protein-coupled receptors (GPCRs), neuromedin u receptor 1 (NMUR1) and neuromedin u receptor 2 (NMUR2), also have identified as FM-3/GPR66 and FM-4/TGR-1 respectively.[12-15] NMU is implicated in a wide variety of physiological functions, including nociception, pituitary hormone secretion, stress responses, HPA (hypothalamic-pituitary-adrenal)

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axis activation and immunomodulation.[16-20] NMS is involved in modulation of the reproductive system, regulation of circadian rhythms, stimulating the HPA axis and stress related behavior.[21] The NMU-NMS-NMUR2 system plays a role in the dynamic control of the gonadotropic axis.[22] The dispersed distribution of NMUR2 in the brain suggests additional functions of NMU and NMS in the CNS. It is present in the ependymal cells lining the third ventricle.[23] These ependymal cells work as a protecting barrier between brain and cerebrospinal fluid.[24] Therefore, this could suggest neuroendocrine and transport functions of NMUR2. In addition, NMUR2 is expressed in the subfornical organ and the vascular organ of the lamina terminalis that are the communication sites between the brain and blood – borne substances.[23] Some studies have recently demonstrated the regulatory role of female sex steroid hormones on NMUR2 expression.[25,26] They have revealed that variation of brain NMS and NMU is due to the phases of the female rat estrous cycle[25,26], however their brain expression after TBI remains unexplored. The purpose of this study was to determine whether changes of estradiol and progesterone during the estrous cycle are associated with difference in blood–brain barrier (BBB) permeability as well as brain levels of NMU, NMS and their receptor expression in experimental TBI.

METHODS

Experiments were performed on female Albino N-Mari rats (200–250 g). The animals were kept in a temperature-controlled (22±2ºC) room in 12-hrs light/12-hrs dark cycle with standard food and water ad libitum. Experiments were carried out in conformity with the national guidelines for animal research. The study was approved by the Ethics Committee (No: EC/KNRC/90-4), Kerman University of Medical Sciences, Kerman, Iran.

Experimental protocols

Animals were divided randomly into 6 experimental groups of 18 animals per group. The sham groups included: (1) proestrus (Sh-P), (2) non-proestrus (Sh-NP) and (3) ovariectomized (Sh-OVX). Similarly, the traumatic groups were as follows: (4) proestrus (TBI-P), (5) non-proestrus (TBI-NP) and (6) ovariectomized (TBI-OVX). The traumatic groups, TBI-P and TBI-NP, underwent trauma during two main stages of estrous cycle – proestrus and non-proestrus respectively. TBI-OVX rats underwent trauma one week after ovariectomy.

The corresponding sham groups underwent false brain trauma under anesthesia. Rats in each group were assigned to subgroups for real-time PCR quantification and western blot analysis, enzyme-linked immunoassay (ELISA) and measurement of Evans blue (EB) extravasation.

Bilateral ovariectomy

Animals were ovariectomized bilaterally under anesthetization with a mixture of ketamine (80 mg/kg) and xylazine (10 mg/kg). A small incision (2 cm) was made in the midline of abdomen, between the umbilicus and vagina. The skin and abdominal muscles were cut and the fallopian tubes were tied by a catgut thread below the ovaries and the ovaries removed. Then the muscles and skin were replaced back and sutured.[27]

Determination of estrous cycle

To monitor the estrous cyclicity, vaginal cytologies were performed daily between 10:00 and 11:00 a.m. for about one week prior to the injury. Smears, obtained with a wet cotton swab, transferred to a glass slide and the resulting samples were stained with the Papanicolaou staining method[28], and observed under light microscopy. The estrous categories were staged according to cytological characteristics classically represent the estrous cycle phases: proestrus phase (round nucleated epithelial cells), estrus phase (anucleated cornified cells), metestrus phase (combination of leukocytes and cornified cells) and diestrus phase (few cells, predominantly leukocytes).

