Original Article

Related Fluoxetine and Methylprednisolone Changes of TNF-α and IL-6 Expression in The Hypothyroidism Rat Model of Spinal Cord Injury

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Abstract

Objective: Spinal cord injury (SCI) is a serious clinical condition that leads to disability. Following primary injury, pro-inflammatory cytokines play an important role in the subsequent secondary events. The thyroid hormone (TH) is known as the modulator of inflammatory cytokines and acts as a neuroprotective agent. Methylprednisolone (MP) is used for the early treatment of SCI. Fluoxetine (FLX), also is known as a selective serotonin reuptake inhibitor (SSRI), has therapeutic potential in neurological disorders. The aim of the present study was to investigate the combined effects of MP and FLX on SCI in the rat hypothyroidism (hypo) model.

Materials and Methods: In this experimental study, 48 male Wistar rats with hypothyroidism were randomly divided into 6 groups (n=8/group): control (Hypo), Hypo+Surgical sham, Hypo+SCI, Hypo+SCI+MP, Hypo+SCI+FLX, and Hypo+SCI+MP+FLX. SCI was created using an aneurysm clip and Hypothyroidism was induced by 6-Propyl-2-thiouracil (PTU) at a dose of 10 mg/kg/day administered intraperitoneally. Following SCI induction, rats received MP and FLX treatments via separate intraperitoneal injections at a dose of 30 and 10 mg/kg/day respectively on the surgery day and FLX continued daily for 3 weeks. The expression levels of tumor necrosis factor-alpha (TNF-α) and interleukin-6 (IL-6) were quantified by Real-time polymerase chain reaction (PCR) and Western blotting. Myelination and glutathione (GSH) levels were analyzed by Luxol Fast Blue (LFB) staining and ELISA respectively.

Results: Following combined MP and FLX treatments, the expression levels of TNF-α and IL-6 significantly decreased and GSH level considerably increased in the trial animals.

Conclusion: Our results show the neuroprotective effects of MP and FLX with better results in Hypo+SCI+MP+FLX group. Further study is required to identify the mechanisms involved.

Keywords: Fluoxetine, Interleukin-6, Methylprednisolone, Tumor Necrosis Factor-Alpha, Spinal Cord Injury

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Introduction

Spinal cord injury (SCI) is recognized as a serious disabling medical condition that could happen to anyone of either gender at any age. Therapeutic options for SCI patients are not successful and they could not return to normal life. Most of the patients have to live with moderate to severe disabilities leading to many economic, social, and health problems in the society (1). Epidemiological studies have shown an increasing incidence of SCI in different societies with around 318 people/million annually suffer from SCI in Iran (2). SCI still remains one of the greatest therapeutic challenges to the health system.

SCI has two pathological phases: primary or mechanical phase, and secondary or inflammatory phase. In the primary phase, mild to severe structural damage happens following initial trauma that in turn leads to neuronal and glial cell membrane disruption, cell death, and axonal degeneration. During this phase, the local blood-spinal cord barrier is also disrupted severely (3). Following the primary injury, a cascade of inflammatory events known as the secondary phase initiates. This phase might continue for weeks or even months by pathological features of subacute and chronic inflammation. The events of this phase dramatically influence the outcome and prognosis of SCI (4).

Various pro-inflammatory cytokines such as tumor necrosis factor-α (TNF-α), interleukin (IL)-1α, IL-1β,

and IL-6 that are released from the inflammatory activated cells play an important role in the progression and severity of the injury (5).

Simultaneous local ischemia and edema in the site of the injury and near area increase intracellular calcium that leads to the disruption of ionic homeostasis and activation of proteases (6). Accumulation of excitatory amino acids such as glutamate, in the extracellular matrix, causes further loss of neurons and glia. The final outcome of all these events is gliosis and cavity formation in the injured part of the spinal cord. Demyelination occurs in the site of injury that could extend far from the site to the adjacent distal and proximal spinal segments (7). Extensive and progressive death of the oligodendrocytes is reported during this phenomenon. The expression of TNF-α increases in neurons, glia, and endothelial cells after SCI. This up regulation mediated by TNFR1 and TNFR2 occurs earlier than the other cytokines (8). Previous studies have demonstrated that inflammatory response following changing vascular permeability causes neighboring undamaged cells to be exposed to harmful molecules such as nitric oxide (NO), reactive oxygen species (ROS), elastase, and matrix metalloproteinase-9 (MMP-9).

The release of TNF-α by microglia leads to glutamate release which in turn causes cell death and demyelination. The glutamate released by the astrocytes also stimulates the microglia to produce more TNF-α (9). Similarly, IL-6 is released by microglia, macrophages, astrocytes, and neurons following SCI leading to more infiltration and activation of microglia, macrophages and astrogliosis (10).

Thyroid hormones (THs) including T3 and T4 play important roles in regulating metabolism, neuronal development, and survival. During the development of central nervous system (CNS), an insufficient level of THs causes histological, biochemical, and behavioral deficiencies. It is shown that the THs are important agents for neuron and glial differentiation (11). Under the influence of T3, the oligodendrocyte precursor cells (OPCs) differentiate to mature oligodendrocytes in the spinal cord and subventricular zone that in turn stimulates myelination. As the oligodendrocytes play an important role in myelination in CNS, any disturbances in their function are important in neurological diseases such as SCI (12). Thus the presence of adequate THs level is important during normal development and pathological conditions, so any type of THs deprivation such as hypothyroidism cases could have a negative impact on the process of development and repair.

Following SCI, the use of corticosteroids is considered as the drug of choice in the early hours after injury. Methylprednisolone (MP) is known for its important inhibitory role during the second phase of SCI (13).

