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

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Abstract- Spinal cord injury (SCI) is a crucial complication that results in neurons degeneration. The SCI lead to triggering of secondary complications such as inflammation that in turn has a key role in neurodegeneration development. The previous studies showed that TNF-α and iNOS genes expression increased significantly 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 estrogen hormone used as a therapeutic agent in rats with SCI. The results showed that injection of 10 μg/kg/12h estrogen hormone reduced the TNF-α and iNOS genes expression significantly and confirmed the role of progesterone in the reduction of inflammation reduce the inflammation. The numbers of intact neurons in Estrogen group were higher than other groups and showed that progesterone has protective effects on neuron death. The BBB test was performed and demonstrated that estrogen is an effective factor in the improvement of locomotor response. Our results suggested that estrogen hormone with anti-inflammatory activity can be an efficient agent for SCI complications therapy.

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

Introduction

Spinal cord injury (SCI) generally happens in the adolescent as a consequence of activity or games-related events; the traumatic SCI happens at around 10–60 cases per one million individuals annually (1). SCI is characterized by a two-step mechanism, the first step is mechanical damage that happens within a few minutes after SCI (2-4) and prompts serious neurological complications, for example, paraplegia and quadriplegia (5),the second step is a secondary damage activated by the initial injury leading to a series of problems such as urinary tract disease; cardiovascular and respiratory dysfunctions lead to morbidity and/or even mortality (up to 16.7%) (5), microvascular damage, edema, demyelination, ischemia, excitotoxicity, electrolyte imbalances, free radical production, inflammation and late apoptotic cell death (2-4). Inflammatory response after trauma has a key role in the secondary step mechanism of injury in SCI (6,7) and intensifies the spreading of the initial injury. On the other hand, it has been proven that reduction of the inflammatory response after a spinal cord injury can cause a decrease secondary damage (8-10).

Overexpression of cytokines, an important mediator of inflammation has been observed following by central nerve system (CNS; brain) damage (11,12).After inducing SCI, the expression of pro-inflammatory cytokines such as interleukin 1 (IL-1) and tumor necrosis factor α (TNF-α) are increased within the first few hours after injury (6,13,14). Up-regulation of TNF-α gene has been observed in spinal cord injury (6,15-17) but the sources of it are not well known. It is stated that damage to the surrounding cells can cause production and secretion of TNF-α (18-20) and inflammatory responses in traumatic injuries are developed and

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followed by increased necrosis (21).

TNF-α activates NF-kB downstream molecule which in turn up-regulate pro-inflammatory genes such as acute-phase proteins, CAMs, iNOS, interleukins, proteases, and others enhance the inflammatory activity (22-26).

Several studies have proven the protective effects of estrogen in the treatment of neurodegenerative disease of CNS such as Alzheimer's and Parkinson (27). The most common type of estrogen in the human body is 17-β-Estradiol; in different models of neurodegenerative CNS disease that can cause inflammation, estrogen has been appeared to be neuroprotective and curative (28-36). Estrogen effects as an antioxidant and anti-inflammatory agent, and the detailed mechanisms are not yet determined (37-41).

The aim of this study was to evaluate the effects of estrogen on the expression of TNF-α and iNOS after induction of spinal cord injury in Wistar male rats.

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 Science rules.

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

1) Sham group: with surgery without causing SCI.

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

3) Estrogen group: with spinal cord injury induced and treated with doses of 10μg/kg/12h estrogen intraperitoneally.

4) Sesame oil group: with spinal cord injury induced and treated with a dose of 1mg/kg/12h sesame oil intraperitoneally.

Spinal cord injury was conducted with weight compression. Briefly, anesthesia, at first, was created by injection of ketamine (80mg/kg) and xylazine (15mg/kg), intraperitoneally. Then the unconscious reflex test was investigated 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 remains intact during a laminectomy. The spinal cord was pressed by a 50 g weight for 5 min using a rectangular plate which is 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 surgery were checked and controlled in range 36-37˚C and after induction of SCI by the mentioned method, the 3-0 Black Silk was used for suture of skin.

After the surgery as prevention of dehydration,1ml ringer solution was injected intraperitoneally To each rat; upon awakening, rats were evaluated neurologically 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, and the bladder was evacuated twice a day manually until normal reflex was recurrent.

