Iranian Journal of Basic Medical Sciences

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IJBMS

Effects of sex steroid hormones on neuromedin S and neuromedin U2 receptor expression following experimental traumatic brain injury

Mohammad Khaksari [1], Fatemeh Maghool [2*], Gholamreza Asadikaram [3], Zahra Hajializadeh [4]

[1] Endocrinology and Metabolism Research, and Physiology Research Centers, Kerman University of Medical Sciences, Kerman, Iran
[2] Neuroscience Research Center, School of Medicine, Kerman University of Medical Sciences, Kerman, Iran
[3] Endocrinology and Metabolism Research, and Physiology Research Centers, Kerman University of Medical Sciences, Kerman, Iran
[4] Neuroscience Research Center, School of Medicine, Kerman University of Medical Sciences, Kerman, Iran

ARTICLE INFO

Article type:
Original article

Article history:
Received: Jul 28, 2015
Accepted: Apr 7, 2016

Keywords:
Estradiol
Neuromedin U
NMS
NMUR2
Progesterone
Traumatic brain injury

ABSTRACT

Objective(s): Neuroprotective effects of female gonadal steroids are mediated through several pathways involving multiple peptides and receptors after traumatic brain injury (TBI). Two of these peptides are including the regulatory peptides neuromedin U (NMU) and neuromedin S (NMS), and their common receptor neuromedin U2 receptor (NMUR2). This study investigates the effects of physiological doses of estradiol and progesterone on brain edema, NMS and NMU as well as NMUR2 expression following TBI.

Materials and Methods: Ovariectomized female rats were given high-and low-dose of female sex steroid hormones through implantation of capsules for a week before trauma. The brain NMUR2 expression, prepro-NMS expression, NMU content, and water content (brain edema) were evaluated 24 hr after TBI induced by Marmarou's method.

Results: Percentage of brain water content in high- and low-dose estradiol, and in high- and low- dose progesterone was less than vehicle (P<0.01). Results show high expression of prepro-NMS in high dose progesterone (TBI-HP) rats compared to the high dose estrogen (TBI-HE), as well as vehicle (P<0.01). NMU content in low-dose progesterone (TBI-LP) group was more than that of vehicle group (P<0.001). Furthermore a difference in NMU content observed between TBI-HP compared to TBI-HE, and vehicle (P<0.05). The NMUR2 mRNA expression revealed an upregulation in TBI-HP rats compared to the TBI-HE group (P<0.001).

Conclusion: Findings indicate that progesterone attenuates brain edema and induces an increase in NMS and its receptor which may mediate the anti-edematous effect of progesterone after TBI.

► *Please cite this article as:*
Khaksari M, Maghool F, Asadikaram GR, Hajializadeh Z. Effects of sex steroid hormones on neuromedin S and neuromedin U2 receptor expression following experimental traumatic brain injury. Iran J Basic Med Sci 2016; 19:1080-1089.

Introduction

Numerous animal studies clearly show that female sex steroid hormones exert potent protective effects against brain damage in animal models of traumatic brain injury (TBI). An initial study showed that pseudopregnant females (with high circulating levels of progesterone) developed almost no post-injury brain edema after TBI (1). Either estradiol or a combination of estrogen and progesterone replacement therapy in ovariectomized (OVX) rats significantly attenuated TBI-induced cerebral edema (2). It has reported that vulnerability in proestrus, the stage of the estrous cycle with high endogenous estradiol and progesterone levels, is lower than in the other stages of estrous cycle (2). In contrast, large brain damage was seen in animals injured at metestrus with low sex steroid hormones production (3). Administration of physiological doses of estradiol or progesterone to the traumatic ovariectomized animals caused a reduction in brain water content through increasing the blood levels of the female gonadal hormones (4). Carswell *et al* reported that brain injury outcomes are improved in proestrus stage compared to the non-proestrus stages of estrous cycle (2).

It has shown that animals with high plasma levels of progesterone at the time of injury had greater improvement than those with low levels of hormone (5, 6). Evidence from Wright *et al* (7) suggests that there is a strong inverse correlation between serum levels of progesterone and the degree of TBI-induced edema. The synthetic as well as circulating endogenous progesterone has been reported to have neuroprotective effects in traumatic female rats (1).

*Corresponding author: Fatemeh Maghool. Neuroscience Research Center, School of Medicine, Kerman University of medical Science, Kerman, Iran. Tel: +98-913 2074423; Fax: +98-3195016799; email: fmaghool@gmail.com; fmaghool@kmu.ac.ir

Neuroprotective effects of female gonadal steroids are mediated through several pathways involving multiple peptides and receptors. A number of peptides and their receptors that are expressed in the brain are regulated during the estrous cycle, and in response to administration of sex steroid hormones. Two of these peptides are including the regulatory peptides neuromedin U (NMU) and neuromedin S (NMS). The widespread distribution of mRNA encoding their common receptor, Neuromedin U2 receptor (NMUR2), throughout the brain (8) suggests that these neuromedins are implicated in numerous biological processes. Evidences from studies showed the implication of NMU and NMS in stress, inflammation**,** and a variety of autonomic and neuroendocrine functions (9-13). The studies also suggest that NMU and NMS may have a protective role in the setting of neurodegenerative disease (14, 15). Okamura *et al* (14) in his study reported that NMS has protective effects against the neurotoxic changes induced by NMDA receptor antagonists. Castro *et al* (16) revealed that NMS attenuates oxidative stress damage in the injured mouse brain. It has indicated that NMU protects neuronal cell viability, and inhibits inflammation-induced memory impairment (15).

