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What are the progesterone-induced changes of the outcome and the serum markers of injury, oxidant activity and inflammation in diffuse axonal injury patients?

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ABSTRACT

To permit appropriate targeted therapy, the present clinical study was aimed to investigate the effects of progesterone on the outcome and the serum markers of injury, oxidant activity and inflammation in diffuse axonal injury (DAI). Forty-eight male DAI patients were divided into two groups (control and progesterone). Progesterone group received progesterone in dose of 1 mg/kg per 12 h for five days. The outcome was investigated using Extended Glasgow Outcome Scale (GOS-E) and functional independence measure (FIM). The markers of inflammation [interleukin-1β (IL-1β), IL-6, transforming growth factor-β1 (TGF-β1)], injury (brain protein of S-100B), and oxidant activity [malondialdehyde (MDA)] were evaluated in the serum of the patients. Higher GOS-E and FIM scores were observed in progesterone group at the six-month follow-up ($P < 0.05$ and $P < 0.01$, respectively). Meanwhile, a reduction in the serum levels of IL-1β, MDA and S-100B was noticed in progesterone group 24 h after injury ($P < 0.05$, $P < 0.001$ and $P < 0.05$, respectively), and there was an increase in serum levels of IL-6 and TGF-β1 ($P < 0.01$ and $P < 0.05$, respectively). Also, lower levels of MDA and S-100B, and higher levels of TGF-β1 were observed in progesterone group six days after injury ($P < 0.05$). According to these findings, progesterone may improve the outcome in DAI patients probably through modulation in the levels of cytokines, and reduction in the injury and oxidant activity.

1. Introduction

Traumatic brain injury (TBI) is a leading cause of death and severe disability around the world and results in large direct and indirect costs to society. Diffuse axonal injury (DAI) is one of the most common types of TBI, and accounts for about half of severe TBIs [1]. Although the clinical management of TBI has been greatly improved for the development of standards of care, no medical treatment has been proven to be effective in reducing death or disability following TBI [2].

TBI results in both primary brain injury immediately caused by an initial impact and secondary brain injury caused by cellular and molecular responses to a primary injury. These responses comprise releasing free radicals, neuroinflammation and apoptosis leading to brain edema and delayed neuronal death which are considered to worsen a primary brain injury and to influence the neurologic outcome of patients [3,4]. Therefore, major opportunity for interventions to limit neurological defect is reversing or preventing a secondary brain injury [5].

As secondary brain injury mechanisms are complex and various, simultaneous targeting of several injury factors using multipotential drugs may improve the outcome of TBI patients [6]. After three decades of extensive research on progesterone in TBI, it is known that this neurosteroid affects multiple mechanisms involved in neuroprotection and repair after various types of brain injury [7,8]. Although the clinical benefit of progesterone has been suggested in three phase II randomized controlled clinical trials for TBI patients [9–11], two phase III clinical trials have not displayed the efficacy of progesterone in TBI [12,13]. The adverse events attributable to progesterone drug have not been reported in TBI patients [10,11].

It seems that TBI studies should be focused on the molecular mechanisms of injury, rather than merely clinical observations. In recent years, TBI studies have been concentrated on whether brain-derived substances detectable in biological fluids could be useful as efficacy

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markers of therapeutic interventions [14]. A blood brain barrier (BBB) disruption causes either entering of peripheral proteins into cerebrospinal fluid (CSF) or leakage of CSF proteins that both can provide biomarkers of TBI [15]. Accordingly, S-100B is best known as a CSF/serum marker of injury in TBI [16,17], and is a calcium-binding protein physiologically produced and released by astrocytes in central nervous system (CNS) [18]. The concentration of this protein increases in CSF and serum following cerebral injuries [19], and can be useful as a serum biomarker of injury in DAI patients [20,21]. Malondialdehyde (MDA) is a noxious product of lipid peroxidation due to acting reactive oxygen species (ROS) which increase BBB permeability in TBI [22], moreover studies have reported a considerable increase in the production of ROS in TBI [23,24]. Because of short half-life of ROS, products of acting ROS including MDA are used for the estimation of ROS [25]. Damage to brain membrane lipids is an early event in brain injury [26]. An increase in brain levels of cytokines in patients with brain injury also causes neuroinflammation and damage of BBB leading to the releasing of cytokines into systemic blood circulation [3,27].

Progesterone reduces lipid peroxidation leading to the maintenance of membrane integrity and stabilization of BBB in experimental brain injury that improves the outcome [28–31]. Moreover, progesterone suppresses inflammation in preclinical models of TBI by modulating cytokine release and by inhibiting immune cell activation and migration [32–34]. Also, it has been previously indicated that progesterone reduces brain edema and BBB permeability following experimental TBI [35], and these effects were associated with reducing interleukin-1β (IL-1β), TNFα, IL-6 and increasing transforming growth factor-β1 (TGF-β1) in the brain [36,37].