Although fluoxetine (FLX) is used as a selective serotonin reuptake inhibitor (SSRI) in depression, recently it has received more attention for its neuroprotective and antioxidant effects. FLX increases functional recovery following SCI by preventing MMP triggered by unknown mechanisms. It is reported that FLX declines gene expression of IL-6 and TNF-α and thus acts as an anti-inflammatory agent too (14).

To the best of our knowledge, the combined effects of FLX and MP on the expression of pro-inflammatory cytokines in SCI, in the absence of THs has not been studied. Therefore, the present study was carried out to find the possible protective effects of MP and FLX combination therapy on functional recovery, inflammation, and oxidative stress in the adult rat hypothyroidism model following SCI.

Materials and Methods

Animal model and groups

This experimental study was approved by the Ethics Committee of School of Medicine Shahid Beheshti Uneversity of Medical Sciences (IR.SBMU.MSP.REC.1395.444). Forty eight adult Wistar male rats (200-250 g) were attained from the Pasteur Institute of Iran and used in this study. The animals were maintained in individual cages in a 12 hours light/dark cycle in a standard housing environment with food and water ad libitum. The animals were randomly divided into six groups (n=8/group): control (Hypo), Hypo+surgical sham, Hypo+SCI, Hypo+SCI+MP, Hypo+SCI+FLX, Hypo+SCI+MP+FLX. In this study, all the methods used were approved by the Committee of Ethics in Animal Research of Iran University of Medical Sciences.

Hypothyroidism induction

To induce hypothyroidism the rats received 10 mg/kg/daily of 6-Propyl-2-thiouracil (PTU, Sigma, St. Louis) in distilled water (DW) intraperitoneally until the animals sacrificed. Three weeks later, ELISA (Diaplus Kit, USA) was done to evaluate the level of circulating T3 and T4 to confirm the model. T3, T4 hormone levels in hypothyroid groups significantly decreased following administration of PTU compared to normal rats from 79.3 ± 0.26 to 18.12 ± 0.43 ng/dl and from 3.17 ± 0.08 to 1.2 ± 0.15 µg/dl respectively.

Spinal cord injury surgery

SCI was performed for all the animals except the control group at the end of 3 weeks. To do SCI, an aneurysm clip (AESCULAP, Germany, Lot Num: 51105502) with a 70 g closing pressure was used (Fig.1A). The animals were anesthetized intraperitoneally by a mixture of ketamine/xylazine (80/10 mg/kg) (Alfasan/Rompun). Under the complete sterile conditions the vertebral column opened, the spinal cord was exposed and injured at the 9th to 10th

thoracic vertebral level. The incision site was sutured and received several washes. To inhibit infection, penicillin (Gibco, USA) was used. Twice daily manual compressing of the urinary bladder was done immediately on the day after surgery and continued until the normal function of the urinary bladder was restored.

Methylprednisolone and fluoxetine administration

MP (SOLU-MEDROL-500 mg, Pfizer Company, Belgium) treatment was administrated (30 mg/kg/IP) at 2, 4, and 6 hours after injury to the animals of Hypo+SCI+MP and Hypo+SCI+MP+FLX groups (15).

FLX (Sigma, St. Louis) treatment was performed (10 mg/kg/IP) immediately after SCI and continued daily for 3 weeks for Hypo+SCI+FLX group. The same dose and timetable were used for the animals of Hypo+SCI+MP+FLX group.

Behavioral analysis

Basso, Beattie, Bresnahan (BBB) behavioral test twenty one scaling based test was used to evaluate the movements of hindlimbs and severity of SCI in the period before surgery, the first day of the injury, and the first, second and third weeks after surgery. The rats with a score >4 were omitted from the study.

Histological study

At 6 weeks, transcardial perfusion and fixation with aldehyde solution of paraformaldehyde 4% and glutaraldehyde (Merck, Germany) 2.5% in PB (0.1 M, pH=7.4) were performed. The spinal cord at the proximal to the distal level of injury was removed, post-fixed, and embedded in paraffin (Merck, Germany). By using a rotary microtome (Leica- rm2235, UK), coronal sections of 5 µ were obtained.

Myelin area assay

To study myelination, Luxol Fast Blue (LFB) staining (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) with cresyl violet (Sigma-Aldrich, Germany) was utilised. Demyelinated regions in the total area of the spinal cord white matter were quantified by using Infinity software (Lumenera Corporation, Canada) (16).

Estimation of the volume of the spinal cord (gray matter)

The total volume of the spinal cord gray matter at two segments above and below the injury site was measured using Cavalieri's method with the following formula:

$$V=\Sigma P\times a/p\times t$$

where "V" is the distance between the sampled sections. The ΣP is estimated using the point-counting method. Where a/p is the area associated with each point projected on the spinal cord tissue (17).

Counting the number of motor neurons and glial cells

We used this technique to count both motor neurons and glial cells of the spinal cord at two segments above and below the injury site using the optical dissector method. The total number of neurons and glial cells was estimated by multiplying the numerical density (Nv) by the total volume (V) (17).

$$N\ (total)=NVv\times V\ (final)$$

RNA extraction and real-time polymerase chain reaction

The animals were sacrificed by decapitation within a few seconds. Under the aseptic conditions, the spinal cord was removed, located in sterile tubes, and snap frozen on dry ice. The measurement of total RNA extraction was done in accordance with the protocol by using RNX-plus (Cinnagen, Iran). The RNA samples were re-suspended in 30 µl of nuclease-free water. The purity of RNA was measured by NanoDrop ND-2000 spectrophotometer (Thermo Fisher Scientific, Wilmington, USA) and gel electrophoresis, with the OD260/OD280 ratio of all RNA samples 1.9-2.0 and OD260/OD230 ratio up to 2. cDNA was synthesized using PrimeScript™ RT reagent Kit (Takara, Japan). The comparative expression levels of genes TNF-α and IL-6 were analyzed in all groups using SYBR green-based real-time polymerase chain reaction (RT-PCR). The expression values of genes were normalized to the hypoxanthine phosphoribosyltransferase (*HPRT-1*) based on the usual procedure in real-time PCR. The primers used were (5′–3′):

HPRT-1-

F: GCTTGCTGGTGAAAAGGACC
R: TCCACTTTCGCTGATGACACA

TNF-α-

F: CCCTCACACTCAGATCATCTTCT
R: CCTTGAAGAGAACCTGGGAGT

IL-6-

F: ACTGCCTTCCCTACTTCACAA
R: AGTGCATCATCGCTGTTCAT

All RT-PCR reactions were performed in triplicate and the fold change was calculated by $2^{-(\Delta\Delta CT)}$ method.