Within the duration of study the rats were considered for 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 color. After 72hours, the rats were killed by normal saline and fixative perfused, and the laminectomy area was cut and extracted for the following analysis.

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

RNA extraction and real-time PCR

Total RNA was extracted from tissue with peq Gold RNA TriFast kit (PeqLab,Germany); the purity and quantity of extracted total RNA were evaluated 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) and cDNA was stored at -80°C after production.

Quantitative real-time PCR (qrtPCR) was carried out using the MyIQsystem (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.value<0.05) was considered statistically significant.

Table 1. Primers and analyzed genes

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

Results

TNF-α gene expression between the groups revealed that the expression of TNF-α in Sham group (0.97) was lower than the other groups in figure 1.

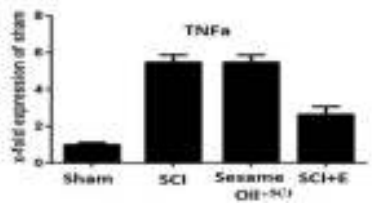

Figure 1. Expression of TNF-α in groups

This result was not statistically significant. Expression of TNF-α in the SCI (which had a spinal cord injury without any treatment) and SCI-estrogen groups were 5.8 and 2.6, respectively, this result was not statistically significant.

INOS gene expression in Sham and SCI groups were 1.1 and 237, respectively. This difference between groups was statistically significant(*P* value<0.o5 :).When spinal cord injury was treated with 20 mg/kg/day of estrogen, the expression level of INOS gene was reduced to 97 as compared to the SCI group which was statistically significant figure 2 (*P* value<0.o5).

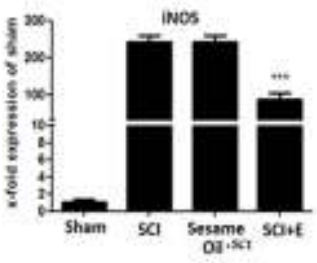

Figure 2. Expression of iNOS in groups

Enumeration of neurons was done using a light optical microscope and optical software. The number of intact neurons was evaluated among the three groups. in the sham group 615, intact neurons were counted. In the SCI and SCI-estrogen groups, intact neurons count was 180 and 360, respectively where the result was not statistically significant.

The intact neurons in SCI were significantly lower than Sham and SCI-estrogen groups' figure 3.

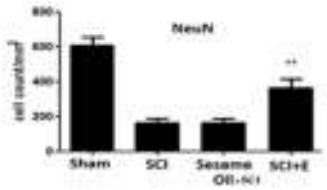

Figure 3. The number of healthy neurons in groups.
** The number of healthy neurons in estrogen group is significantly higher than SCI group

In all rats, the mature neurons were stained by immunohistochemical method in all rats figure 4. Stained slides of spinal cord showed that after induction of SCI in rats, the density of positive NeuN neurons was significantly reduced and following estrogen hormone therapy, the density of NeuN positive cells significantly increased in spinal cord (*P*.value<0.05) and neuronal density in SCI and sesame oil group reduced significantly (*P*.value<0.05).

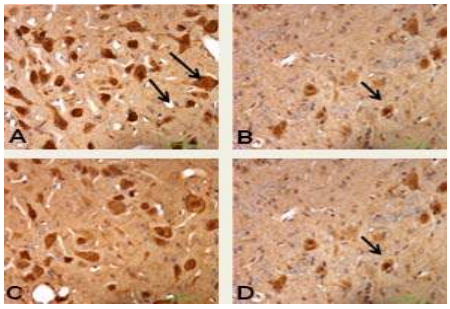

Figure 4. Photomicrograph of the spinal cord after immunohistochemical preparation for NeuN antibody
A: Sham; B: SCI; C:SCI + Estrogen; D: sesame oil. NeuN positive neurons are identified as brown cells. Scale bar:50 micrometers. Magnification: ×400

Discussion

Spinal cord injuries that lead to an irreparable neurological deficit, unfortunately, affect mainly younger people. The mechanism of this disease is not completely understood for this reason; its specific drug still has not been approved, and patients involved deal with many problems. Recently, the sexual hormones are

used for SCI therapy there for estrogen related damages as a treatment to attenuate the spinal cord injuries was evaluated.