Since the results of the performed clinical trials of progesterone in TBI are paradoxical, the effect of progesterone on the neurological outcome of DAI patients remains unknown [9–13]. Also, TBI biomarkers can indicate appropriate therapeutic strategies to minimize secondary brain injury and improve the development of individualized treatment, thereby reducing poor outcome [38]. Thus, considering the above, we designed a clinical trial to determine the effects of the early administration of progesterone on the outcome, and injury, oxidant activity and inflammation markers in moderate and severe DAI patients. To this end, firstly, the outcome was assessed using Extended-Glasgow Outcome Scale (GOS-E) and functional independence measure (FIM) at a three- and six-month follow-up. Secondly, injury (S-100B), oxidant activity (MDA) and inflammation (IL-1β, IL-6 and TGF-β1) markers were evaluated using serum collection at the time of admission, and 24 h and six days after injury.

2. Materials and methods

2.1. Patients and study design

The study conducting and reporting were according to Good Clinical Practice and CONSORT Guidelines [39] (Fig. 1). The study protocol was approved by ethics committee of Kerman University of Medical Sciences (K/92/579) and registered in Iranian Registry of Clinical Trials (www.irct.ir, CT2014042017356N1). This prospective, single-blind study was performed in the trauma main center of Kerman province, called Shahid Bahonar Hospital from May 2013 to July 2015.

The male patients with a non-penetrating TBI were selected on the basis of eligibility and exclusion criteria by a physician that was not informed to study design. The eligibility criteria were Glasgow Coma Scale (GCS) score of 12 or less, DAI using computed tomography (CT) scan, admission within four hours after injury, and 18 to 60 years of age. The exclusion criteria were a life expectancy of less than 24 h, a prolonged hypoxemia (partial pressure of arterial oxygen, <60 mmHg), hypotension (systolic blood pressure, <90 mmHg), selection for surgery, craniotomy, presence of other diseases and spinal cord injury at the time of randomization, and other traumas during DAI. An informed consent was taken from the patients' relatives.

In the current study, forty-eight male patients who were selected on the basis of eligibility and exclusion criteria were randomly placed in case (received progesterone) or control (DAI) groups. The sample size was estimated by PASS and NCSS software using values from relevant studies [11]. Randomization was performed using random digit

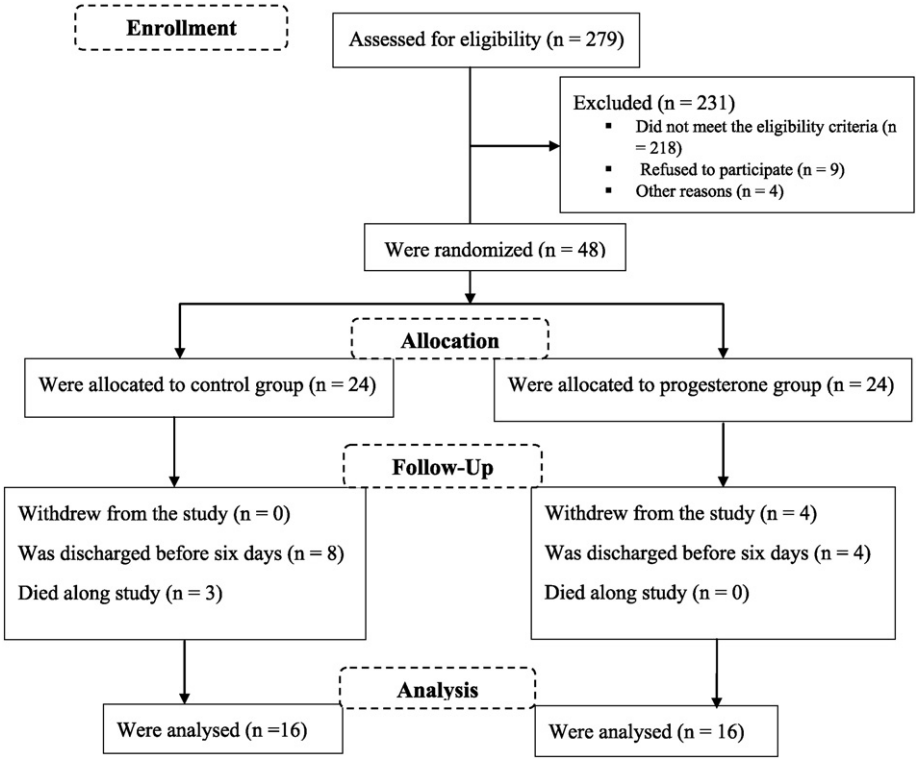

Fig. 1. Diagram of the phases of a parallel randomized trial for two groups of DAI patients (study enrollment, randomization, follow-up, and data analysis). n: number of patients.

numbers. A CT scan was performed for all the patients at admission time [11].

Blood samples were drawn at three time points (3 mL of blood in each time) from all the patients. To obtain the value of serum markers in time of injury, a blood sample was drawn at the time of admission. The second and third blood samples were drawn at times of 24 h and six days after injury, respectively. We performed the evaluation of serum markers at times of 24 h and six days after injury because it has been shown that activation of the inflammatory cascade in injured area does not reach its peak before 24 h in TBI [40] and also, because of the period of progesterone administration. The blood samples were centrifuged at 4000 *g* for 10 min to collect serum. The serum markers that were index of injury (S-100B), oxidant activity (MDA), and inflammation (IL-1β, IL-6 and TGF-β1) were evaluated in the present research by an experimenter who was blind to the samples of the study.