Western Blot

The animals were sacrificed with a lethal dose of the ketamine and xylasine; subsequently the spinal cord was rapidly removed and frozen in liquid nitrogen and kept at -80°C until use. Afterward the samples were homogenized by an ice-cold lysis buffer containing Radioimmunoprecipitation assay buffer (RIPA) with protease inhibitor cocktail in a ratio of 1:10 for 1 hour and centrifuged (Eppen dorf, Hamburg, Germany) at

12000×g for 20 minutes at 4°C. The supernatant was removed and preserved. The concentration of protein was analyzed by using a Thermo Scientific NanoDrop 1000 spectrophotometer (Thermo Scientific, USA), and aliquots of 100 µg of protein for each sample. Then protein was denatured through a sample buffer containing TRIS-HCL, glycerol, bromophenol blue and mercaptoethanol at 95°C for 5 minutes.

The proteins were transferred to Hybond-PTM membrane and blocked with 5% non-fat milk. Subsequently, they were stained with anti- TNF-α and anti- IL-6 monoclonal antibodies (Sigma-Aldrich, Germany) and incubated with primary rabbit anti-rat diluted 1:300 (Abcam, USA), followed by a secondary alkaline phosphatase-conjugated anti-mouse antibody (Sigma-Aldrich, Germany) at a ratio of1:10000 for 1 hour. Bands were detected using the chromogenic substrate 5-bromo-4-chloro-3-indolyl phosphate in the presence of nitro blue tetrazolium. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibody (Sigma-Aldrich, Germany) was used to detect the endogenous standard for normalization. The bands of all of groupswere analyzed according to molecular weight. The details has been describd priviousley (18).

Glutathione measurement

The assay was based on the glutathione (GSH) and 5, 5′-dithiobis (2-nitrobenzoic acid) (DTNB) reaction. The resultant yellow color was measured spectrophotometrically at wave lengths of 412 nm with an ELISA Reader (Statfax 100, USA). Measurement of serum GSH concentration was estimated from standard GSH levels with colormaterial assay kit (zellbio ELISA kit by Cat No: ZB-GHS-96A., MD, and Germany). The results were represented as nmoles of GSH/ mg of protein.

Statistical analysis

Data were analyzed and presented as mean ± SEM using SPSS 19 (IBM SPSS, UK). Statistical analysis was performed using the Graph Pad Prism version 8 (Graph Pad Software Inc., San Diego, CA). The difference among the groups was analyzed by One-way ANOVA followed by Tukey's post-hoc multiple comparison test. P<0.05 was considered to designate a statistically significant value.

Results

Neurological functional score of BBB test

The motor function of both hindlimbs of SCI animals was estimated using BBB behavioral test in order to locomotor recovery measurement for 3 weeks after SCI (Fig.1B). As it is shown in Figure 1B, the result of the BBB test for the animals of surgical sham and control groups was normal. The BBB result for the SCI animals was less than score 4. Following treatment significant (P<0.05) increase in the BBB score was detected in the animals of Hypo+SCI+MP and Hypo+SCI+FLX, compared to the animals of SCI group, suggesting functional improvement (11.80 ± 0.90/10.32 ± 0.71 vs. 7.5 ± 0.80). Better functional recovery (P<0.01) was observed in the animals of Hypo+SCI+MP+FLX (16.2 ± 0.92).

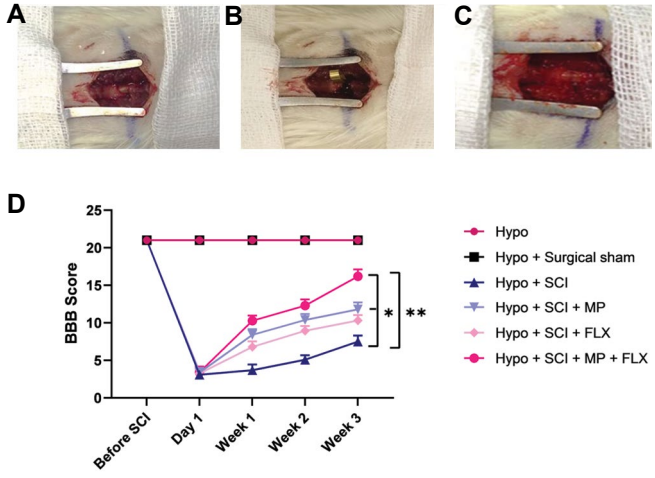


Fig.1: Spinal cord injury model and BBB test. **A.** Spinal cord compression injury was done with an aneurysm clips. **B.** Laminectomy was made at T9-10 vertebral level. Compression model created by a clip. **C**. The injured site was observed as a cyanosis line**. D.** BBB Score in different groups to confirm the model of spinal cord injury. Data were expressed as mean ± SD. *; Significant difference between Hypo+SCI group vs. Hypo+SCI+MP and Hypo+SCI+FLX groups (P<0.05), **; Significant difference between Hypo+SCI group compared to Hypo+SCI+MP+FLX group (P<0.01), BBB; Basso Beattie Bresnahan, Hypo; Hypothyroidism, SCI; Spinal cord injury, MP; Methylprednisolone, and FLX; Fluoxetine.

