Effect of Progesterone Therapy on TNF-α and iNOS Gene Expression in Spinal Cord Injury Model

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Abstract- Spinal cord injury (SCI) as a destructive crash result in neurons degeneration. The SCI lead to the onset of biochemical and molecular cascades such as inflammation that in turn has a key role in neurodegeneration development. The previous studies demonstrated the role of TNF-α and iNOS genes in intensifying the process after SCI. As a consequence, these genes overexpression intensify the inflammation and neuron degeneration process. In the present study, 32 male Wistar rats were chased and divided into four groups of eight. The SCI were induced in three groups and another group used as a sham. The progesterone hormone used as a therapeutic agent in rats with SCI. The results showed that injection of 10 μg/kg/12h progesterone hormone reduced the TNF-α and iNOS gene expression significantly and confirmed the role of progesterone in the reduction of inflammation. Also, the numbers of intact neurons in progesterone group were higher than other groups that demonstrated the protective effects of progesterone on neuron death. The BBB test was performed and demonstrated that progesterone is an effective factor to the improvement of locomotor response. These results of the study confirmed the anti-inflammatory activity of progesterone hormone and suggested that it can be used as a therapeutic factor for SCI.

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Keywords: Spinal cord injury; Tumor necrosis alpha; Inducible nitric oxide synthase; Progesterone; Iran

Introduction

Traumatic spinal cord injury (SCI) may be caused by the traumatic crash to the spine that dislocates or compresses one or more of vertebrae. SCI has two mechanisms for initiating the injury in the spinal cord that leads finally to loss of neurons which can have bad consequences (1,2); SCI complications include complete loss of motor and sensory function (3,4). Besides, the ventral horn motoneurons show early degeneration and chromatolysis happens after SCI (primary death) which usually depends on the severity and type of injury (3,5,6).

Neuronal death continues after the onset of spinal cord injury for hours which is called secondary death and consists of series of cellular, molecular and biochemical pathways (7). These pathways could lead to cell death by different ways for example increased expression of TNF-α is seen in one of these pathways. Previous studies have evaluated the role of TNF-α in mechanism of SCI; the level of TNF-α rapidly increases after SCI and leads to initiation of neurons apoptosis in vitro (8,9) also, it is documented that TNF-α production reduces and accelerates functional recovery after SCI by the injection of IL-10 (as a very strong anti-inflammatory cytokine) (10). Thus, Lee *et al*., (2000) showed that TNF-α can act as an external signal of apoptosis after spinal cord injury (11), but the main mediators of apoptosis related to TNF-α have not yet been determined. The number of studies have focused on the role of nitric oxide (NO) in oxidative stress, neuronal injury, and degeneration of neurons (12-15) and demonstrated that TNF-α pathway leads to over-expression of the nitric oxide synthase (NOS) gene.

Yune *et al*., (2003) observed a peak of increased expression of TNF-α one hour after induction of SCI and after 4 hours saw a significant increase in the

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expression of NOS (16). As a consequence, the nitric oxide (NO) is overproduced and increase the potential severity of damages in spinal cord injury. To prove this hypothesis, the neuronal NOS were inhibited by 3-bromo-7-nitroindazole, and it was found that the severity of the injury was significantly reduced after spinal cord injury (17). Also, Yune *et al*., (2003) observed greatly reduced NOS expression after the injection of antibodies against TNF-α (16).

Neuroprotective effects of steroid hormones have been documented in numerous studies (18,19). The number of these steroids is increasing every day such as estrogen (20) and progesterone (21) which show the severity of CNS damage reduction and improving neuronal recovery after injury.

The curative effect of progesterone is confirmed after spinal cord injury and progesterone promotes recovery of neuronal injury in the spinal cord after SCI (22). Thomas *et al*., (1999) have demonstrated that locomotor activity of rats treated with progesterone is better than the control group, and also white matter damage was significantly lower (22). Protective mechanisms of progesterone have not yet fully known, but it is possible that the protective effects of progesterone are involved in inhibition of free radicals (23), regulation of selective genes expression or excitotoxicity (24).

Treatment of traumatic brain injury by progesterone affects various factors like reducing the NF-kB (Transcriptional regulator protein of inflammation and apoptosis factors) (25,26), reduction of TNF-α expression (an inflammatory factor that initiate various signaling pathways leading to apoptosis and demyelination) (25,27,28) and overexpression of Bcl2 (anti-apoptotic gene).