GOS-E and FIM were assessed at three and six months after injury. GCS, body temperature, heart rate, respiratory rate, blood pressure, and blood oxygen saturation were recorded during stay in hospital. And, laboratory tests including hematology, coagulation profile and biochemistry were performed daily until one week after injury.

Progesterone (Aboureihan Pharmaceutical Company, Iran) in case group was received intramuscularly 1 mg/kg every 12 h, for five days [11], and all of the study patients received standard clinical management protocol for TBI [41]. The adverse events of progesterone administration were checked in progesterone group according to Table 1.

2.2. Evaluation of outcomes

The primary outcome was analyzed according to GOS-E and FIM scores six months after injury. A GOS-E score of 1 indicates death, 2 indicates a vegetative state, 3 or 4 indicates a severe disability, 5 or 6 indicates a moderate disability, and 7 or 8 indicates a good recovery [42]. An FIM score is a measure of dependence in 18 domains (13 motor and 5 cognitive) [43]. Motor domains are scaled 1 to 7, except two domains (sphincters control) that are scaled 1 to 2. Cognitive domains are scaled 1 to 3, except two domains (conception and expression) that are scaled 1 to 6. The minimum FIM score is 18, and the maximum is 102.

The secondary outcome was assessed using GOS-E and FIM scores three months after injury. A GOS-E useful score shows an evaluation of function and dependence, and an FIM score determines cognitive function, motor function, and daily activities [11]. Thus, in the present study, the functional outcome was evaluated using GOS-E and FIM scores.

2.3. Determination of serum level of MDA

MDA is a well-known indicator of lipid peroxidation, and its level was obtained using the thiobarbituric acid method [44] and using a standard curve prepared by tetramethoxypropane. Briefly, the serum was precipitated by 10% trichloroacetic acid (TCA), and the pink color resulting from thiobarbituric acid reaction, as index of lipid peroxidation, was measured at 535 nm [31]. The level of MDA was expressed as micromoles per milliliter (μmol/mL).

2.4. Assay of serum levels of cytokines (IL-1β, IL-6 and TGF-β1)

Enzyme-linked immunosorbent assay (ELISA) kits of IL-1β (sensitivity range: 3.91–250 pg/mL), IL-6 (sensitivity range: 9.38–600 pg/mL), and TGF-β1 (sensitivity range: 31.3–2000 pg/mL) relevant to human were purchased (DuoSet ELISA Development System, R&D Systems, Minneapolis, USA) and the manufacturer's protocols were followed. The serum levels of cytokines in all the samples were assessed in triplicate. Intra- and inter-assay precision tests were also performed, and the accuracy of these kits was quantified by recovery experiments. It was indicated that about 90%–98% of a recombinant human cytokine standard can be determined. The serum levels of IL-1β and IL-6 were quantified as picograms of antigen per milliliter of serum (pg/mL), and TGF-β1 as nanograms of antigen per milliliter of serum (ng/mL).

Table 1
Adverse events checked in progesterone-administered patients with DAI.

Thromboembolism
Deep vein thrombosis (DVT)
Pulmonary thromboembolism (PTE)
Blood pressure

2.5. Assay of serum level of brain protein (S-100B)

The serum level of S100B protein was measured by a commercially human S-100B ELISA kit (sensitivity range 41–2000 pg/mL) ((BioVendor, Brno, Czech Republic). A quantitative assay was done using the secondary antibody labeled with peroxidase. After adding a peroxidase substrate to microplate, a colored product was produced to read in a spectrophotometer. The serum level of S-100B was expressed as picograms per milliliter (pg/mL).

2.6. Determination of serum level of progesterone

The serum level of progesterone was measured using human progesterone ELISA kit (Ideal Tashkhis, Iran) (sensitivity range: 0–60 ng/mL) according to the manufacturer's protocol. Inter- and intra-assay coefficients of variations were 9 and 5.7%, respectively.

2.7. Statistical analysis

The data were expressed as mean ± SEM, or frequency and percentage. The data normality was checked by Shapiro–Wilk's test. There was normal distribution only for variables of TGF-β1, MDA, S100 and GOS. But all of the quantitative variables were expressed as mean ± SEM for simplicity and clarity. A mixed design analysis of variance (ANOVA) was performed to evaluate an interaction between the times of the variables and the groups. The comparison of the variables among the times was analyzed using Friedman test and two way repeated measures ANOVA after checking normality. When sphericity was violated, Greenhouse–Geisser correction was used (interaction between groups and times, $P < 0.001$). Therefore, this comparison was analyzed by paired t-test or Wilcoxon test according to normality. The comparison between the groups for the quantitative variables was performed by two independent-tests or Mann–Whitney-U-test after checking normality. And, the qualitative variables between the groups were compared by Chi-square test or Fisher's exact test after checking normality. At $P \leq 0.05$, the difference was considered significant.