Histological study and cavity formation

Histological changes including cavity formation, density, morphology of the ventral horn neurons and remarkable demyelination were evaluated. These parameters are shown in Figure 2. All treated SCI animals were compared with control and surgical sham groups. A significant demyelination (P<0.01), larger and more cavities in SCI animals were noted compared to the all three treated groups (45.51 ± 13.84 vs. 20.14 ± 5.53/28.27 ± 3.04/17.5 ± 7.48). As Figure 2 shows, among the treated groups, better results were observed in the animals of the Hypo+SCI+MP+FLX group (P<0.05).

Stereological Cavalieri method for gray matter volume

By using the Cavalieri method, the total volume of the gray matter of the spinal cord at two segments above and below the injury site was calculated in all groups. As shown in Figure 3A, there was a significant (P<0.05) difference between SCI animals compared to the treated groups (2.66 ± 0.61 vs. 3.21 ± 0.34/3.20 ± 0.27/3.81 ± 0.25 nm^3) with better results for the combination treatment by MP and FLX.

Stereological counting of motor neurons and glial cells

The total number of motor neurons and glial cells in the ventral horns of the spinal cord at two segments above and below the injury site were counted separately. Quantitative stereological analysis revealed a significant decrease (P<0.01) in the number of motor neurons in the SCI group (without treatment) compared to the treated groups (84.62 ± 10.20 vs. 136.23 ± 10.92/126.48 ± 11.19/147.37 ± 11.91 nm^3), the hypo and sham groups (P<0.01, 84.62 ± 10.20 vs. 179.08 ± 18.87/163.23 ± 20.01 nm^3). Better results were seen for the animals of Hypo+SCI+MP+FLX (Fig.3B). The number of glial cells in all treated groups was significantly lower than SCI animals. Among the three treatment groups the number of these cells was lower but not significant in Hypo+SCI+MP+FLX animals compared to MP and FLX

alone (Fig.3C).

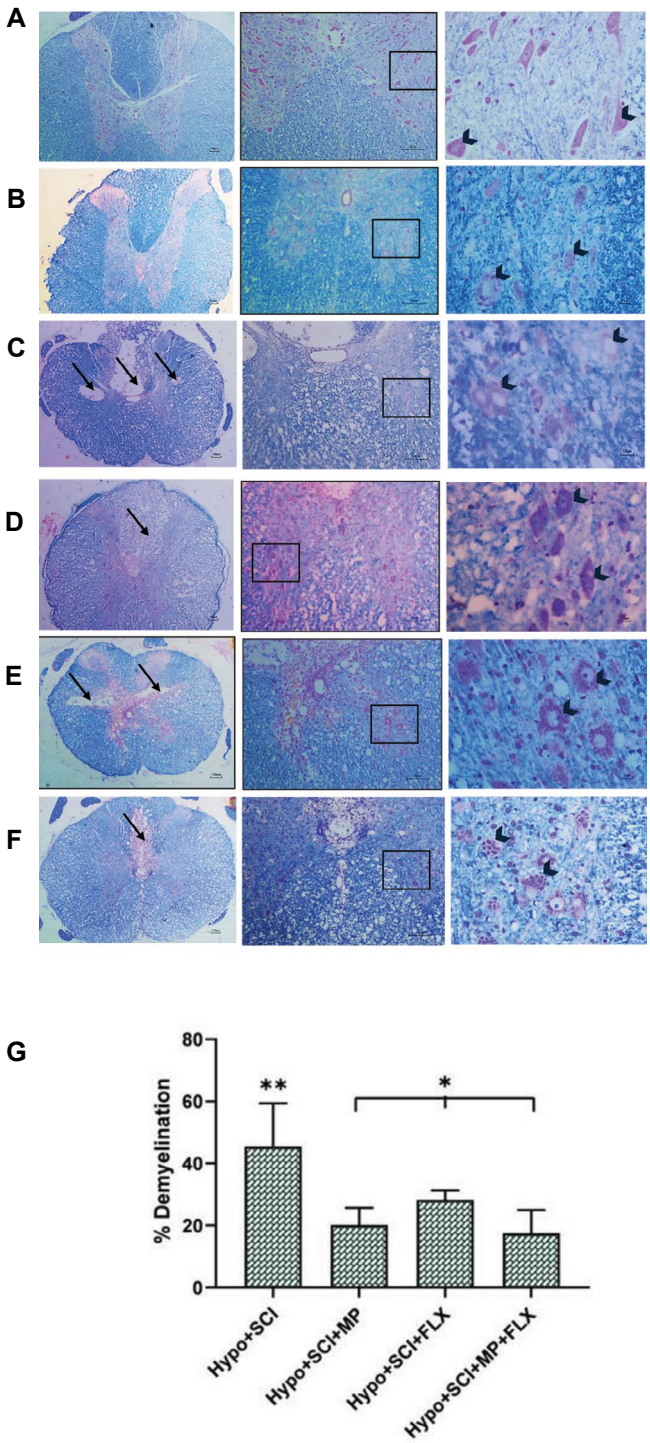


Fig.2: LFB stained from a cross section of the spinal cord. There was no histological alteration in **A.** Hypo group, **B.** Surgical sham group. Cavity formation 3 weeks after SCI was observed in **C**. Hypo+SCI group. In **D.** Hypo+SCI+MP, **E**. Hypo+SCI+FLX, **F.** Hypo+SCI+MP+FLX groups cavity area decreased compared to Hypo+SCI group. Arrow indicated cavitation. Ventral horn neurons reveal normal morphology in Hypo, Surgical sham, Hypo+SCI+MP, Hypo+SCI+FLX, Hypo+SCI+MP+FLX groups vs. Hypo+SCI group that shows few nissl bodies (scale bar: 10 µm). Arrowhead indicated nissl bodies. **G.** Percentage of demyelination in SCI groups. Data were expressed as mean ± SD. *; Significant difference between treatment groups (P<0.05), **; Significant difference between Hypo+SCI group vs. treatment groups (P<0.01), LFB; Luxol Fast Blue, Hypo; Hypothyroidism, SCI; Spinal cord injury, MP; Methylprednisolone, and FLX; Fluoxetine.