Briefly, SCI resulted in permanent damages by reduction and loss of the structural proteins of myelin which include NBP, PLP, and MOG; for this reason, mature oligodendrocytes are not able to induce myelin repair after injury (29-32), but Labombarda *et al*., (2009) demonstrated that after 3 days, remyelination begins when spinal cord injury was treated by progesterone (33).

The present study investigated the therapeutic effects of progesterone after the induction of spinal cord injury. Therefore in this study, TNF-α and iNOS expression and the effects of progesterone on the number of healthy neurons were evaluated.

Materials and Methods

This study was conducted on 12 weeks old male Wistar rats weighing 250 to 300 g (Pasteur, Iran). The animals were housed in cages and had free access to water and standard food for three days under controlled temperature with the light and dark cycle, 12h for each of them. Animal handling was performed in accordance with the guidelines of Iranian animal ethics society, Tehran University of Medical Sciences rules.

After being matched according to body weight, the rats were allocated to four groups of 8:

1) Sham group: with surgery without causing SCI.

2) SCI group: with spinal cord injury induced and without any treatment.

3) Progesterone group: with spinal cord injury induced and treated with a dose of 10 mg/kg/12h progesterone with sesame oil as a solution intraperitoneally.

4) Sesame oil group: with spinal cord injury induced and treated with sesame oil alone.

All study groups and gene expression were analyzed by Real-Time PCR.

Spinal cord injury was conducted with weight compression; briefly, anesthesia was first created by injection of ketamine (80 mg/kg) and xylazine (15 mg/kg), intraperitoneally. Then the unconscious reflex test was performed for confirmation of narcosis by severe pinch, and the surgical area was shaved, and rats were placed in the stereotactic frame. Laminectomy was performed at T10 vertebra by fine rongeur tool as the Dura mater was to remain intact during a laminectomy. The spinal cord was pressed by a 50 g weight for 5 min using a rectangular plate which was longitudinally oriented over the spinal cord; the plate had an area of 11.0 mm2 (2.2×5.0 mm) and a concave shape that ensured equal distribution of the pressure on the spinal cord tissue. The rats body temperature during the surgery were checked and controlled in the range of 36-37˚C and after induction of SCI by the mentioned method, the 3-0 Black Silk was used for suture of skin.

1ml ringer solution was intraperitoneally injected to each rat after the surgery for prevention of dehydration. Upon awakening, rats were neurologically evaluated and monitored for food and water uptake and urine output for 72h. The prophylactic antibiotics or analgesics were not used to prevent any possible interaction with the experimental therapy; the bladder was evacuated twice a day manually until a normal reflex was recurrent.

The functions of the posterior limb were scored between 0-21 (complete paralysis to normal) by Basso Beattie Bresnahan (BBB) test and finally rats with a score more than one were excluded from the study and only rats, with a score less than one, were selected for

the following analysis.

During this study, the rats were given special care, such as emptying the bladder, attenuation of pain, wound sterilization, adding the multivitamins in water and fruit to the diet, as well as urine appearance was checked for its color. After 72 hours, the rats were killed by normal saline perfusion and the laminectomy area was cut and extracted for the following analysis.

RNA extraction and real-time PCR

Total RNA was extracted from tissue with peqGold RNA TriFast kit (PeqLab, Germany) and the purity and quantity of extracted total RNA were evaluated by using spectrophotometer (Nanodrop 1000, PeqLap, Germany). Agarose gel electrophoresis (1%) was used for checking the RNA integrity and after confirming the quality and quantity of RNA, 1µg of it was used for cDNA synthesis by Qiagen kit (Invitrogen, Germany); cDNA was stored at -80°C after production.

Quantitative real-time PCR (qrtPCR) was carried out by using the MyIQ system (Biorad, Germany) and PCR kit.

The qrt-PCR was performed with incubation at 95°C for 5 min followed by 40 cycles of denaturation at 94°C for the 30s; Annealing at 66°C for 35s at 72°C, Extension for 30s at 72°C and then fluorescence was measured.

A list of used primers and analyzed genes is given in Table 1. Statistical analysis was carried out by SPSS v.21.A; $P<0.05$ was considered statistically significant.