3. Results

3.1. Study patients

A total of 279 male patients with non-penetrating TBI were screened during the study. And, the patients ($n = 48$) meeting the eligibility criteria were randomly placed in progesterone ($n = 24$) or control ($n = 24$) group. The patients who stayed in the hospital for less than six days were excluded from the study due to their unavailability (25%). Also, four patients (8.33%) who were in progesterone group withdrew from the trial before the completion of the drug administration period. Thus, the data were available for 32 patients (66.67%). In control group, one patient died in the three-month follow-up, and two patients died in the six-month follow-up. The mortality of the patients was attributed to heavy head injury. These three patients who died in control group were inserted in the analysis. In contrast, there was no mortality in progesterone group (Fig. 1). The demographic and clinical characteristics of progesterone and control groups in the admission

Table 2
Comparison of the clinical and demographic characteristics between progesterone and control groups at the admission time.

Characteristics	Control (n = 16)	Progesterone (n = 16)	P value
Age (years); mean ± SEM	30.75 ± 3.4	28.44 ± 1.74	0.84
Injury time to randomization (min); mean ± SEM	84.81 ± 9.93	69.36 ± 19.38	0.98
GCS (score); mean ± SEM			
Total	7.75 ± 0.52	7.5 ± 0.55	0.81
Moderate	10.4 ± 0.51	10.5 ± 0.87	>0.99
Severe	6.5 ± 0.28	6.5 ± 0.34	0.98
Cause of DAI; n (%):			>0.99
Motor vehicle	15 (93.8%)	16 (100%)	
Fall	0 (0.0%)	0 (0.0%)	
Other	1 (6.2%)	0 (0.0%)	

The data are also presented as n (%); n: number of patients; GCS: Glasgow coma scale; DAI: diffuse axonal injury.

time are presented in Table 2. These characteristics did not differ significantly between the two groups.

3.2. Progesterone effect on outcome

The comparison of the outcome between progesterone and control groups is shown in Table 3. A quantitative analysis of GOS-E was performed between treatment and control groups. At the admission time, GOS-E score was not different between the two groups ($P = 0.78$). Also, GOS-E score between the two groups was not significantly different at the three-month follow-up ($P = 0.16$). But, GOS-E score of the patients who took progesterone was higher than that in control group at the six-month follow-up ($P < 0.05$).

FIM score was not different between the two groups at the admission time ($P = 0.18$). Meanwhile, three months after the injury, FIM score was not different between progesterone and control groups ($P = 0.21$). However, higher score of FIM was found in progesterone-administered DAI patients in comparison with control group six months after the injury ($P < 0.01$).

A significant increase in GCS score was observed in each of progesterone and control groups six days after the injury and the discharge time in comparison to the admission time. There was a significant increase in GOS-E and FIM scores in each of progesterone and control groups at the times of three and six months after the injury in comparison to the admission time. Meanwhile, a significant increase in GOS-E and FIM scores was observed only in progesterone group at the time of six months after the injury in comparison to the time of three months. There was no significant difference in body temperature, heart and respiratory rates, blood pressure, blood oxygen saturation, and laboratory testing between progesterone and control groups. No serious adverse effects were found attributable to the study drug.

3.3. Progesterone effect on serum level of MDA

The effect of progesterone on the serum level of MDA in DAI patients is shown in Fig. 2. A significant difference in MDA level was not seen between control and progesterone groups at the time of admission ($P = 0.07$). However, 24 h and six days after the injury, a reduction in MDA level was indicated in progesterone group (0.88 ± 0.23 μmol/mL and 2.00 ± 0.23 μmol/mL, respectively) compared to control group (2.56 ± 0.28 and 2.66 ± 0.22 μmol/mL, respectively) ($P < 0.001$ and $P < 0.05$, respectively). MDA reduced in progesterone group compared to control group 65.62% and 24.66% at 24 h and six days after the injury, respectively (Fig. 2). There was a significant increase in MDA level in control group at the times of 24 h and six days after the injury compared to the admission time ($P < 0.01$). Also, a significant decrease in MDA level in progesterone group was found 24 h after the injury compared to the times of admission and six days after the injury ($P < 0.05$).

3.4. Progesterone effect on serum levels of cytokines (IL-1β, IL-6 and TGF-β1)

The effect of progesterone on the serum levels of cytokines in DAI patients is shown in Fig. 3. The serum level of IL-1β is illustrated in Fig. 3A. A significant difference in IL-1β level was not seen between control and progesterone groups at the times of admission and six days after the injury ($P = 0.38$ and $P = 0.23$, respectively). But, a reduction in IL-1β level was revealed in progesterone group (22.62 ± 6.2 pg/mL) in comparison to control group (95.96 ± 30.20 pg/mL) 24 h after the injury ($P < 0.05$). IL-1β reduced in progesterone group in comparison to control group 76.42% at 24 h after the injury (Fig. 3A). A significant decrease in IL-1β level was found in progesterone group during the evaluation in comparison to the admission time ($P < 0.01$, at 24 h and six days). Whereas, a significant decrease in IL-1β level was revealed in control group at six days after the injury in comparison to the times of admission and 24 h after the injury ($P < 0.05$).

The effect of progesterone on the serum level of IL-6 in DAI patients is illustrated in Fig. 3B. A significant difference in IL-6 level was not seen between control and progesterone groups at the times of admission and six days after the injury ($P = 0.85$ and $P = 0.74$, respectively). At 24 h

Table 3
Comparison of the outcome between progesterone and control groups three and six months post-DAI.