Induction of TBI

The diffuse TBI was induced by the Marmarou weight drop model[29], using the TBI induction apparatus (made by Kerman University of Medical Science, Physiology Department). Briefly, animals were anesthetized (mix of 1-2% isoflurane, 66% N2O and 33% O2 gas), a stainless steel disc (10 mm diameter) was placed between bregma and lambda sutures and a 450 g weight inside a 2-m tall Plexiglas pipe dropped onto the disc. After TBI induction, the animals were connected to a respiratory pump (TSE animal respirator, Germany) and following the restoration of spontaneous respiration, disconnected from the pump and returned to their cages.

Evaluation of blood–brain barrier integrity

BBB permeability was measured by the Evans blue method with a slight modification at 5 hrs

after TBI. Briefly, rats were injected intravenously with Evans blue dye (2 ml/kg) at 4 hrs after trauma and allowed to circulate for 1 hr and then perfused with normal saline 1 hr later. Rats were then decapitated, and the brains were quickly removed, and homogenized in 1 ml of 0.1 mol/L phosphate buffered saline (PBS). Trichloroacetic acid was then added to it and the samples were centrifuged at 1000 g for 30 min and the supernatant was analyzed at 610 nm with a spectrophotometer (UV/VIS, Spectrometer, UK). The amount of extravasated EB was expressed as µg/g brain tissue.[6]

Hormone measurements

Blood samples were collected before the trauma induction, centrifuged (2500 rpm for 10 min), and stored at -80 °C until analysis. Serum was analyzed using quantitative enzyme-linked immunoassay Kits for 17β-estradiol (E2) and progesterone (P4) (Dia Metra, Italy). The limit of P4 assay sensitivity was 0.1 ng/ml and 8.68 pg/ml for P4 and E2 respectively. The inter- and intra-assay coefficients of variations were 9 and 5.7% for P4 and ≤10% and ≤9% for E2, respectively.

RNA and protein extraction

In this study the whole brain was used for extraction, not particular regions, to observe the general changes of of NMS, NMU and NMUR2 expression in the brain. Samples were homogenized and organized for both mRNA and protein processing. RNA was isolated using TRIzol reagent (TRI Reagent, Sinagen Co, Tehran, Iran), according to the manufacturer's instructions. Following recovery of RNA from the aqueous phase, soluble proteins were separated from the organic phase by successive precipitation.[30] Protein concentration was estimated according to the Bradford method.[31] The purity of the RNA was assessed at 260 and 280 nm and the 260/280 ratio above 1.8 was considered as an indicator of RNA purity.

Enzyme-linked immunoassay (ELISA)

We have developed an indirect sandwich ELISA technique to measure NMU content of the brain. Briefly, 100 µl the NMU-23 (Santa cruz Biotechnology) goat polyclonal antibody, diluted 1:50 in a coating buffer (100 mM bicarbonate/carbonate buffer, pH 9.6), were applied to coat the wells of Elisa 96-well microtiter plates (SPL Life Sciences) except three wells as a negative

control at 4ºC overnight. After coating, the wells were washed three times with PBS containing 0.05% Tween 20 and blocked with 1% bovine serum albumin (BSA) in PBS for 90 min at 37ºC to diminish nonspecific binding. The wells were washed as done before and incubated for 2 hrs at 37ºC with 100 µl aliquots of protein samples and washed again. Subsequently, the primary anti-NMU diluted 1:100 in PBST was added to each well for 2 hrs at 37ºC. After washing 3 times with PBST, a donkey anti-goat peroxides conjugate secondary Ab (1/10000 dilution; Santa Cruz Biotechnology) was added. Following 2 hrs incubation, the wells were again washed 4 times with PBST and 100 µl of substrate TMB (3,3V, 5,5V-tetramethylbenzidine) was added. After an appropriate development time, the reaction was quenched using 100 µl of 0.15 M H2SO4 and optical density (OD) was assessed at 450 nm with an ELISA reader (DRG Eliza-Mat 2000).

cDNA synthesis

Total RNA was reverse transcribed into cDNA using total RNA sample mixed with random hexamer (Pars tous biotechnology, Iran) according to the manufacturer's recommendations as follows: RNA sample (1 µg) mixed with random hexamer (2 µl) was incubated at 65°C in a thermal cycler for 5 min, and chilled on ice. Afterward, 10 µl of RT Premix (Pars tous biotechnology, Iran) was added, and the mixture was incubated for 10 and 60 min at 25°C and 40°C respectively. The reaction was stopped by heating at 70°C for 10 min and cooled to 4°C.