In the current study, we investigated the probable effects of the administration of physiologic doses of 17β-estradiol and progesterone on cerebral edema, NMS, NMU, and NMUR2 mRNA expression in ovariectomized female rats following traumatic brain injury.

Materials and Methods

Adult female Albino N-Mari rats (200–250 g body wt) were used in this study. Animals were kept in 12-hr light/12-hr dark cycle with standard food and water *ad libitum*. The experiments were conducted in conformity with the national guidelines for animal experiments. All procedures were approved by the Ethics Committee of Kerman University of Medical Sciences, Iran (approval number: EC/ KNRC/90-4).

Experimental protocols

Animals were ovariectomized one week before trauma and randomly allocated into 10 groups of 6:

The sham groups were as follows: proestrus (P), non-proestrus (NP) and ovariectomized (OVX); and the traumatic groups were included: proestrus (TBI-P), non-proestrus (TBI-NP), high estradiol (TBI-HE), high progesterone (TBI-HP), low estradiol (TBI-LE), low progesterone (TBI-LP), ovariectomized (TBI-OVX), and vehicle (Veh).

TBI-P and TBI-NP rats underwent trauma during two main phases of estrous cycle - proestrus and nonproestrus - respectively. TBI-OVX animals underwent trauma one week after ovariectomy. The corresponding sham animals underwent false brain trauma under anesthesia.

In hormone replacement groups animals received silastic tubing filled with high doses of 17β-estradiol (TBI-HE) and progesterone (TBI-HP) after OVX, and TBI-LE and TBI-LP animals received silastic tubing filled with a low doses of 17β-estradiol and progesterone respectively. In vehicle group rats were inserted with vehicle (sesame oil) capsules.

Animals in each group were assigned to subgroups for water content assessment, western blot analysis, and real-time PCR quantification, as well as enzyme-linked immunosorbent assay (ELISA).

Bilateral ovariectomy and capsule implantation

One week before TBI, rats were anesthetized with a mixture of ketamine/xylazine (80/10 mg/kg IP). A small incision (2 cm) was made in the abdomen, between the umbilicus and vagina. After cutting the skin and abdominal muscles, the fallopian tubes were tied by the absorbable catgut sutures below the ovaries and the ovaries were removed. The muscles and skin were then sutured (17). The loose skin below the neck was cut immediately following ovariectomy and a tunnel was dissected caudally, and silastic capsules were implanted subcutaneously. Then the skin was sutured (18).

Preparation of the capsules

Silicone tubules (Dow Corning Corp, Midland, MI) were filled with 17β-estradiol (E2) dissolved in sesame oil (inner/outer diameter: 1.575/3.175 mm and 30 mm in length) 180 µg/ml (TBI-LE) or 1 mg/ml (TBI-HE), plugged at each end with five mm of wooden applicator sticks and fixed with silastic adhesive. This paradigm produces serum levels of E2 that are correspond to basal and proestrus serum levels of hormone respectively (19). In vehicle group capsule containing vehicle (sesame oil) was implanted. In TBI-HP group, animals were implanted with three silastic capsules (1.55 mm ID, 3.18 mm OD 40 mm in length, Dow Corning Corp, Midland) each capsule was 30 mm long and packed with crystalline progesterone and the TBI-LP group received two 20 mm lengths of crystalline hormone (20, 21). The serum hormone levels produces by this paradigm are about 40–50 ng/ml, and 10–20 ng/ml in TBI-HP and TBI-LP groups which are equivalent to proestrus and basal levels of progesterone found during estrous cycle, respectively (19, 22).

Determination of estrous cycle

The female rat estrous cycle consists of four stages: a proestrus stage with an elevated levels of estrogen and progesterone, and non-proestrus phase which includes estrus, diestrus, and metaestrus stages. As part of this study, animals were classified into proestrus (high hormone levels) or non-proestrus (low hormone levels) groups. For the determination of estrous cycle stage, vaginal

smears were taken with a cotton swab daily between 10:00 and 11:00 a.m. Smears were then placed on a slide and examined using light microscopy (40_ objective lens), and estrous categories were classified based on standard cytological criteria of each of the stages of the estrous cycle (23).