Outcome	Control (n = 16) mean ± SEM	Progesterone (n = 16) mean ± SEM	P value
Glasgow outcome scale:			
At admission	2.06 ± 0.06	2.00 ± 0.00	0.78
Three months post-trauma	4.5 ± 0.56	5.63 ± 0.54	0.16
Six months post-trauma	5.13 ± 0.68	6.81 ± 0.36	<0.05[#]
Functional independence measure:			
At admission	21.25 ± 0.60	19.56 ± 1.24	0.18
Three months post-trauma	75.00 ± 7.96	88.00 ± 5.38	0.21
Six months post-trauma	69.53 ± 11.42	94.44 ± 4.45	<0.01[##]
Glasgow coma scale:			
At admission	7.75 ± 0.52	7.50 ± 0.55	0.81
Six days post-trauma	10.81 ± 0.97	11.81 ± 0.73	0.54
At discharge	12.92 ± 0.87	13.69 ± 0.44	0.75

n: number of patients; DAI: diffuse axonal injury.
[#] $P < 0.05$: significant difference between progesterone group and control group six months after the injury.
[##] $P < 0.01$: significant difference between progesterone group and control group six months after the injury.

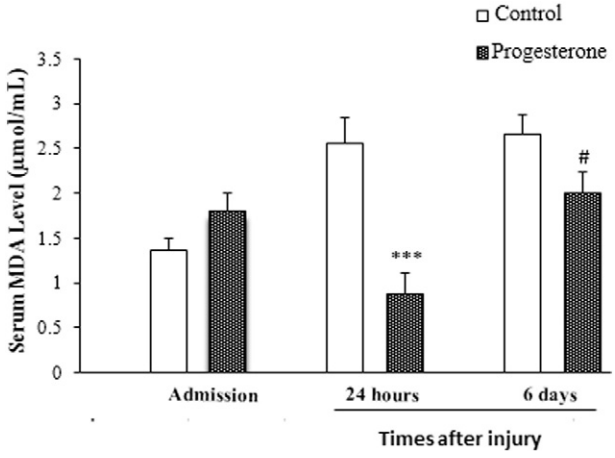

Fig. 2. The temporal profile of serum malondialdehyde (MDA) level in progesterone-administered and control patients with diffuse axonal injury (DAI). The data are expressed as mean ± SEM. ***$P < 0.001$ vs. control group at 24 h after the injury; [#]$P < 0.05$ vs. control group at six days after the injury.

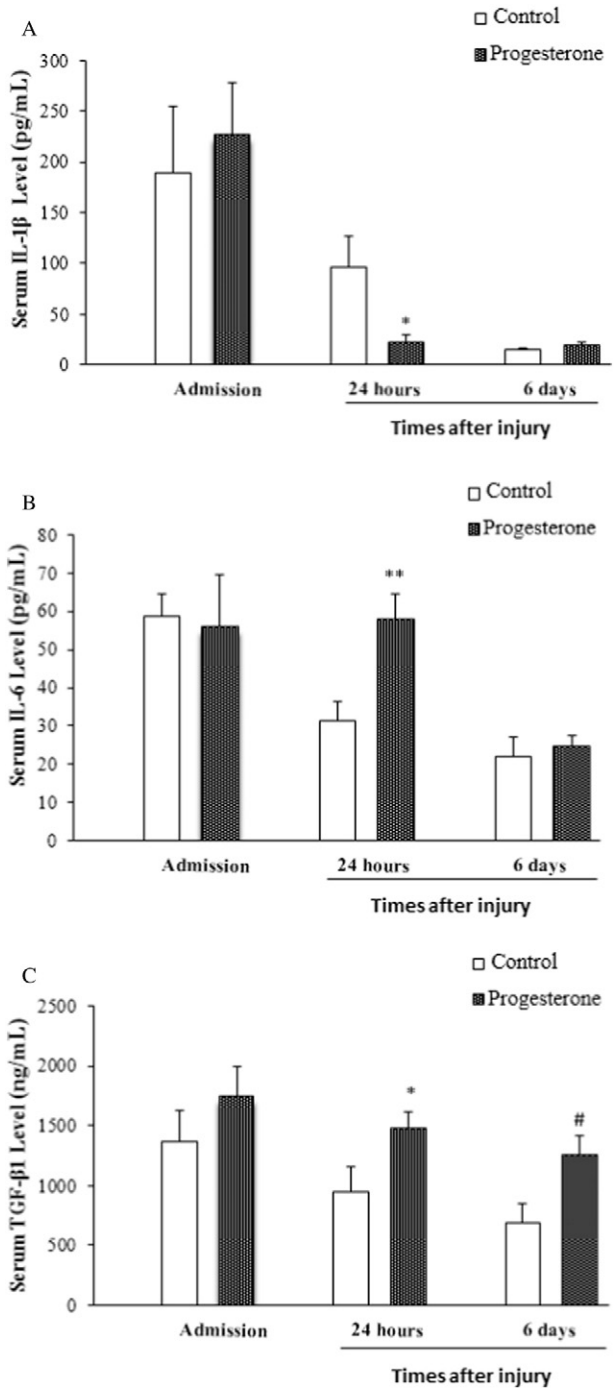

Fig. 3. The temporal profile of serum IL-1β, IL-6 and TGF-β1 levels in progesterone-administered and control patients with diffuse axonal injury (DAI). The data are expressed as mean ± SEM. A) *P < 0.05 vs. control group at 24 h after the injury. B) **P < 0.01 vs. control group at 24 h after the injury. C) *P < 0.05 vs. control group at 24 h after the injury; #P < 0.05 vs. control group at six days after the injury.