Real-time PCR quantification

Quantitative Real Time PCR was carried out using the Corbett Life Science (Rotor-Gene 6000) System as follows: 94°C for 10 min (1 time); 94°C for 30 s, 60°C for 30 s, and 72°C for 30 s (40 times); and 72°C for 8 min (1 time). 2 µL of a 5-fold diluted cDNA were added in each PCR reaction with a final volume of 20 µL. Reactions were performed with a mix (Absolute QPCR SYBR Green ROX; ABgene, Portsmouth, NH) containing SYBR Green I dye (SinaClon, Karaj, Iran), DNA polymerase, dNTPs and MgCl2. Primers for quantitative RT-PCR (qRT-PCR) were: NMUR2 forward: 5´- GATGAATCCCTTGAGGCGAA -3´ and reverse: 5´-ATGGCAAACACGAGGACCAA -3´ (101 bp; NM_022275.2). The specificity of the PCR products was confirmed by acquiring the melting curve with a linear temperature transition

at 1°C/s from 50°C to 90°C. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were applied as an endogenous reference gene. The cycle threshold (CT) levels were set above background noise and during the exponential phase as previously described (32). The relative expression ratio expressed as N-fold difference (decrease or increase) of the genes, calculated by a randomization test performed in the relative expression software tool (REST) for Rotor-Gene©.[32] The results were considered at $p < 0.05$.

Western blot

Western blotting was carried out to analyze prepro-NMS protein expression at 24 hrs following TBI. Aliquots of tissue protein samples were mixed with Laemmli buffer (0.125 M TrisHcl, 0.004% bromphenol blue, 10% 2-mercaptoethanol, 20% glycerol, 4% Sodium dodecyl sulfat (SDS)), heated at 95ºC for 5 min, and electrophoresed on 15% polyacrylamide gel and then transferred onto a polyvinylidenedifluoride (PVDF) membrane (Roche, Mannheim, Germany). The membrane was stained with the Ponceau S staining solution to confirm the successful transferring of proteins to the membrane. Non-specific bindings were blocked by immersing the membrane in 2% non-fat dry milk in TBST (0.1 M PBS, 0.05% Tween-20) at 4°C overnight, and immunolabeled with rabbit anti-NMS primary antibody (1:2,000 dilution, Phoenix Pharmaceuticals Inc, Belmont, CA) at 37ºC for 1 h. Afterward, the membranes were washed in TBST [Tris (10 mM, pH 8.0), NaCl (150 mM, Tween-20 (0.05%)] three times on a roller mixer for 10 min and incubated for 1 h with goat anti-rabbit immunoglobulin G peroxidase –conjugated (Santa Cruz Bio- technology) at a1:10,000 dilution, at room temperature followed by washing for 4 times in TBST. Protein bands were detected by an enhanced chemiluminescence advanced kit (Roche, Mannheim, Germany) and visualized on autoradiographic X-ray film. To verify identical protein loading and/or normalize for protein amount, membranes were striped with the stripping buffer and reblotted with mouse anti- β-actin antibody (1:1000 dilution, Sigma, USA) as the loading control. The bands were semi-quantitated using Image J. (National Institutes of Health, USA). Statistical analysis was carried out using Graphpad Instat TM software (San Diego, CA, USA).

Statistical analysis

Data were analysed using one way analysis of variance for comparison among groups followed by Tukey (HSD) post hoc for pairwise comparisons. A $p < 0.05$ was considered statistically significant.