Table 1. Primers and analyzed genes

Name of gene	Forward primer	Reverse primer
TNF-α	GGAGGGAGAACAGCAACTCC	TCTGCCAGTTCCACATCTCG
iNOS	TGGTTGAGGGGACTGGATTT	CCAACTCTGCTGTTCTCCGT

Results

In this study TNF-α gene expression within 4 groups was determined, and the results showed that this gene expression in Sham group was about 0.97 which was lower than the others. Furthermore, gene expression in both SCI and SCI-progesterone groups were 5.8 and 3.9, respectively where this result did not have any effect on the recovery of rats.

Considering iNOS gene expression among 4 groups revealed that this gene expression in Sham group was about 1 which had a significant difference in comparison with this gene expression in SCI group that suffered from spinal cord injury ($P<0.05$). Also, this gene expression in SCI group was 247. According to the results obtained in diagram 2, it was determined that after spinal cord injury treatment with 10 mg/kg/day doses of progesterone, this gene expression level was significantly reduced as compared to the SCI group ($P<0.05$). iNOS expression was about 65 in progesterone group.

According to the results obtained in figure 3, the number of intact neurons was evaluated with Optica microscope and Optica software among the studied groups. The number of intact neurons in Sham and SCI group were 615 and 180 respectively. This result was statistically significant ($P<0.05$); the number of intact neurons were 405 in progesterone group as compared to sham group, and the results were statistically significant ($P<0.05$).

The mature neurons were stained by immunohistochemical methods in all rats. Spinal cord stained slides determined that the density of positive NeuN neurons significantly reduces after induction of spinal cord injury and the density of positive neurons significantly increases in the spinal cord after treatment by progesterone hormone and the placebo group was exactly like the SCI group.

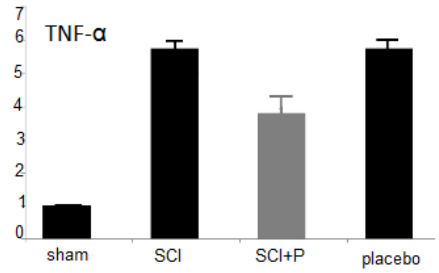

Figure 1. Expression of TNF-α in the spinal cord after SCI

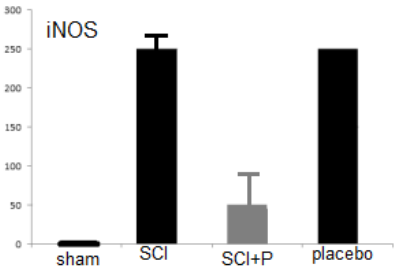

Figure 2. Expression of iNOS in the spinal cord after SCI

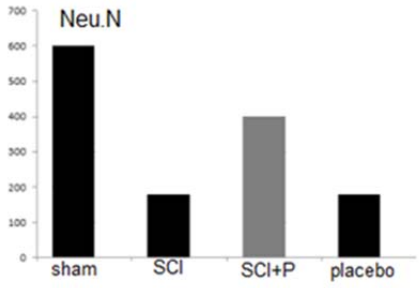

Figure 3. Number of healthy neurons in the spinal cord after SCI

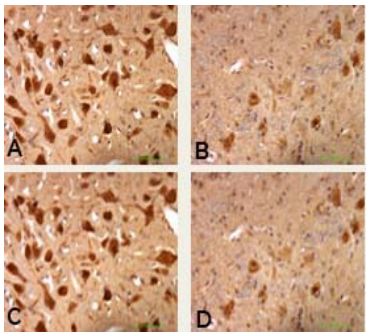

Figure 4. Photomicrograph of the spinal cord after immunohistochemical preparation for NeuN antibody. A: Sham; B: SCI; C: SCI + Progesterone; D: sesame oil. NeuN positive neurons are identified as brown cells. Scale bar:50 micrometers. Magnification: ×400

Discussion

SCI resulted in intense damages in people, and there is no cure for SCI; although the mechanisms for this injury are not fully identified, the role of inflammation and oxidative damage were specified in the process (34-37). The Inflammatory cytokines interferon γ, TNF-α, and IL-1β are needed to induce expression of NOS in transcription level. The transcription factors STAT1 (for interferon) and NF-kB (for TNF-α and IL-1β) are intermediate NOS activation (38-40). According to the previous data, directly and indirectly, anti-apoptotic roles of progesterone have been shown. In the present study, the impact of SCI was investigated on TNF-α and iNOS expression and also the protective effects of progesterone was evaluated after SCI including which reduced the expression of inflammatory genes and increased the number of healthy neurons.