after the injury, a significant increase in IL-6 level was revealed in progesterone group (58.05 ± 6.50 pg/mL) in comparison with control group (31.18 ± 5.20 pg/mL) (P < 0.01) that this increase was 86.18% (Fig. 3B). A significant decrease in IL-6 level was found in control group during the evaluation in comparison with the admission time (P < 0.01 and P < 0.001, at 24 h and six days, respectively). Furthermore, significant decrease in IL-6 level was observed in progesterone group at the time of six days after the injury in comparison with the times of admission and 24 h after the injury (P < 0.01).

The effect of progesterone on the serum level of TGF-β1 in DAI patients is illustrated in Fig. 3C. A significant difference in TGF-β1 level was not seen between control and progesterone groups at the time of admission (P = 0.29). At 24 h and six days after the injury, a significant increase in TGF-β1 level was found in progesterone group (1474.04 ± 145.18 ng/mL and 1254.54 ± 162.83 ng/mL, respectively) compared to control group (952.32 ± 204.25 and 689.85 ± 162.14 ng/mL, respectively) (P < 0.05) that these increases were 54.8% and 81.78%, respectively (Fig. 3C). A significant decrease in TGF-β1 level was observed in progesterone group at the time of six days after the injury compared to 24 h after the injury (P < 0.05).

3.5. Progesterone effect on serum level of brain protein (S-100B)

The effect of progesterone on the serum level of S-100B in DAI patients is shown in Fig. 4. A significant difference in S-100B level was not observed between control and progesterone groups at the time of admission (P = 0.41). In contrast, 24 h and six days after the injury, a significant decrease in S-100B level was found in progesterone group (196.38 ± 47.42 pg/mL and 170.53 ± 36.43 pg/mL, respectively) in comparison with control group (334.47 ± 38.99 and 307.63 ± 50.1 pg/mL, respectively) (P < 0.05). S-100B reduced in progesterone group compared to control group 41.29% and 44.57% at 24 h and six days after the injury, respectively (Fig. 4). A significant decrease in S-100B level was revealed in control and progesterone groups during the evaluation in comparison to the admission time (P < 0.01, at 24 h and six days).

3.6. Serum level of progesterone

The effect of progesterone on serum level of progesterone in DAI patients is shown in Table 4. A significant difference in progesterone level was not seen between control and progesterone groups at the time of admission (P = 0.16). But, progesterone level in patients who received progesterone was higher than that in control group at 24 h and six days after the injury (P < 0.001). Unlike control group, a significant increase in progesterone level was revealed in progesterone group during the evaluation compared to admission time (P < 0.05 and P < 0.001, at 24 h and six days, respectively).

4. Discussion

To our knowledge, the present study is the first study to evaluate the effect of progesterone on the outcome and the serum markers of brain

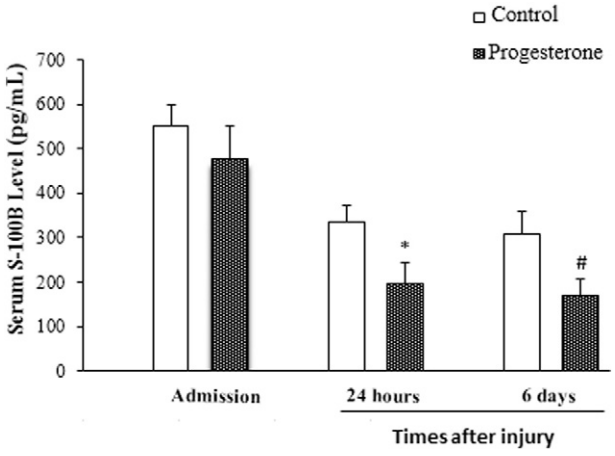

Fig. 4. The temporal profile of serum S-100B level in progesterone-administered and control patients with diffuse axonal injury (DAI). The data are expressed as mean ± SEM. *P < 0.05 vs. control group at 24 h after the injury; #P < 0.05 vs. control group at six days after the injury.

Table 4
The temporal profile of serum progesterone level in control and progesterone-administered patients with DAI.

Serum progesterone level (ng/mL)	Control (n = 16) mean ± SEM	Progesterone (n = 16) mean ± SEM
At admission	12.14 ± 0.94	14.45 ± 1.31
24 h after injury	4.50 ± 0.84	19.42 ± 1.05***
Six days after injury	6.55 ± 1.40	20.90 ± 0.56###

n: number of patients; DAI: diffuse axonal injury.
*** P < 0.001 vs. control group 24 h after the injury.
P < 0.001 vs. control group six days after the injury.

injury, oxidant activity and inflammation in DAI patients. The results of the current study showed that, firstly, the neurologic outcome (using GOS-E and FIM) improved in progesterone-administered DAI patients. Secondly, a modulation in the levels of cytokines (using IL-1β, IL-6 and TGF-β1), and a reduction in oxidant activity (using MDA) and injury (S-100B) markers were revealed in these patients. Meanwhile, we observed death cases only in control group. It is necessary to state that the adverse events attributable to the progesterone administration were not seen in our research similar to the others [9–11].