RESULTS

BBB permeability

The level of Evans blue extravasation was identical in the different groups of non-traumatic animals. Traumatic-ovariectomized rats demonstrated a significant increase ($p < 0.001$) in Evans blue extravasation relative to sham-ovariectomized (Figure 1). Indeed, TBI-OVX animals indicated a significant increase ($p < 0.001$) in BBB permeability relative to that in traumatic-proestrus rats. Similarly, traumatic non-proestrus animals had a significant increase in BBB permeability relative to traumatic-proestrus ($P < 0.05$) after TBI.

Serum hormone levels

As shown in Figure 2A, the serum level of E2 in traumatic-proestrus rats was significantly higher compared with that in the traumatic non-proestrus group ($p < 0.001$). Furthermore, the serum P4 level was significantly lower in TBI-NP rats ($p < 0.001$) relative to that in TBI-P group (Figure 2B).

Western blot analysis of prepro-NMS expression after traumatic injury

Western blot analysis showed higher expression of prepro-NMS in Sh-NP rats compared to the Sh-P group ($p < 0.05$, Figure 3) while at 24 hrs after TBI, it showed significantly higher expression in TBI-P animals in comparison to TBI-NP group ($p < 0.05$). Prepro-NMS expression was increased in TBI-P rats in compared to Sh-P ($p < 0.001$) as well as TBI-OVX animals ($p < 0.001$). However, no difference was observed between TBI-OVX and TBI-NP groups. There was no significance difference between TBI-OVX compared to Sh-OVX group. While the prepro-NMS expression was higher in Sh-NP compared with Sh-OVX group ($p < 0.01$), no difference was observed between Sh-P and Sh-OVX rats.

NMU content after traumatic injury

Figure 4 illustrates that NMU content in Sh-P and Sh-NP groups was more than Sh-OVX rats ($p < 0.05$). NMU content in TBI-P and TBI-NP groups was significantly more than that of TBI-OVX group ($p < 0.05$, $p < 0.01$ respectively), whereas
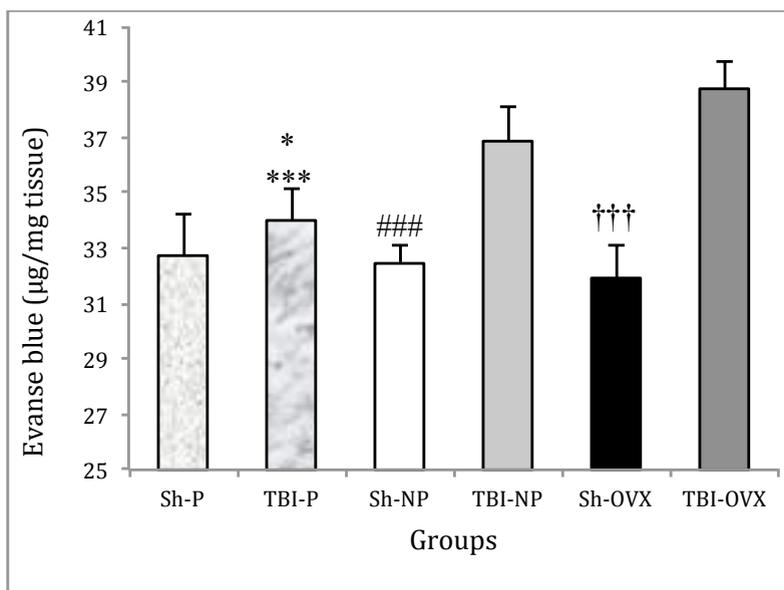
Figure 1. Changes in blood-brain barrier permeability before trauma and 24 hrs following TBI.
Sh-P, sham proestrus; TBI-P, traumatic proestrus; Sh-NP, sham non-proestrus; TBI-NP, traumatic non-proestrus; Sh-OVX, sham- ovariectomized; TBI-OVX, traumatic ovariectomized.
*Significant difference from TBI-NP, p<0.05; ***Significant difference from TBI-OVX, p<0.001; ### Significant difference from TBI-NP, p<0.001; †††Significant difference from TBI-OVX, p<0.001; n=6/ group.

there was no significant difference between Sh-P and TBI-P groups as well as between Sh-NP and TBI-NP rats.

