Journal of Neuroimmunology 333 (2019) 476958

Contents lists available at ScienceDirect

Journal of Neuroimmunology

journal homepage: www.elsevier.com/locate/jneuroim

The therapeutic effect of platelet-rich plasma on the experimental autoimmune encephalomyelitis mice

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ARTICLE INFO

Keywords:
Platelet-rich plasma
Multiple sclerosis
Experimental autoimmune encephalomyelitis
Growth factor
Inflammation
Myelination

ABSTRACT

The use of growth factors is considered to be one of the promising therapeutic strategies for multiple sclerosis (MS). Various studies have shown that platelet-rich plasma (PRP), a bioproduct of concentrated platelets, contains a variety of growth factors such as insulin-like growth factor 1 (IGF-1), platelet-derived growth factor (PDGF), epithelial growth factor (EGF), and transforming growth factor β (TGF-β). The therapeutic roles of PRP, with regard to a wide range of growth factors, on the nervous system have been shown in a limited number of studies. This study aimed to investigate the therapeutic effect of PRP in experimental autoimmune encephalomyelitis (EAE) mouse model of MS. PRP was prepared and intrathecally injected into the EAE mice. The EAE scoring test, the modified neurological severity score (mNSS) test, luxol fast blue and hematoxylin and eosin staining, real-time PCR, and western blotting were used for studying the effect of PRP on the motosensory function, remyelination, inflammatory cell infiltration, gliosis, and inflammatory cytokines expression. PRP administration in treated animals improved the functional abilities, remyelination, and oligodendrogenesis compared to the EAE mice. Furthermore, high numbers of microglia, astrocytes and infiltrating inflammatory cells and also the expression of proinflammatory cytokines were reversed after PRP therapy. In conclusion, these data suggest the PRP as a potential candidate for MS treatment.

1. Introduction

Platelet-rich plasma (PRP) is a fraction of blood with a high content of concentrated platelets. PRP can be potentially used for regenerative medicine and tissue healing due to a large number of growth factors secreting from the platelet intracellular granules (Chen et al., 2018). PRP contains numerous growth factors such as platelet-derived growth factor (PDGF), fibroblast growth factor (FGF), insulin-like growth factor-1 (IGF-1), and vascular endothelial growth factor (VEGF). Moreover, PRP includes some neurotrophic factors such as nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), and neurotrophin-3 (NT3) (Swift et al., 2006). In addition to its therapeutic applications in maxillofacial reconstruction, wound healing, and bone tissue regeneration, recent studies have shown a growing interest in PRP for treating central nervous system (CNS) diseases (Shen et al., 2009). Several studies have reported the therapeutic effect of PRP in the animal model of spinal cord injury (SCI) (Chen et al., 2018). It was also demonstrated that PRP has therapeutic effects on peripheral nerves (Sánchez et al., 2017). In addition, another study manifested that PRP application enhances myelin regeneration in interrupted sciatic nerve (Shen et al., 2009). Moreover, PRP is also used in inflammatory diseases. It has been widely used for treating knee osteoarthritis and resulted in significant clinical improvements (Meheux et al., 2016). PRP has presented anti-inflammatory effects after its intra-articular injection in osteoarthritis mice (Khatab et al., 2018).

Multiple Sclerosis (MS), one of the most common neurological disorders, is depicted by demyelination in the CNS. Trying to boost remyelination development is a prospective strategy for treatment of MS. Studies have demonstrated that survival, differentiation, and proliferation of oligodendrocytes, the CNS myelin-forming cells which are damaged during MS, are widely controlled by growth factors. Given that apoptosis of oligodendrocytes leads to demyelination and neurodegeneration, recent research in this field has focused on the oligodendrocytes protection with the goal to accelerate myelin regeneration. The application of growth factors for MS treatment has been addressed by a number of researchers. For example, it was reported that IGF-1 treatment can promote remyelination in an animal model of MS (Yao et al., 1995). In addition, it has been shown that the application of exogenous growth factors in PRP such as IGF, TGF-β, and EGF has neurotrophic effects in neurogenic diseases and CNS damage (Kojima

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https://doi.org/10.1016/j.jneuroim.2019.04.018
Received 6 March 2019; Received in revised form 30 April 2019; Accepted 30 April 2019