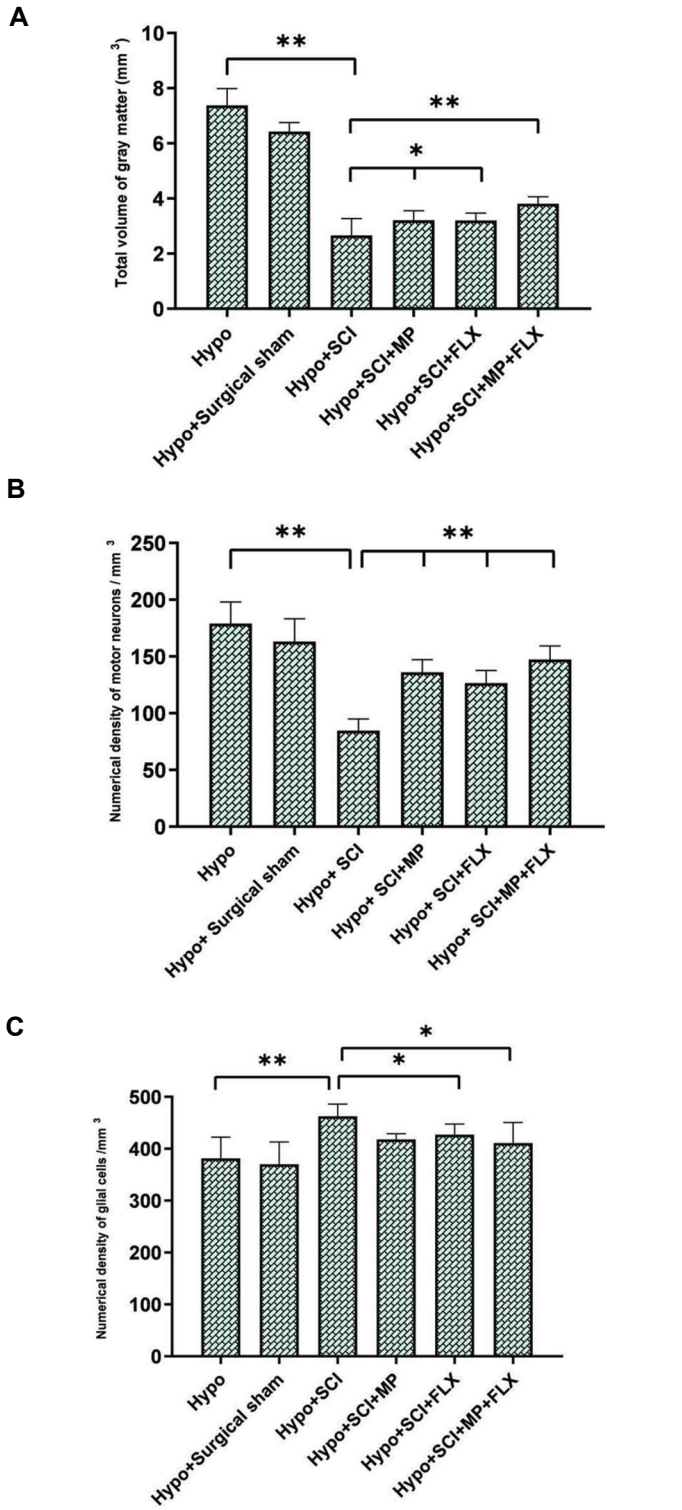
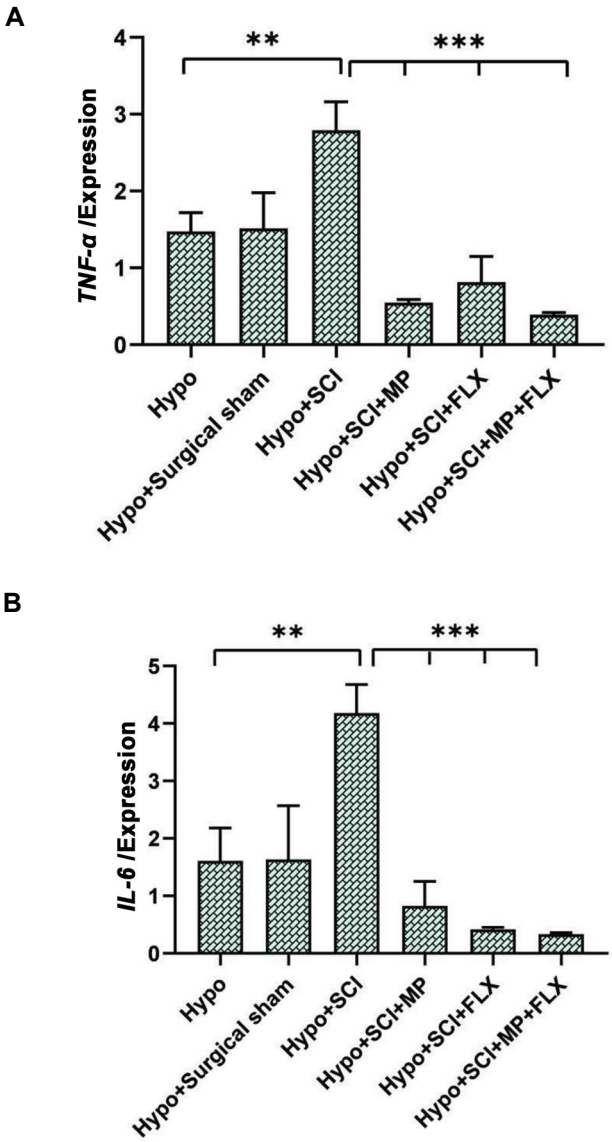