Gene expression of NMUR2 after traumatic injury

The real-time PCR analysis of the NMUR2 gene expression showed a 1.9 fold increase in the
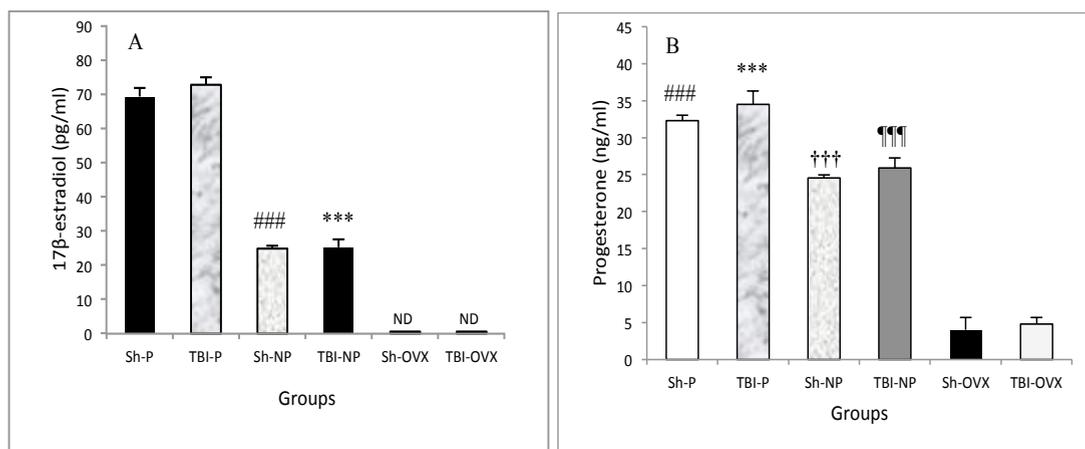