The effects of progesterone injection were evaluated on the expression of TNF-α gene. TNF-α gene expression results in this study indicated that there was no spinal cord injury in Sham group, and this gene expression level was very low which indicates a lack of inflammation in this group. On the other hand, this gene expression in SCI group had noticeably increased where this increase was significant in comparison with Sham group. This result revealed that spinal cord injury induction causes inflammation and TNF-α gene expression increases. Thus it is likely that other cytokines and interleukin levels engaged in inflammation increase, too.

The results of this study were in line with previous results (41) (42,43). Also, Yune *et al*., (2003) observed a maximum levels of TNF-α one hour after SCI induction (16).

These results were in line with the results of Young B. Lee *et al*., (2000) where they observed that the amount of TNF-α increased an hour after spinal cord injury. They observed that treatment of SCI by antibodies against TNF-α significantly reduced the TUNEL positive cells as compared to the control group (11). Zendedel *et al*., at 2015 showed that TNF-α gene expression significantly reduce by injection of SDF-1to rats with SCI (44). Some studies have demonstrated that TNF-α was overexpressed around the contused area, 3 to 24 h after SCI (45,46) and the first inflammatory cytokine mRNA detected after SCI was TNF-α (47,48). Upregulation of TNF-α leads to intensify neuronal cell death in the rat spinal cord (49); however, some studies reported the anti-apoptotic properties of TNF-α (50-52).

Also in progesterone group after creating spinal cord injury in rats, prescribing progesterone with 10 mg/kg/day dose; this gene expression level was reduced more than SCI group which is an indicative of inflammation and probably spinal cord injury decrease, therefore, progesterone probably has protective effects against spinal cord injury and it can be assumed that it has stimulatory effects on its repair. TNF-α expression level decrease shows a significant difference in progesterone group than the others; this report provides evidence that is in line with results of previously reported findings. Drew *et al*., (2000) have demonstrated that production of pro-inflammatory cytokines TNF-α and INF-γ was suppressed by progesterone injection after SCI (53).

Considering of iNOS gene revealed that by creating injury in the spinal cord, the expression of iNOS significantly increased, and this result suggested that final product of iNOS gene (nitric oxide, NO) may be involved in the mechanism of spinal cord injury. These study results were supported by previous studies that showed the role of NO in inflammation and SCI (11).
Besides, this study data are compatible with Young B. Lee *et al*., (2000) who reported that SCI could lead to overexpression of NOS gene and the inhibition of NOS causes a significant reduction of apoptotic cells (11). On

the other hand, some researcher reported the role of TNF-α in activation of NF-kB and in turn overexpression of iNOS, and finally they produce the NO as a mediator of inflammation (11,12,54). Some studies reported that the iNOS gene is involved in the inflammatory process in CNS and estrogen pretreatment reduces iNOS expression and NO production from activated microglia (55-57). Yune *et al*., (2003) were observed a peak of increased expression of iNOS 4 hours after induction SCI (16).

The effect of progesterone post-treatment on iNOS expression are not completely clear, but in the present study, it was determined that progesterone post-treatment reduces the expression of iNOS.

In this study, for better understanding the therapeutic progesterone that significantly affects the created spinal cord injuries; the number of intact neurons was counted in different groups. The results obtained showed that the number of intact neurons was so high in Sham group with no spinal cord injury, but when the spinal cord injury was created, the number of these neurons decreased. There was a significant difference between the two groups. This result proved that the number of intact and active neurons were much lower in spinal cord injury state than the normal one. When progesterone was used for spinal cord injury treatment after a certain treatment period, the number of intact neurons was increased significantly, but the number of intact neurons had not increased to the extent of Sham group. However, the number of intact neurons in the placebo group was exactly like SCI group. This result determines that progesterone had an appropriate therapeutic effect on the increase of a number of neurons and spinal cord injury treatment.

The results of this study are in accordance with Du S *et al*., (1999) results which noted that SCI significantly reduced the number of neurons (58). Young B. Lee *et al*., (2000) concluded that TNF-α is likely an external message for apoptosis in neurons and probably NO is the major mediator of apoptosis mechanism, thus; inhibition of NOS can reduced apoptosis in neurons (11).

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