Hormone analysis

Blood samples of tail vein were collected in glass tubes before the trauma induction and centrifuged at room temperature at 2500 rpm for 10 min, and then stored at -80 ˚C until analysis. 17β-estradiol (<9% intra-assay coefficients of variations) and progesterone (5.7% intra-assay coefficients of variations) were measured by quantitative enzyme-linked immunoassay kit (Dia Metra, Italy). The minimum detection limit was 20 pg/ml and 0.2 ng/ml, for estradiol and progesterone respectively. The hormone levels less than the detection limit were considered undetectable.

Induction of TBI

Diffuse TBI was induced by the Marmarou method (24), by means of a TBI induction apparatus (made by the Physiology Department, Kerman University of Medical Science). Briefly, after animal anesthetization (gas mixture of isoflurane (1-2%), N_2O (66%) and O_2 (33%)), a 450 g weight was dropped from a 2 meters height through a Plexiglas guide tube onto the stainless steel disc fixed to the rat's skull. After trauma induction, the animals were connected to a respiratory pump (TSA animal respirator, Germany). They were placed in their cages following restoration of spontaneous respiration and stabilization.

Brain edema assessment

Brain water content was measured 24 hr after the trauma induction using the dry-wet weight method (25). Briefly, animals were anesthetized and their brains were removed and weighed to obtain their wet weight (WW). The tissue then was dried at 100 °C for 72 hr in an incubator (Memmert, Germany), and reweighed to obtain the tissue dry weight (DW).

The brain water content percentage was calculated as: (WW−DW)/WW×100%.

Measurement of brain NMU content

An indirect sandwich ELISA was developed to measure the rat brain NMU content. Briefly, brain tissue homogenated in phosphate-buffered saline (PBS, pH 7.2) and centrifuged at 13,000 rpm for 10 min. The supernatant used for ELISA assays. 100 µl of the NMU- goat polyclonal antibody (Santa-cruz Biotechnology), diluted to 1:50 in a coating buffer (100 mM carbonate/bicarbonate buffer, pH 9.6), was used to coat the wells of microtiter plates (SPL Life Sciences) except for three wells as control wells and incubated at 4 ˚C overnight. Then the wells were washed with PBS containing 0.05% Tween 20 (PBST) for three times and blocked with 1% bovine serum albumin (BSA) in PBS at 37 ˚C for 90 min to diminish nonspecific binding. Afterward, they were incubated in a 1:100 dilution of primary anti-NMU in PBST at 37 ˚C for 2 hr, washed for 3 times in PBST and incubated for 2 hr with a donkey anti-goat peroxidase conjugate secondary Ab diluted 1/10000 in PBST (Santa Cruz Biotechnology).

Washing was done with PBST for 4 times and 100 µl of TMB (3,3V, 5,5V-tetramethylbenzidine) as a substrate was added. The reaction was quenched using 0.15 M H_2SO_4 (100 µl) after appropriate development time and the optical density (OD) was determined with an ELISA reader (DRG Elisa-Mat 2000) at 450 nm.

RNA extraction and cDNA synthesis

RNA was isolated from the animal brain tissue using Trizol reagent (Cinnagen Co, Tehran, Iran), in accordance with the manufacturer's instructions. Briefly, samples were homogenized in a guanidine-thiocyanate buffer. After phenol/chloroform extraction (26), the RNA was precipitated using isopropanol. Once the supernatants were discarded the pellets were washed with 100% ethanol and centrifuged and the supernatant were discarded. Pellets were air-dried and resuspended in diethylpyrocarbonate (DEPC) - treated water. The purity of the RNA was assessed at the absorbance ratio of 260/280 nm and the ratio above 1.8 was considered acceptable.

For the real-time RT-PCR, the extracted RNA was reverse transcribed into cDNA. Briefly, RNA sample (1 µg) mixed with random hexamer (2 µl, Pars Tous Biotechnology, Iran) was incubated in a thermal cycler for 5 min at 65 °C, and chilled on ice. Subsequently, RT Premix solutions (10 µl, Pars Tous Biotechnology, Iran) was added, and it was incubated at 25 °C and 40 °C for 10 and 60 min respectively. The reaction was terminated by heating at 70 °C for 10 min, followed by chilling at 4 °C.

Real-time PCR quantification

The Corbett Life Science (Rotor-Gene 6000) System was used for quantitative real time PCR. The cycle protocol was: preincubation at 94 °C (10 min) followed by 40 cycles at 94 °C (30 sec), 60 °C (30 sec), 72 °C (30 sec) with a final extension at 72 °C for 8 min. Two µl of cDNA (5-fold diluted) was used in each PCR reaction mixture with a final volume of 20 µl. Reactions were carried out with a SYBR®green Master Mix (ABgene, Portsmouth, NH) containing DNA polymerase (Thermostart; Portsmouth), $MgCl_2$, dNTPs and SYBR Green I dye (SinaClon, Karaj, Iran). NMUR2 Primers (Bio Basic Inc., Canada) used were as follows: NMUR2forward: 5´-GATGAATCCCTTGAGGCG-AA-3´ and reverse: 5´-

ATGGCAAACACGAGGACCAA -3′ (101 bp; NM_022275.2) Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were applied as an endogenous reference gene. The specificity of the PCR products was verified by acquiring the melting curve with a linear transition of temperature from 50 ˚C to 90 ˚C at 1˚C/sec. Real-time RT-PCR was run in triplicate. Due to the different amplification efficiency of NMUR2 and GAPDH, the Pfaffl method of the Relative Expression Software Tool© (REST©) for Rotor-Gene© (27), which was developed in order for an easier calculation of relative quantification examination in real-time RT-PCR was employed for the Pair Wise Fixed Reallocation Randomization Test ©.