Figure 2. Serum hormone levels in different experimental groups (mean±SEM).
Sh-P, sham proestrus; TBI-P, traumatic proestrus; Sh-NP, sham non-proestrus; TBI-NP, traumatic non-proestrus; Sh-OVX, sham- ovariectomized; TBI-OVX, traumatic ovariectomized.
###Significant difference from Sh-P, p<0.001; ***Significant difference from TBI-P, p<0.001; ND, not detectable (**A**). ###Significant difference from Sh-NP and Sh-OVX, p<0.001; ***Significant difference from TBI-NP and TBI-OVX, p<0.001; †††Significant difference from Sh-OVX, p<0.001; ¶¶¶Significant difference from TBI-OVX, p<0.001 (**B**). n=6/group.
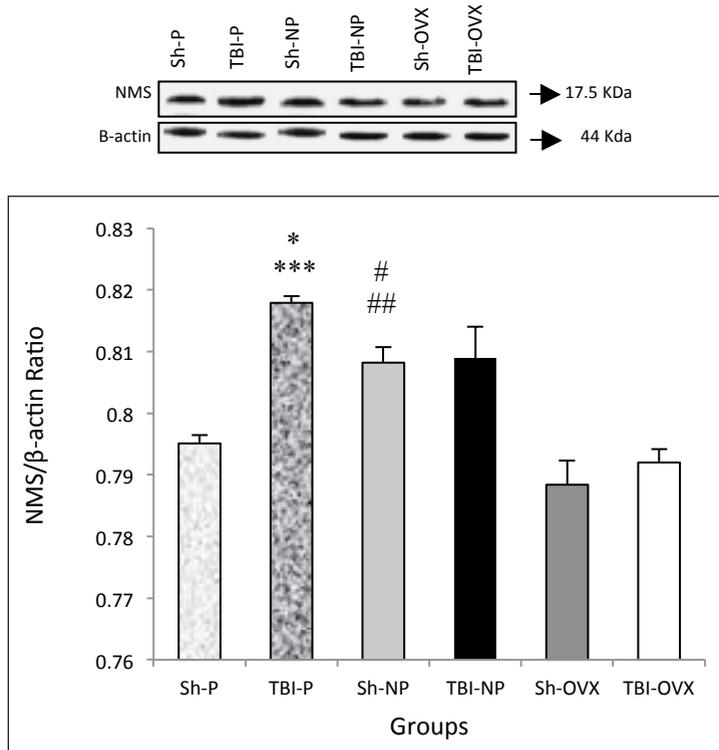
Figure 3. Western blot analysis of preproNMS protein expression at 24 hrs following brain injury.
Sh-P, sham proestrus; TBI-P, traumatic proestrus; Sh-NP, sham non-proestrus; TBI-NP, traumatic non-proestrus; Sh-OVX, sham- ovariectomized; TBI-OVX, traumatic ovariectomized.
*$p<0.05$ vs TBI-NP; ***$p<0.001$ vs Sh-P and TBI-OVX; #$p<0.05$ vs Sh-P; ##$p<0.01$ vs Sh-OVX.
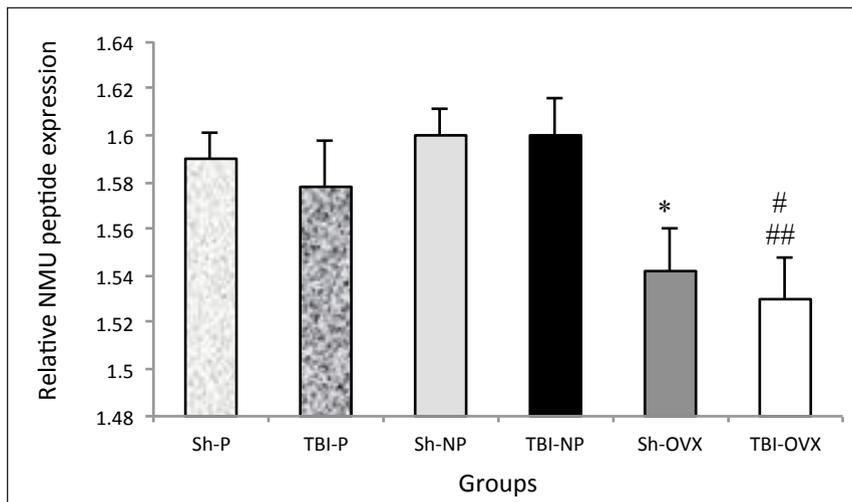Data are expressed as mean ± S.E.M.

Figure 4. Comparative levels of brain NMU content at 24 hrs following brain injury.
Sh-P, sham proestrus; TBI-P, traumatic proestrus; Sh-NP, sham non-proestrus; TBI-NP, traumatic non-proestrus; Sh-OVX, sham- ovariectomized; TBI-OVX, traumatic ovariectomized.
#$p<0.05$ vs TBI-P; ##$p<0.01$ vs. TBI-NP; *$p<0.05$ vs Sh-NP and Sh-P.
Y-axis indicates ELISA absorbance Units.
Data are expressed as mean ± S.E.M.

Table 1: Relative expression of the NMUR2 gene after multiple comparisons between experimental groups

	VS.	Sh-P	TBI-P	Sh-NP	TBI-NP	Sh-OVX	TBI-OVX
Experimental groups	Sh-P	-	-	-	-	1.13	-
	TBI-P	3.1[b]	-	-	2.26[a]	-	4.39[c]
	Sh-NP	1.9[a]	-	-	-	2.84[a]	-
	TBI-NP	-	-	1.2	-	-	1.75[a]
	Sh-OVX	-	-	-	-	-	-
	TBI-OVX	-	-	-	-	0.8	-

Note: Values represent fold change in the ratio of gene expression between the NMUR2 gene in various groups, normalized by GAPDH as an endogenous reference gene. [a]$p<0.05$, [b]$p<0.01$, [c]$p<0.001$.
Sh-P, sham proestrus; TBI-P, traumatic proestrus; Sh-NP, sham non-proestrus; TBI-NP, traumatic non-proestrus; Sh-OVX, sham- ovariectomized; TBI-OVX, traumatic ovariectomized.