Fig.3: Stereological assessment method for spinal cord tissue. **A.** The Cavalieri method for gray matter volume was used for the total residual volumes of gray matter. *; Significant difference between Hypo+SCI group vs. Hypo+SCI+MP and Hypo+SCI+FLX groups (P<0.05) and **; Significant difference between Hypo+SCI group vs. Hypo, Surgical sham and Hypo+SCI+MP+FLX groups (P<0.01). **B.** Stereological counting of motor neurons. The number of surviving neurons was lower in the Hypo+SCI group than in the Hypo and surgical sham groups (P<0.01). **; Significant difference between Hypo+SCI group vs. treatment groups (P<0.01). **C.** Stereological counting of glial cells. The number of glial cells in all treated groups was significantly lower than Hypo+SCI animals. Data were expressed as mean ± SD. *; Significant difference between Hypo+SCI group vs. Hypo+SCI+FLX and Hypo+SCI+MP+FLX groups (P<0.05), **; Significant difference between Hypo+SCI group vs. Hypo and surgical sham groups (P<0.01), Hypo; Hypothyroidism, SCI; Spinal cord injury, MP; Methylprednisolone, and FLX; Fluoxetine.

Real-time polymerase chain reaction

RT-PCR was used to identify the expression of *TNF-α* and *IL-6* mRNA. The expression of *TNF-α* and *IL-6* significantly increased in the SCI group in comparison to the control and sham groups (P<0.01). Following treatment with MP, FLX and the combination of both the expression of *TNF-α* and *IL-6* significantly decreased with a greater reduction in animals receiving both FLX+MP (P<0.001, Fig.4A, B).



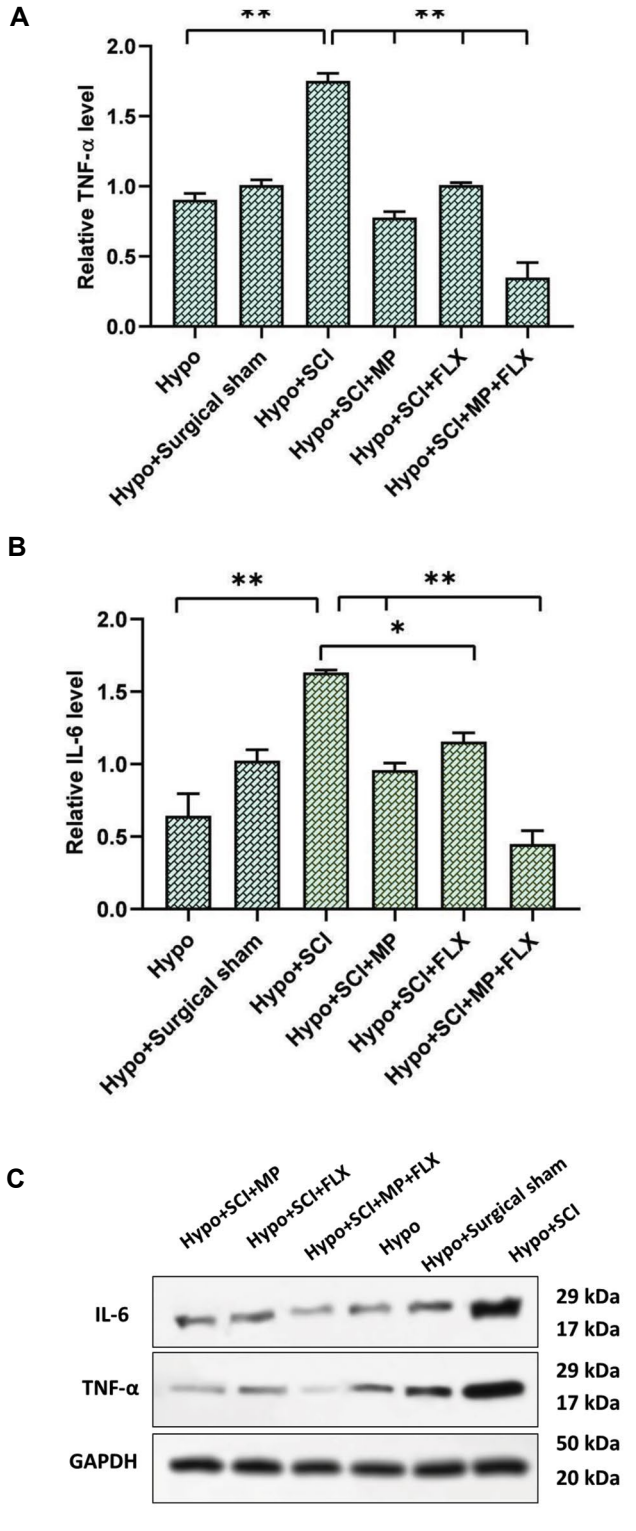


Fig.4: Real-time PCR for the expression of *TNF-α* and *IL-6* mRNA. **A.** The expression of *TNF-α* was increased remarkably in Hypo+SCI group compared to Hypo and surgical sham groups (P<0.01). Following treatment, the *TNF-α* expression reduced significantly compared to Hypo+SCI group (P<0.001). Between treated groups better results were seen in Hypo+SCI+MP+FLX group. **B.** The expression of *IL-6* was increased considerably in Hypo+SCI group compared to Hypo & Surgical sham groups (P<0.01). The *IL-6* expression after treatment decreased significantly compared to Hypo+SCI group (P<0.001). Among treated groups better results were seen in Hypo+SCI+MP+FLX group. **; Significant difference between Hypo+SCI vs. Hypo group, ***; Significant difference between Hypo+SCI vs treatment groups, PCR; Polymerase chain reaction, TNF-α; Tumor necrosis factor-alpha, IL-6; Interleukin-6, Hypo; Hypothyroidism, SCI; Spinal cord injury, MP; Methylprednisolone, and FLX; Fluoxetine.