In the current research, only TBI patients of DAI type were selected. Most of the promising therapeutic strategies of the experimental animal studies have failed in the clinical studies of TBI [45]. And, a human TBI is a heterogenic disease making it difficult to compare and interpret the data of the individuals [46]. So, only DAI patients were recruited into the study to minimize the effects of variations between the TBI patients. DAI is one of the most common types of TBI [1] and is a progressive process initiated by the tensional forces of the injury that gradually results in early focal axonal alteration and delayed disconnection [47]. Despite the normal findings on CT scan, DAI is one of the most serious brain injuries.

We observed the outcome recovery in progesterone group in the long term as FIM and GOS-E scores were higher than those in control group six months after the injury. Interruption of the cascade of the molecular and cellular responses following the initial impact in TBI presents an important target to reduce the secondary brain injury. Meanwhile, TBI is not an event affecting only the brain itself. Regardless of its etiology, a brain injury has to be considered as a complicated systemic event that affects the function of many organs in addition to the brain [48]. It seems that multipotential drugs should be considered to modulate the multiple injury mechanisms in TBI with respect to the complexity and diversity of the secondary injury mechanisms [6]. Progesterone has pleiotropic effects that may interrupt the injury cascade in TBI. And, there are more than 200 articles demonstrating the neuroprotective effects of progesterone resulting from studies in four species including humans, and in 22 different brain injury models including TBI. Overall, the preclinical studies have shown that progesterone administration results in a reduction in neuronal loss and cerebral edema, and an improvement in the outcome [7,35,36]. Since there are few clinical studies investigating the effect of progesterone in TBI while showing contrary results [9–13], performing more clinical trials seems necessary.

The results of three single-center clinical trials evaluating the effect of progesterone on the outcome are in line with our result on the outcome [9–11]. A reduction in the mortality of progesterone-administered TBI patients was reported in the research performed by Xiao et al. and Wright et al. [10,11], but we found the death cases only in control group. The results of the above investigations suggest the neuroprotective effect of progesterone in TBI that needs further research to be proved. In contrast, the results of two multicenter clinical studies evaluating the effect of progesterone are inconsistent with the result of our study on the outcome [12,13]. The large clinical trials often failed with no clear reasons, and this failure is not limited to the trials of TBI [45,49]. Nonetheless, some reasons for these different results can be proposed including the difference in dose of drug and onset of drug administration, and especially eligibility criteria of studies. [48]. In addition, there are several models of injury in TBI that may have different pathologic mechanisms with distinct inflammatory responses [46]. Also, different patients may have specific genetic and epigenetic histories and respond differently to a similar treatment. Finally, the variability in patients' routine care in different sites, and preexisting conditions and individual characteristics of patients may play a role in the response to a similar treatment and lead to different results in similar clinical studies [50,51]. So, dividing the TBI patients in a large clinical trial into three categories of mild, moderate and severe may not be suitable to determine their response to a treatment [48].

Progesterone affects the expression of approximately 500 genes involved in regulating inflammation, apoptosis and vascular remodeling which support the multipotential properties of progesterone [52]. The multiple neuroprotective mechanisms of progesterone are reduction of brain edema [53], oxidative stress [29], inflammation [33], excitotoxicity [54], apoptosis and myelin repair [32]. The investigation of the cellular and biochemical pathways affected by progesterone shows the reasonable explanation for its efficacy in neuronal injury.

An intact BBB prevents the diffusion of most water-soluble molecules over 500 Da [55]. However, a BBB disruption causes the appearance of brain-related proteins in the systemic circulation [19]. And, the assessment of TBI biomarkers improves the development of an individualized treatment [38]. Thus, in the present study, the effect of progesterone on both the brain damage and the oxidant activity of DAI patients was determined by the assay of S-100B and MDA levels in the serum, respectively. Interestingly, a concomitant reduction of S-100B and MDA levels was found in progesterone-administered patients 24 h and six days after the injury. The reduction of MDA by progesterone has previously been reported in experimental studies in line with our study [31,56]. But, to the best our knowledge, there has not yet been any clinical trial assessing the effect of progesterone on S-100B and MDA biomarkers in TBI.

There is a considerable increase in the production of free radicals in TBI [23,24], and the brain is greatly vulnerable to injury-induced oxidative damage, so oxidative stress plays a leading role in the pathology of TBI [57]. Because the direct measurement of free radicals is difficult, an alternative method of the assessment is measuring lipid peroxidation by their products such as MDA [58]. Lipid peroxidation induced by oxidative damage is a phenomenon that induces alterations in cellular membranes. The highest level of MDA is detected thirty minutes after brain trauma which is maintained elevated 72 h after the injury onset [26,59,60]. In the present study, an increase in MDA level was maintained in control group during six days of the evaluation. This difference may be because of the difference in the type of TBI and the time of taking sample for measurement of MDA in the studies.