Sh-NP ($p < 0.05$) compared to the Sh-P group. In addition, NMUR2 mRNA was significantly upregulated (3.1 fold increase, $p < 0.01$) in TBI-P than that in the Sh-P rats (Table. 1). However at 24 hrs after TBI, the results revealed unchanged expression levels between TBI-NP and Sh-NP groups. On the other hand, NMUR2 mRNA expression was significantly downregulated in Sh-OVX rats related to the Sh-NP group ($p < 0.05$, Table. 1) as well as in TBI-OVX compared to the TBI-NP group ($p < 0.05$). Whereas, the elevated expression of NMUR2 mRNA was found in TBI-P rats compared with the TBI-NP (2.26 fold increase, $p < 0.05$).

DISCUSSION

The current study showed that Evans blue extravasation were reduced in traumatic-proestrus rats and that the sex steroid hormone levels at the time of injury could be the reason for the lower BBB permeability seen in this group. Moreover, we provided evidence that the expression of NMS protein and its receptor is affected by the stage of estrous cycle and that it may be contributed to prevention of edema formation. The main findings of present study are (1) prepro-NMS expression was higher in proestrus than in non-proestrus stage after TBI and ovariectomy caused a significant reduction in its expression; (2) NMU levels in ovariectomized animals were significantly less than those in normal cycling rats; however, its levels were not significantly different between the proestrus and the non-proestrus groups; (3) the expression of NMUR2 mRNA in proestrus rats increased more than that of in non-proestrus

group following trauma.

In our work, a weight-drop model of TBI initially explained by Marmarou *et al.*[29] was used. Damage caused by this model is similar to the surface brain injury observed in humans.[33] Recent study indicated that the cerebral edema in traumatic proestrus rats is less than that in the non-proestrus.[11] A profound attenuation of cerebral edema and Evans blue extravasation has been reported by the administration of estradiol or progesterone due to an increase in concentrations of gonadal hormones.[4,6] Female rats with low progesterone levels or no hormone had less improvement than those with high levels of hormone at the time of injury.[34,35] Consistent with these studies, our results showed that BBB permeability significantly reduced in traumatic proestrus rats compared with that in the TBI-OVX and TBI-NP animals. This can attributed to the differences in female sex steroid hormone levels between the animal groups. Female gonadal hormones have various neuroprotective effects such as anti-inflammatory[5], antioxidant[36], and vasodilatory activities that may reduce BBB permeability and edema development[37] and limiting cellular necrosis and apoptosis.[38,39] Furthermore, ovarian steroids modulate the expression, synthesis and release of several neuropeptides in response to multiple physiological or pathological stimuli.[40]

NMU and NMS are regulatory peptides with diverse functions, including stress response, regulation the gonadotropic axis and immunomodulation. In the present study, in spite of lower expression of prepro-NMS protein in the sham-proestrus compared to the non-proestrus

rats, a significant increase in its expression was seen in TBI-P compared to that in TBI-NP rats following trauma. Likewise, NMUR2 mRNA was lower in proestrus stage than in non-proestrus. But after trauma, NMUR2 gene expression was significantly increased in TBI-P group compared with that in TBI-NP rats. This increase in receptor expression can enhance the effects of NMS in the brain.

It has been shown that in female rats, the hypothalamic expression of NMS gene fluctuates in a cycle-dependent way, with the highest expression at proestrus and the least value during the estrous stage.[22,25] In another study the expression of NMS mRNA in the hypothalamus and pituitary of the pig was the highest in the proestrus stage and the least in the estrus.[26] The same pattern of expression is also reported for NMUR2 gene in the pig brain.[26] The discrepancy between their and our findings may be due to difference in timing of sample collection after estrous cycle determination. In their studies, tissue sampling was conducted after vaginal smear examination, whereas in our study, tissue collection was performed at 24 hrs after estrous cycle staging. Furthermore, there are no published reports of brain NMS changes following traumatic brain injury, so we cannot compare our results with others.