Fig.5: Western blot analysis was used to show the expression of TNF-α, IL-6. **A.** The expression of TNF-α in Hypo+SCI group was increased compared to other groups (P<0.01). Significant decline was seen in treated groups compared to other groups (P<0.01). **B.** As it is shown the expression of IL-6 in Hypo+SCI group significantly was higher than other groups (P<0.01). Treatments lead to significant reduction of IL-6 expression with a better result that was seen in Hypo+SCI+MP+FLX, Hypo+SCI+MP, and Hypo+SCI+FLX respectively. **C.** Western blots of IL-6 and TNF-α in surgical sham and Hypo groups compared to the Hypo+SCI group and treated groups. *; Significant difference between Hypo+SCI vs Hypo+SCI+FLX, **; Significant difference between Hypo+SCI vs other groups, TNF-α; Tumor necrosis factor-alpha, IL-6; Interleukin-6, Hypo; Hypothyroidism, SCI; Spinal cord injury, MP; Methylprednisolone, and FLX; Fluoxetine.

Western blot

Western blot was used to assess the expression level of TNF-α and IL-6 proteins. In the Hypo+SCI group, the expression of both proteins was significantly higher than other groups, and a significant decline was seen following treatment with MP, FLX, and MP+FLX ($P<0.01$). A significant difference for TNF-α and IL-6 was observed between the Hypo+SCI group compared to Hypo and Sham groups ($P<0.01$, Fig.5A). The expression of IL-6 in the SCI group compared to Hypo+SCI+FLX animals was significantly ($P<0.05$) and other treatment groups ($P<0.01$). The lowest significant expression of IL-6 was seen in Hypo+SCI+FLX animals ($P<0.05$, Fig.5B). Western blot was detected to confirm the expression level of TNF-α and IL-6 proteins in the injury site of the spinal cord in all groups (Fig.5C).

Glutathione measurement

To evaluate the neuroprotective effects of MP and FLX, the level of GSH in the spinal cord at the level of two segments above and below injury was studied. As Figure 6 shows, the level of GSH significantly decreased in SCI compared to sham and control groups ($P<0.05$). Following treating with MP, FLX solely, and MP + FLX the level of GSH increased significantly with no differences among treated groups ($P<0.01$).

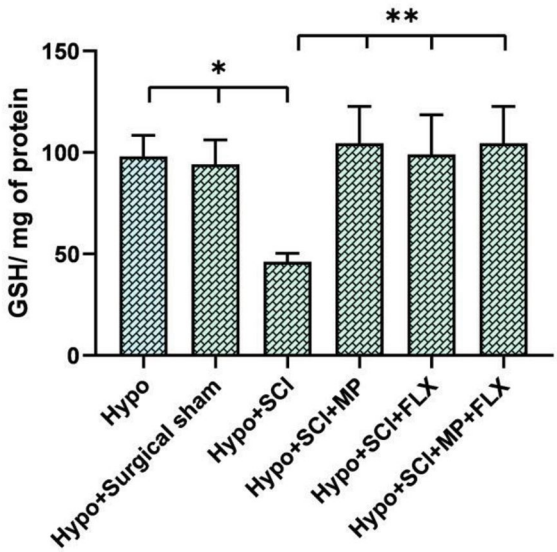


Fig.6: The level of GSH following spinal cord injury. In Hypo+SCI group, the activity level of GSH was significantly declined. Whereas rats treated with FLX and MP revealed a significant increase ($P<0.01$) compared to Hypo+SCI rats. The level of GSH in Hypo+SCI was significantly decreased ($P<0.05$) compared to Hypo and sham groups. *; Significant difference between Hypo+SCI vs. Hypo and sham groups, **; Significant difference between Hypo+SCI vs treatment groups, GSH; Glutathione, Hypo; Hypothyroidism, SCI; Spinal cord injury, MP; Methylprednisolone, and FLX; Fluoxetine.

Discussion

In the present study, for the first time, we studied the functional and histological changes and recovery process following SCI in the absence of THs in the rat hypothyroidism model. THs are mainly metabolic hormones. Lack of adequate level of these hormones in the neuro embryonic period results in histological, biochemical and behavioral abnormalities as well as deficiency in the nervous tissue (19). THs have neuroprotective effects that continue throughout life (20). Accordingly, we hypothesized that any decrease in the level of THs might lead to an increase in the vulnerability of the nervous system injuries. In order to evaluate this hypothesis, we induced SCI in rats with hypothyroidism to study the degeneration and repair processes in the absence of THs.

The pathology following of SCI is complex and includes two continuous primary and secondary steps. The secondary step determines the final outcome of repair and therapy (21). The acute inflammation that happens in this phase leads to a chronic process accentuating the symptoms of SCI. Because of the events of this phase, most of the therapeutic strategies focus on suppressing inflammatory cytokines mediators (22). The expression and activity of various cytokines such as TNF-α changes at the site of injury. The early expression and up-regulation of TNF-α in the site of are considered valuable markers in SCI (23). So the suppression of TNF-α activity could be considered as a strategic therapeutic procedure.