This study has shown that TNF-α expression in Sham group without SCI was very low (0.97) indicating a lack of triggering an inflammatory reaction in this group but the expression of TNF-α in SCI group was significantly higher (5.8) than the Sham group. The study investigated the effects of SCI on the expression of the inflammation-associated gene (TNF-α), and it has been shown that SCI could lead to the onset of inflammation and overexpression of TNF-α; it is likely that the expression of other cytokines and inflammation associated interleukins increase after SCI. In the present study the increase of TNF-α expression after SCI was in line with results of previously reported findings by Jan Xu *et al*., (1998) (42), Bartholdi *et al*., (1997) (6), and Yakovlev *et al.,* (1994) (16). Zendedel *et al*., at 2015 showed that TNF-α gene expression significantly reduce by injection of SDF-1to rats with SCI (43).

It was also found that in estrogen group the expression of TNF-α was decreased as compared to SCI group, which probably reflect a reduction of inflammation in this group. This study data suggest that estrogen has a protective effect against spinal cord injury and may stimulate the repair of it. The expression of TNF-α in the estrogen group. There was a significant difference between the two groups.

The results of this study were in line with the results of Young B. Lee *et al*., (2000). In which the amount of TNF-α increases an hour after spinal cord injury (44). They observed that treatment of SCI by antibodies against TNF-α significantly reduced the TUNEL positive cells as compared with to the control group (44). This study has shown that estrogen has a curative effect on SCI related inflammation, and these results are in consistent with the results of the some studies that evaluated the effect of some drugs on SCI related inflammation (27,42,45,46).These studies show that an increase in TNF-α expression is an early event after SCI starting within hours after injury.

After induction of spinal cord injury in rats, the iNOS expression in the SCI group increased significantly as compared to Sham group. This result suggested that final product of iNOS gene (nitric oxide, NO) may be involved in the mechanism of spinal cord injury and confirmed the previous studies that showed the role of NO in inflammation and SCI (44).

Furthermore, the treatment of spinal cord injury by estrogen lead to a significant reduction of expression of iNOS as compared to SCI group (P.value<0.05). The reduced level of iNOS expression after treatment with estrogen could confirm protective effects of estrogen in the treatment of spinal cord injury. These findings are consistent with an anti-inflammatory role of estrogen in SCI (42,47). Besides, data are compatible with Young B. Lee *et al*., (2000) that reported the SCI could lead to overexpression of NOS gene, and the inhibition of NOS causes a significant reduction of apoptotic cells (44). 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 (44,48,49).

Some studies reported that the iNOS gene is involved in the inflammatory process in CNS and estrogen pretreatment reduces both the iNOS expression and NO production from activated microglia (50-52).

In the present study, the numbers of healthy neurons were counted in different groups to have a better understanding of effects of estrogen therapy on spinal cord injuries. This result indicates that Sham group, which had no spinal cord injury, had very high number of healthy neurons, but when the injury was induced the number of healthy neurons was significantly decreased compared to the Sham group (P.value<0.05). These results proved that the number of healthy and active neurons in the SCI is much lower than the normal. These results are in accordance with the previous studies that reported the role of TNF-α overexpression on increasing neuron apoptosis and decreasing the number of healthy neurons (44).

In this study estrogen was used for the treatment of spinal cord injury. After a period of treatment, the number of healthy neurons significantly increased as compared to SCI (P.value<0.05), but the number of healthy neurons in the estrogen group was lower than Sham group. These results showed that estrogen treatment could lead to an increase in a number of healthy neurons and could cure the spinal cord injury. This study results confirmed the Du S *et al*., (1999) results which noted that SCI significantly reduced the number of neurons (53). Young B. Lee *et al*., (2000)concluded that TNF-α is likely an external message of apoptosis in neurons, and NO is the major mediator of apoptosis mechanism. Thus, inhibition of NOS can reduced apoptosis in neurons (44).

This study data suggest that estrogen could significantly increase the number of a healthy neuron as a consequence of the reduction of TNF-α and iNOS genes expression after induction of SCI. In the current report, this study showed that both TNF-α and iNOS indirectly have a key role in the death of neurons.

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