The release of NMS under stressful conditions has been well documented[41] that this increase is due to activation of the HPA axis.[42] In proestrus rats, with relatively high levels of estradiol and progesterone, stress induces higher adrenocorticotropin (ACTH) and corticosterone responses than the other stages of the estrous cycle. On the other hand, it has revealed that NMS suppresses physiological parameters changes induced by acute stress such as oxidative stress damage.[43] So, it is probable that the observed increase in NMS in traumatic proestrus group reduced brain edema following TBI.

It has demonstrated that NMS stimulates the proopiomelanocortin (POMC) system in the brain.[44] α- MSH represses the increase of intracerebral proinflammatory cytokines such as TNF-α and IL-1β following brain injury.[45,46] These cytokines have a major role in brain dema formation following TBI.[47,48] Thus, another probable mechanism that needs further study involves alterations in α-MSH secretion, which can be implicated in anti-edematous effect of NMS.

NMS might reduce brain edema via a decrease in oxidative damage. It has reported that intracerebroventricular injection of NMS suppressed the oxidative damage in rat brain.[43] Further, it has suggested that NMS has some neuroprotective effects.[49] Another study showed that NMS attenuates the oxidative damage to brain lipids and proteins.[50]

A further possible mechanism by which NMS could affect brain edema is through the activation of progesterone and influencing the expression of its receptor in the brain. Moreover, progesterone has been shown to diminish lipid peroxidation and BBB permeability through multiple pathways including of antioxidant enzymes upregulation and by increasing the free radical scavengers levels in the brain.[36]

In this study a significant increase in NMUR2 gene expression was detected in proestrus group following trauma. Its expression in brain regions in contact of cerebrospinal fluid[51] and its up-regulation in proestrus group after trauma, representing its possible function in the regulation of water transport and edema formation.

Vigo *et al.*[25] reported that in female rat brain, the NMUR2 mRNA expression is induced by progesterone. Progesterone can affects edema formation through its action on the vasopressin secretion and cerebrospinal fluid production.[52] NMUR2 coexpression with vasopressin in neuronal populations involved in osmotic balance maintenance[53] suggesting that progesterone could modulate vasopressin secretion through the pathways involving activation of NMUR2.

In another part of the present study, the brain content of NMU in sham non-proestrus rats exhibited a trend to increase compared to that of in proestrus; however, it was not statically significant. The data also showed a pronounced decrease in NMU content following ovariectomy which is in agreement with the data from Vigo *et al.*, and this may be attributed to the elimination of sex hormones or related to other unknown regulatory factors.[25] However, twenty-four hrs after trauma, no difference in brain NMU content was found between the sham and traumatic groups. Since NMU levels did not change at different stages of estrous cycle, it can be concluded that this peptide may not contribute to the different neuroprotective effects seen between the proestrus and the non-proestrus stages. A small part of the PVN parvocellular neurons express NMU mRNA. Moreover, CRF decreases neuronal excitability in a great number of NMU mRNA expressing neurons.[54] Thus, it can be stated that there is a bi-directional interaction between NMU and the HPA axis following TBI. On one hand, NMU activates the HPA axis, and on the other, CRH suppresses the secretion of NMU. This might explains the

non-significant changes of this neuropeptide after trauma.

In conclusion, the study showed that BBB permeability was reduced in traumatic-proestrus animals than that of the non-proestrus. Furthermore, the current study indicated that TBI resulted in alterations in prepro-NMS and its receptor expression in proestrus rats. The increase in NMS and its receptor in the proestrus stage following TBI could be possibly attributed to the increased level of sex steroid hormones in this stage. It could also be the result of the effect of other regulatory factors downstream of sex steroid hormones. On the other hand, NMS and its receptor NMUR2 may mediate the antineuroinflammatory effects of sex steroids hormones after trauma, although the exact underlying mechanisms remain to be fully elucidated. Further studies using NMUR2 antagonist or NMUR2 knock-out animals are needed to demonstrate a direct link between NMU/NMS-NMUR2 and the protective effect of female sex steroids against brain edema after TBI.

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