Decrease of the TNF-α expression by using CoQ10 following SCI reported by Hassanzadeh et al. (18). MP has the same effects on the expression of TNF-α in the primary phase (24). The decrease of TNF-α and nitric oxide expression in SCI following administration of atomoxetine was reported (25). Similarly, our data confirmed the decrease of TNF-α expression in the injured spinal cord tissue following administration of MP.

It is shown that TNF-α plays important roles in myelin degeneration, apoptosis, and astrocyte toxicity. Moreover, it stimulates neutrophils to release IL-8 which in turn leads to a strong inflammatory response (9). TNF-α hyperactivity destroys tight junctions of BBB and BSCB proteins, increases the permeability, and activates nuclear factor-kappa B (NF-κB) signaling (26). Regarding the role of TNF-α, the results of the present research showed a remarkable increase in the expression of TNF-α in Hypo+SCI group that is in line with other researches. IL-6 is considered one of the pro-inflammatory mediators that plays an important role in the SCI secondary phase. Overexpression of IL-6 leads to cellular events that strengthen the inflammatory process. IL-6 releases from microglia/macrophages, astrocytes and neurons following SCI and consequently binds to its receptors and enhances gliosis (10). IL-6 involves in various inflammatory reactions steps. IL-6 leads to the activation and infiltration of neutrophils, monocytes, macrophages, and lymphocytes. Curcumin administration decreased the level of IL-6 after SCI reported by Ni et al. (27). A remarkable reduction in iNOS, IL-6, and IL-10 by MP administration in the SCI model was shown by Li et al. (28). Our study showed the effectiveness of MP and FLX on reducing IL-6 expression. The result of the combined

use of FLX and MP+FLX was better than MP alone suggesting both MP and FLX, have anti-inflammatory effects via inhibiting TNF-α expression. Although using MP for the SCI cases is not new, its specific mechanism of action is not fully understood.

Based on other reports it seems that MP acts via various mechanisms including inhibiting the free oxygen radicals generation, resisting the peroxidation of lipids, improving microtubule circulation, reducing intracellular calcium influx, preserving the blood spinal cord barrier, reducing vasogenic edema and maintaining the excitability of neurons (29). It also increases spinal cord blood flow, changes the electrolyte concentration, decreases the expression of iNOS, IL-6, IL-10, and TNF- α , inhibits endorphin release, reduces free radical availability, and the inflammatory response (30). Fluoxetine, a selective SSRIs, is widely prescribed in the treatment of depression. New evidence suggest that SSRIs enhance neural plasticity, neurogenesis and synaptogenesis (31, 32). FLX has anti-inflammatory, antiapoptotic and antioxidant abilities (33). Novio et al showed neuroprotective effects of FLX against microglial activation due to neurotoxicity (34). Antioxidant potential of FLX on cerebral inflammation was shown by Kalogiannis et al. (35). The anti-inflammatory effect of FLX was reported in various experimental studies.

Antidepressants like FLX inhibit the secretion of pro-inflammatory cytokines such as IL-1b, IL-2, TNF-α, and IFN-γ, the proliferative activity of T cells and the cytotoxic activity of natural killer cells (36). The effect of FLX on activated microglia was shown by Liu et al who reported that FLX promotes reduction in IL- 6, TNF-α, and nitric oxide.

The molecular mechanism by which FLX acts is at least partially through reducing transcription levels IL-6 and TNF-α mRNA. In addition, it may act via inhibiting the phosphorylation of MAPK as a signaling pathway of pro-inflammatory cytokines and by activating nuclear factor kappa B.

Regarding the role of microglia in the early phase of SCI, it is believed that the mentioned effects of FLX on activated microglia play an important role in inhibiting the progression of inflammation (14). An increase in Akt, CREB, BDNF, Bcl-2 and BAD mRNA in the rat brain following administration of FLX and olanzapine was shown by Reus et al. (37). Although the anti-oxidant action of FLX is not fully known, it seems that FLX acts through the direct effects on mitochondria via suppression of ROS production (38).

Based on the results of our study GSH level increased in groups that received FLX. The effect of FLX on the increase of GSH level in the mice's cerebral cortex was reported by Moretti et al. (39). The combination use of FLX with non-steroidal drugs such as indomethacin, celecoxib, and ibuprofen, on suppression of the inflammation was reported (40).

In this study, for the first time the results of administration of MP and FLX, either alone or combined were reported. Although FLX was used with non-steroidal drugs, we think that the combination of FLX with corticosteroid drugs such as MP might have better therapeutic effects than either being used alone or with non-steroidal drugs. How the combination use of MP+FLX leads to better results is not clear to us and we did not study the possible mechanisms, however, MP and FLX may act synergistically with almost the same antioxidant, anti-inflammatory, and anti-apoptotic pathways but further research is needed.

Conclusion

To our knowledge, this research is the first experimental evidence evaluating the therapeutic effect of MP with FLX in traumatic SCI in the absence of THs. It seems that the combination of MP and FLX increased functional recovery besides the beneficial anti-inflammatory and anti-oxidant effects on injured spinal cord tissue. Our results confirmed neuroprotective effects of MP and FLX with better results for Hypo+SCI+MP+FLX group. However, more research needs to be done to identify the exact possible action mechanisms.

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Authors' Contributions

S.B.J., M.S., M.N.; Participated in study design, evaluation, performed editing and approving the last version of this manuscript for submission. A.Z., M.S; Contributed to all experimental work, data, and statistical analysis. M.A.A; Participated in data collection and evaluation. F.F.F; Was responsible for study design and evaluation. S.H.; Performed spinal cord injury model in this study and drafting. E.E.; Conducted molecular experiments and RT-qPCR analysis. M.H.F; Contributed in interpretation of doses of drugs and data. M.S; Contributed extensively in the conclusion and also in the finalization of the manuscript and approved the final draft. All authors read and approved the final manuscript.

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