The relative expression ratio expressed as N-fold differences of the gene expression.

Extraction of protein

TRI reagent protein extraction protocol was used to acquire protein of the reagent organic phase. An aliquot (0.3 ml) of ethanol (100%) was added to the organic phase of the reagent at the time of sampling extraction, and the samples were stored at -20 ˚C until protein extraction.

The samples were defrosted and centrifuged (2000 g, 4 ˚C, 5 min) and incubated with isopropanol (1.5 ml) for 10 min at room temperature. Then it centrifuged (12000 g, 4 ˚C, 10 min) and the supernatant was poured out. The protein-containing pellets were then washed with the solution of 95% ethanol (200 µl) containing 0.3 M guanidine hydrochloride. Each wash was followed by centrifuging for 5 min at 7500 g. The protein pellets were incubated in 100% ethanol (20 min, 25 ˚C) and centrifuged at 7500 g (5 min, 4 ˚C).

After discarding the ethanol, the pellets were dried at room temperature, and dissolved in 1% sodium dodecyl sulfate (SDS) at 50 ˚C (28). The samples were then centrifuged (10,000 g, 4 ˚C, 10 min). The protein content was evaluated by the Bradford protein assay protocol (29).

Western blot

Western blotting was performed to evaluate prepro-NMS protein expression in the brain tissues at 24 hr following TBI. Samples were mixed with Laemmli buffer (0.125 M Tris HCl, 10% 2-mercaptoethanol, 0.004% bromophenol blue, 4% SDS, 20% glycerol), heated at 95 ˚C (5 min), and electrophoresed on polyacrylamide gel (15%) and transferred to a polyvinylidenedifluoride (PVDF) sheet (Roche, Mannheim, Germany). The Ponceau S staining solution was used to verify the transferring of proteins to the membrane. The membrane were immersed in a 2% non-fat dry milk in TBST (0.05% Tween-20, 0.1 M PBS) overnight at 4 ˚C in order to block nonspecific binding sites and immunolabeled with a 1:2,000 dilution of the antibody (rabbit anti-NMS IgG, Phoenix Pharmaceuticals Inc, Belmont, CA) for 1 hr at 37 ˚C (30). Afterward, the membranes were washed 3 times for 10 min in TBST [Tris (10 mM, pH 8.0), Tween-20 (0.05%), NaCl (150 mM,] on a roller mixer and incubated for 1 hr with goat anti-rabbit IgG peroxidase–conjugated at a1:10,000 dilution (Santa Cruz Biotechnology), followed by washing for 4 times in TBST. Protein bands were revealed by enhanced chemilu-minescence kit (Roche, Mannheim, Germany). The membranes were then striped and reblotted with mouse anti-β-actin antibody (1:1000, Sigma, USA) as a loading control. Image J (National Institutes of Health, USA) was used for analysis of band intensities.

Statistical analysis

Data were analyzed using one way analysis of variance for comparison among groups followed by LSD test for pairwise comparisons. GraphPad InStat tm Software (San Diego, CA, USA) was applied for Western blot analysis. A $P<0.05$ was considered statistically significant.

Results

Brain edema

Figure 1a illustrated that the percentage of brain water content in TBI-OVX was significantly more than those of the OVX group ($P<0.001$). Furthermore, TBI-P group showed less brain edema compared to the TOVX ($P<0.01$) group. Indeed, TBI-NP animals indicated a significant increase ($P<0.001$) in brain water content relative to that in NP group, whereas no difference in the cerebral water content was observed between TBI-NP and TBI-OVX groups.

The effect of different doses of female gonadal hormones on brain water content following trauma compared to the untreated animals is shown in Figure 1B. The percentage of brain water content in TBI-LE and TBI-LP was less than those of Veh ($P<0.05$) group. Furthermore, the brain water content in TBI-HE) and TBI-HP groups was statistically less than Veh group, while there was no significant difference in cerebral edema between TBI-HE, TBI-HP, and TP groups. Moreover, there was not any significant difference in the brain water content between TBI-LE and TBI-LP, and TNP groups.