S-100B has been noticed as an outcome marker in DAI [20,21]. An increase in S-100B may reflect either the glial damage or the astrocytes reactions to neural injury, referred to as reactive astrogliosis [61,62]. Meanwhile, it has been reported that an increase in serum level of S-100B is associated with the poor outcome and the extensive brain injury [19,20]. The highest level of S-100B is noticed at the first hours of TBI, and its delayed increase is associated with the secondary brain damage and the unfavorable outcome [20,21] as the highest level of S-100B was observed at the first four hours of injury in our study. In addition, an increase in S-100B of control group compared to progesterone group during the evaluation was associated with poor outcome in the current study. Thus, it is suggested that progesterone may improve the outcome in DAI at least in part by decreasing oxidant activity leading to suppressing injury and BBB permeability. However, this requires further research.

In the present study, the cytokines levels (IL-1β, IL-6 and TGF-β1) were measured. Decreasing level of IL-1β and increasing level of IL-6 were indicated in progesterone group 24 h after the injury. A reduction in progesterone-caused IL-1β has also been reported in experimental studies [63,64]. BBB abnormalities can be induced by the inflammatory

cascade in TBI [65]. Meanwhile, IL-1β rises rapidly after the brain injury, and the nervous damage resulting from TBI is inhibited following reduction of IL-1β [66]. An increase in IL-6 following progesterone administration in the current study is in agreement with the study performed in animal TBI [67]. It has been shown that IL-6 has a dual action both inflammatory and anti-inflammatory. IL-6 exerts its anti-inflammatory effects via an induction in IL-1Ra (interleukin-1 receptor antagonist) synthesis and a reduction in TNF-α synthesis [68]. In addition, IL-6 and TNF-α constitute a feedback loop; then TNF-α induces IL-6 production, and later IL-6 in turn inhibits TNF-α production. It has been reported that pretreatment with IL-6 reduced the mortality in experimental septic shock, indication of a protective role of this cytokine [69].

Unlike decreasing in control group, there was increasing level of TGF-β1 in progesterone-administered patients during our assessment. The increasing TGF-β1 in progesterone group is in agreement with the reports of Khaksari et al. and also Gibson et al. in animal TBI [36,64]. It has been reported that the anti-inflammatory actions of TGF-β1 are dominant, and an anti-inflammatory effect of TGF-β1 is probably controlling the production of IL-1β, TNF-α and free radicals of oxygen [70]. To the best of our knowledge, there has not yet been any clinical trial assessing the effect of progesterone on cytokines in TBI. Yet, it is supposed that the modulation of cytokines may be one of other mechanisms of neuroprotective effect of progesterone in the current study. Further research seems necessary in this respect, though.

Interestingly, unlike a decrease in control group, there is an increase in the serum level of progesterone in progesterone group during evaluation of the serum markers which is in line with another study [36]. Therefore, it is supposed that changes in the serum level of markers in progesterone group are attributable to progesterone effect.

In the present research, progesterone group had higher FIM and GOS-E scores in comparison to control group. Also, this group indicated a reduction of the serum levels of IL-1β, MDA and S-100B and an elevation of the serum levels of IL-6, TGF-β1 and progesterone compared to control.

The authors according to findings of present study suggest that a free radical-induced damage to lipids, an increase in IL-1β, and a decrease in IL-6 and TGF-β1 may be involved in the pathogenesis of the secondary brain injury of DAI. Also, serum S-100B may be considered as a marker of this injury. One of ROS sources in TBI can be macrophages/microglia and neutrophils activated by an inflammatory process initiated following the initial injury [24]. And, it has been suggested that free radicals and inflammatory cascade may increase BBB permeability leading to brain edema in TBI [71,72] whereby increasing the serum levels of S-100B in extensive brain injury [17,19].

The present study has several limitations. First, the number of the patients in the analyses was small because of selecting only DAI patients to arrest the heterogeneity of TBI, whereas greater numbers may demonstrate more differences between the two groups. Second, the markers were evaluated using serum rather than CSF and parenchymal interstitial fluid that disadvantages the investigation due to the lack of specificity to the brain tissue and the low sensitivity to the early injury. Despite these limitations, firstly, it is the first clinical study investigating the effect of progesterone on the outcome of DAI patients associated to their serum markers of injury and inflammation. Secondly, we investigated the serum markers that are rationally involved in CNS injury including the markers of inflammation, injury and oxidative stress [73].

According to the findings of the current study, it is suggested that the outcome recovery in progesterone-administered DAI patients may be resulted from the neuroprotective effect of progesterone. And, this effect may be mediated by the anti-inflammatory and anti-oxidant properties of progesterone. Although these findings are encouraging, further studies with larger number of patients are needed to decide on the usage of progesterone therapy. According to the results of this study, oxidative stress and inflammation seems to be involved in DAI pathogenesis. So, further studies are required to better understanding of the delicate interplay between the cytokine and oxidant response, and the type of cells that respond to these mediators in TBI.

Disclosure

The authors declare no conflict of interest.

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