Serum hormone measurements

As shown in Table. 1, the E2 serum level in TBI-P rats was significantly higher in comparison with that in the TBI-NP group ($P<0.001$). Furthermore, the serum level of P4 in TBI-NP rats was significantly lower ($P<0.0601$) relative to those of TBI-P group. Moreover, low and high doses hormone therapy produces serum levels of female gonadal hormones were equivalent to the basal and proestrus levels of that observed in rat estrous cycle, respectively. There were significant differences in E2 level between the rats

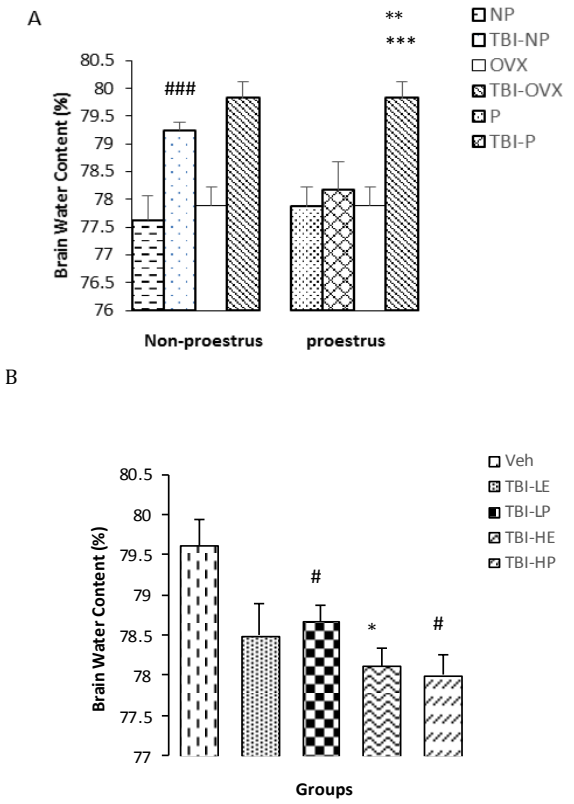

Figure 1. The brain water content (%) in different experimental groups before and after traumatic brain injury (n =6). The data are represented as mean±SEM. ###*P*<0.001 vs NP; ***P*<0.01 vs TBI-P; ****P*<0.001 vs OVX (A). #*P*<0.05 vs Veh; ##*P*<0.01 vs Veh; **P*<0.05 vs Veh; ***P*<0.01 vs Veh (B). TBI-LE (Traumatic+low estradiol), TBI-HE (Traumatic+high estradiol), TBI-LP (Traumatic+low progesterone), TBI-HP (Traumatic+high progesterone), Veh (vehicle)

treated with different doses of 17β-estradiol as well as in P4-treated rats and TOVX group.

The plasma levels of progesterone in traumatic-LP and HP rats was higher than of in TBI-OVX group. Whereas, the differences of progesterone between TBI-HP and TBI-P as well as between TBI-NP and TBI-LP groups were not significant. There was also a significant difference in serum levels of 17β-estradiol between the animals treated with low and high doses of estradiol and Veh group.

Immunoblot analysis

Changes in the expression of prepro-NMS is shown in Figure 2A, for the sham-operated and OVX rats in proestrus and non-proestrus, and traumatic groups before and after trauma.

The prepro-NMS expression decreased in non-proestrus animals after the removal of the ovaries, so that it was significantly lower in OVX group compared with that in the sham-operated animals (*P*<0.05). However, there were not found significant difference in prepro-NMS expression between the sham and other experimental groups.

In proestrus rats there was no significant difference in the expression of prepro-NMS between the sham and OVX group. It means that the removal of ovaries had no effect on expression of this protein. On the other hand, protein expression in traumatic animals whose ovaries has left intact has significantly increased compared to traumatic-ovariectomized group (*P*<0.01). The differences among the other experimental groups were not significant.

Figure 2B shows high expression of prepro-NMS in TBI-HP rats compared to the TBI-HE (*P*<0.05) as well as Veh group (*P*<0.01). However, no difference was observed between TBI-HE, and Veh groups. Prepro-NMS expression was also increased in TBI-LP in comparsion to the Veh groups (*P*<0.05). There was no significance difference between TBI-LE in comparison to the Veh group.

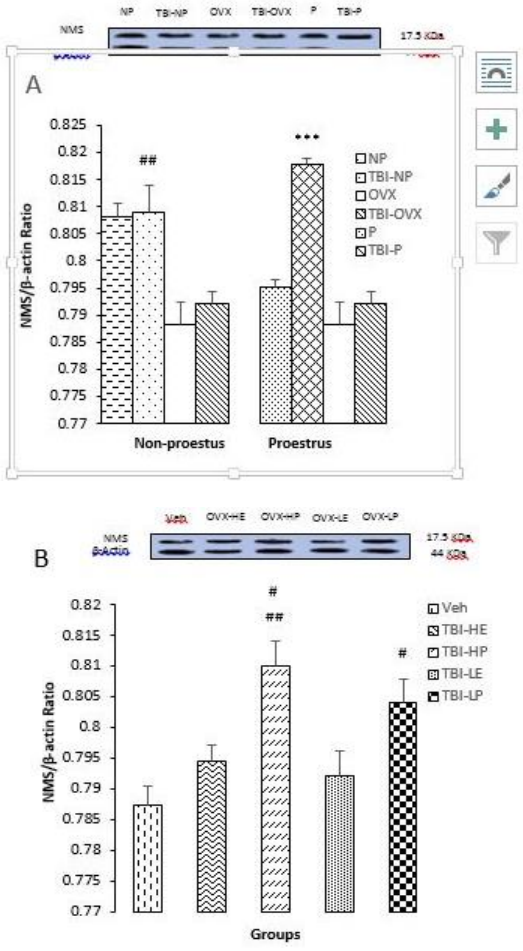

Figure 2. Western blot analysis of prepro-NMS protein expression before and after traumatic brain injury; ****P*<0.001 vs P and TBI-OVX. ##*P*<0.01 vs OVX (A). #*P*<0.05 statistical difference between TBI-HP and TBI-HE; ##*P*<0.01 statistical difference between TBI-HP and Veh. #*P*<0.05 statistical difference between TBI-LP and Veh (B). Data are expressed as mean±SEM. P (proestrus), TBI-P (traumatic proestrus), NP (non-proestrus), TBI-NP (traumatic non-proestrus), OVX (ovariectomized), TBI-OVX (traumatic ovariectomized). TBI-LE (traumatic+low estradiol), TBI-HE (traumatic+high estradiol), TBI-LP (traumatic+low progesterone), TBI-HP (traumatic + high progesterone), Veh (vehicle)

Table 1. Serum hormone levels (mean±SEM)

Group	17β-estradiol (pg/ml)	Progesterone (ng/ml)
TBI-NP	25.23±2[a]	25.92±1.3[a]
TBI-P	72.59±2.1	34.5±1.8
TBI-LE	21.48±0.6[b]	-
TBI-HE	67.21±3.8	-
TBI-LP	-	23.42±0.5[c]
TBI-HP	-	38.33±2.7
TBI-OVX	ND	4.8±0.9[d]
Veh	ND	-

TBI-NP, traumatic non-proestrus; TBI-P, traumatic proestrus; TBI-LE, low estradiol; TBI-HE, high estradiol; TBI-LP, low progesterone; TBI-HP, high progesterone; TBI-OVX, traumatic ovariectomized; Veh, vehicle. [a]*P*<0.001 compared to the TBI-P group; [b]*P*<0.001 compared to the TBI-HE, [c]*P*<0.001 compared to the TBI-HP group; [d]*P*<0.001 compared to the TBI-LP,TBI-HP,TBI-P, TBI-NP; ND, Not detectable. n = 6/group

ELISA data analysis showed no significant difference between P and TBI-P as well as between NP and TBI-NP rats (data are not shown). Figure 3 shows that NMU content in TBI-LE rats was more than Veh group (*P*<0.05). NMU content in TBI-LP group was also significantly more than that of Veh group (*P*<0.001). Furthermore, a significant difference in NMU content was observed between TBI-HP compared to TBI-HE, and Veh group (*P*<0.05).

Changes in the expression of NMUR2 mRNA is shown in Table 2, in proestrus and non-proestrus, for the sham, ovariectomized and traumatic groups before and after trauma.

The expression of NMUR2 mRNA decreased in non-proestrus animal after the removal of the ovaries, so that the expression of this protein in OVX group was significantly lower than that of in the sham-operated group (*P*<0.05). In addition, expression of this protein in the traumatic group was higher than ovariectomized traumatic group (*P*<0.05).

There was no significant difference in proestrus animals in NMUR2 mRNA expression between the sham and group OVX groups. It means that the removal of the ovaries had no effect on expression of NMUR2 gene.

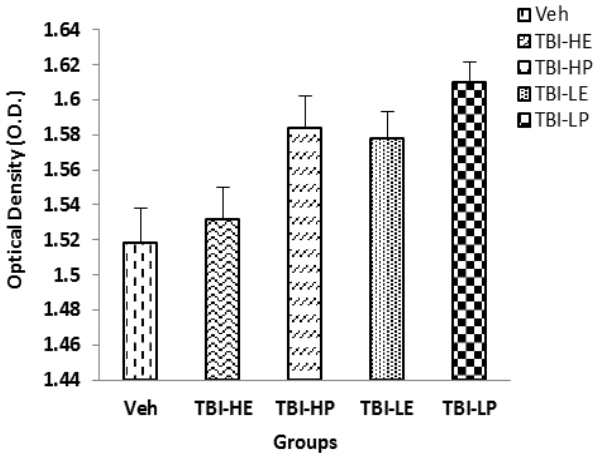

Figure 3. Comparative content of NMU at 24 hr after brain injury (n =6). # *P*<0.05 vs. TBI-HE and Veh. ###*P*< 0.001 vs Veh, *P<0.05 vs Veh. The values along the Y-axis represent ELISA absorbance Units. Data are expressed as mean±SEM. TBI-LE (traumatic + low estradiol), TBI-HE (traumatic + high estradiol), TBI-LP (traumatic + low progesterone), TBI-HP (traumatic + high progesterone), Veh (vehicle)

The expression of this receptor was also significantly increased in traumatic animals with intact ovaries compared to ovariectomized traumatic group (*P*<0.01). There was no significant difference between the other groups.

The real-time PCR analysis of the NMUR2 mRNA expression revealed an upregulation in TBI-HP rats (*P*<0.001, Table. 3) compared to the TBI-HE group. In addition, NMUR2 mRNA was significantly upregulated (4.8 fold increase, *P*<0.01) in TBI-LP than that in the TBI-LE rats. On the other hand, NMUR2 mRNA expression was significantly downregulated in Veh related to the TBI-LP (*P*<0.01) as well as TBI-HP groups (*P*<0.001). While, no significant difference was found between TBI-HE and TBI-LE rats compared with the Veh group.

Table 2. Relative NMUR2 gene expression in different experimental groups

Experimental groups \ VS.	P	NP	OVX	TBI-P	TBI-NP	TBI-OVX
TBI-NP	-	1.2	-	-	-	1.75[a]
TBI-P	3.15[b]	-	-	-	2.25[a]	4.39[c]
TBI-OVX	-	-	0.8	-	-	-
P	-	-	1.13	-	-	-
NP	1.9[a]	-	3.1[a]	-	-	-
OVX	-	-	-	-	-	-

Experimental groups \ VS.	Veh	TBI-HE	TBI-HP	TBI-LE	TBI-LP
TBI-LP	-	-	3.14[b]	-	-
TBI-LE		-	-	-	4.8[b]
TBI-HP	-	-	-	-	-
TBI-HE	-	-	12.46[c]	-	-
Veh	-	-	15.2[c]	-	3.65[b]

Values represent relative fold changes in NMUR2 gene expression between experimental groups, normalized by GAPDH as an internal control. [a]*P*<0.05, [b]*P*<0.01, [c]*P*<0.001. P (proestrus), NP (non-proestrus), OVX (ovariectomized), TBI-P (traumatic-proestrus), TBI-NP (traumatic non-proestrus), TBI-LE (traumatic + low estradiol), TBI-HE (traumatic + high estradiol), TBI-LP (traumatic + low progesterone), TBI-HP (traumatic + high progesterone), TBI-OVX (traumatic-ovariectomized), Veh (vehicle)

Discussion

In the present study, female rats were subjected to a diffuse model of TBI to determine whether the administration of physiological doses of female gonadal hormones associated with the changes in NMU, NMS and their receptor in the rat brain following brain trauma. The results showed that the administration of high physiologic doses of 17β-estradiol and progesterone equivalent to those found in proestrus stage of estrous cycle, attenuated cerebral edema after TBI. The observed effect can be attributed to the high serum levels of sex steroid hormones. The findings of the present study are: 1) prepro-NMS expression is greater in TBI-HP treated group relative to TBI-HE treated rats. 2) Both low hormone-treated groups have similar increase in brain NMU content compared to the OVX rats. 3) NMUR2 mRNA expression is upregulated in TBI-HP and LP-treated rats respect to corresponding groups treated by different doses of 17β-estradiol.

In our study, NMUR2 expression was significantly decreased in animals following ovariectomy. Furthermore, administration of progesterone elevated plasma levels of hormone to the physiological concentrations in OVX rats, resulted in the higher expression of prepro-NMS and NMUR2 compared to the both estradiol-treated and traumatic-untreated groups. Our results are in consistent with study of Yang *et al* (31) who showed that the expression of NMS mRNA and NMUR2 were highest in the proestrus stage in hypothalamus and pituitary of the pigs. The increase in NMS protein and its receptor in TBI-HP treated rats is interesting although its significance is unclear. It might be related to the neuroprotective effects of progesterone against cerebral edema formation in traumatic animals.

The activity of NMS is about 10 times stronger than that of the NMU, suggesting more important role of NMS in the brain (32). The rat NMUR2 gene is mostly expressed in the specific regions of the brain, such as ependymal layer along the wall of the third ventricle (8). This region is believed to play an important protective role in terms of edema formation (33). NMUR2 mRNA is also found in brain structures which lack a blood-brain barrier (BBB) such as subfornical organ as well as the vascular organ of the lamina terminalis (34). Expression pattern of the NMUR2 gene in the regions in contact with the cerebrospinal fluid and its increment in progesterone- treatment rats is consistent with the hypothesis that its ligand, NMS, may mediate protective effects of progesterone in reducing edema improvement.

Lipid peroxidation and free radical production are demonstrated to increase BBB permeability and edema formation (35, 36). Progesterone has been reported to reduce lipid peroxidation through multiple pathways including upregulation of antioxidant enzymes and increment the levels of free radical scavengers (37). The upregulation of prepro-NMS in progesterone-treated group may be another mechanism by which progesterone induces antioxidant effects. It has been suggested that intraventricular injection of NMS attenuates oxidative stress in the CNS (16). It has also been shown that NMS reduces lipid peroxidation and cerebral tissue damage (16). Since in the present study, estrogen did not make a difference in the expression of these neuropeptides and their receptor, other mechanisms can contribute its neuroprotective effect such as reducing the pro-inflammatory cytokine IL-1β or increasing the anti-inflammatory cytokine TGF-β (38), increase in antioxidant enzyme superoxide dismutase activity (39), or through the effect on both estrogen receptors α and β (40).

It has proven that NMS stimulates proopiomelanocortin (POMC) system in the arcuate nucleus of the brain (41). POMC is the precursor of α-MSH, a neuropeptide with regulatory feature of the immune system, that is involved in some regulatory effects of NMS in the CNS (41). It is demonstrated that α-MSH suppresses the expression of pro-inflammatory cytokines including interferon gamma (IFN-γ), tumor necrosis factor-alpha (TNF-α), interleukin-1, 6 and 8 (42, 43). These cytokines play important role in the development of cerebral edema after TBI (44, 45). In addition, α-MSH induces the expression of interleukin-10 that displays significant anti-inflammatory effects following brain injury (46). NMS acts as a part of a neurotransmitter system that is activated in response to the stress and has modulatory effects on stress responses. Animal studies demonstrate that NMS is released in response to the stressful stimuli. There are evidences that NMS increases both CRH and POMC mRNA expression (47). Since α-MSH is one of the production of POMC, it is likely that progesterone induces increase in α-MSH through an increase in NMS, which would in turn mediates the suppressive effect of progesterone on pro-inflammatory cytokines production such as IL1-β and TNF-α (38).

NMUR2 is expressed with vasopressin in the PVN and supraoptic nuclei (SON) (48). NMUR2 mRNA expression in specific neural populations can show that progesterone may modulate vasopressin secretion in part through the mechanisms involving changes in NMS and NMUR2 gene expression (49). It has reported that vasopressin plays an important role in water balance and control of intracellular volume in the brain (50). Therefore progesterone may modulate vasopressin secretion through an up-regulatory effect on NMUR2 expression (51). Through this way, it may control the water balance and edema formation after traumatic brain injury.

In another part of this study an increase in brain NMU content was found in the low hormone-treated

groups compared to that in the traumatic-untreated group. It means that both sex steroids in low doses can increase NMU content in the rat brain. This result is consistent with the study of Vigo *et al* (52) who found hormone capsule implantation for one week in ovariectomized rats induces an increase in the NMU mRNA expression to the levels found in cycling rats.

However, with administration of high doses of 17β-estradiol and progesterone, which is equivalent to the hormone levels in the proestrus phase of rat estrous cycle, the incremental content of this neuropeptide was only observed in progestsrone-replaced group. These results suggested the dose-dependent effects of estradiol on the rat cerebral NMU content. It means that estradiol may uses NMU-involved signaling pathways in low doses, and other signaling factors in high concentration in the brain.

The results of the present study are not consistent with the results of Vigo *et al* who has reported high levels of NMU in proestrus phase when the estradiol level is high (52). The probable reason for this discrepancy is that, in that study the effects of both gonadal hormones in proestrus stage were evaluated, while we investigated the effects of these hormones on the brain NMU content separately.

It is not clear how the brain edema is affected by the changes in NMU content. Previous studies has shown an anti-inflammatory effect for NMU which is mediated by an increase in hippocampal brain drive neurotrophic factor (BDNF) in response to lipopolysaccharide (LPS) induced neuroinflammation (15). Although this peptide has no effect on interleukins, it may inhibits the signaling pathways downstream of cytokines that are involved in the development of cerebral edema including cyclooxygenase-2 (COX-2), reactive oxygen species (ROS) and nitric oxide (NOS). These findings have suggested the anti-inflammatory action of NMU in the CNS. However, NMU is expressed in monocytes, dendritic cells and B cells in peripheral tissues and induces degranulation of mast cells in the acute phase of inflammation. It has suggested that NMU is one of the important activators induce inflammatory mediator release from mast cells (53). Therefore, it can deduced that these two neuropeptides as well as their receptor may mediate neuroprotective effects of progesterone following TBI, although their mediating role in neuroprotective effects of estrogen seems to be less probable.

Conclusion

In summary, our study showed that progesterone administration increased prepro-NMS and NMUR2 expression which were concurrent with its protective effect in traumatic animals. It is likely that such changes are involved in the induction of neuroprotective effects of progesterone. The mechanisms underlying this effect require further investigation.

Acknowledgment

We thank Dr Majid Fasihi Harandi and Mr Beydolah Shahouzehi for their technical support.

Conflict of interest

The authors declare that there is no conflict of interests. The results described in this paper were part of student thesis. This work was supported financially by the Kerman Neuroscience Research Center and Kerman Physiology Research Center, Kerman, Iran.

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