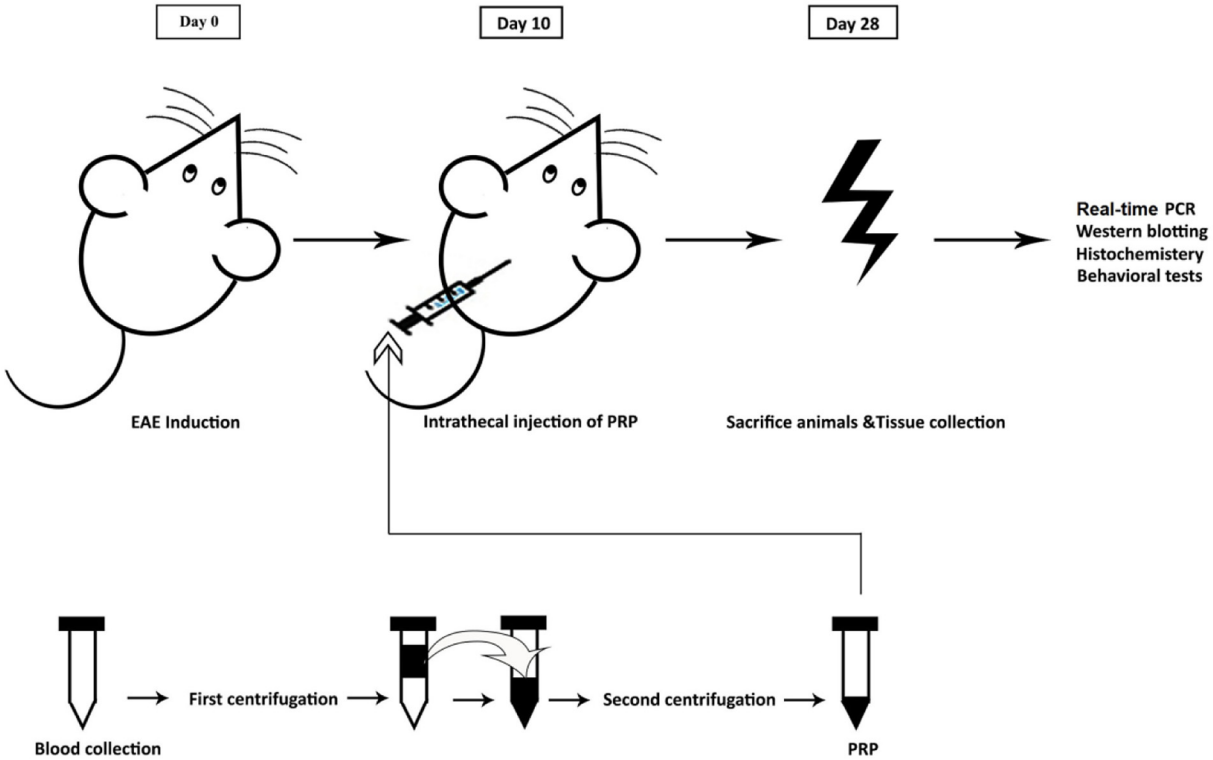

Fig. 1. flowchart pictures the experiments of the study. Six mice were in each group. Depending on the test, the experiments were repeated three times or more. EAE: experimental autoimmune encephalomyelitis; PRP: platelet-rich plasma.

and Tator, 2002; Wang et al., 2000). The presence of neurotrophic and growth factors in PRP and its therapeutic effects in CNS injuries led us to investigate the effect of PRP administration in MS. For this purpose, PRP was injected intrathecally into the experimental autoimmune encephalomyelitis (EAE) mouse model of MS and, then, the effect was evaluated at day 28 of the study.

2. Methods and materials

2.1. Experimental design

Experimental design of our study is depicted in Fig. 1. PRP was provided from the blood of healthy C57BL/J mouse. EAE model was induced in C57BL/J mice and PRP was injected intrathecally after clinical score 1 was observed in EAE mice between days 10 and 14 following immunization. At the day 28, the mice were sacrificed and the lumbar part of spinal cords was analyzed by histological assessment, real-time PCR, and western blotting. In addition, the weight and behavioral changes were evaluated daily in the animals. The experiments were repeated and reproducibility established.

2.2. Preparation of PRP

Fresh blood (1 ml) with 3.8% sodium citrate (9:1) was harvested from healthy C57BL/6 mice through the right atrium under deep anesthesia. In the first phase of enriching platelets, the blood was centrifuged at 1200 rpm for 15 min and the supernatant was collected. In the second phase, the collected supernatant was centrifuged at 1500 rpm for 15 min. Then, the platelet pellet formed at the bottom was harvested with the lower one-third of the plasma and suspended (Dhurat and Sukesh, 2014). PRP samples were analyzed in an automatic counter (Model Sysmex F-820). The average platelet concentration was $12.65 \pm 1.32 \times 10^5$/ml in the final PRP.

2.3. EAE induction and PRP injection

Ten-week-old female C57BL/6 mice were purchased from the Pasture Institute (Tehran, Iran). All animal experiments were approved by the Animal Care Committee of Tehran medical university. Immunization of C57BL/6 was induced by subcutaneous injection of the equal volumes of 300 μg of the myelin oligodendrocyte glycoprotein peptide 35–55 (MOG 35–55, K, J Ross-Petersen ApS, Denmark) diluted in 100 ml phosphate-buffered saline (PBS) and complete Freund's adjuvant (CFA; Sigma, USA) containing 5 mg/ml heat-killed *Mycobacterium tuberculosis* (H37Ra) (Difco, Detroit, MI). After that, the mice received an intraperitoneal injection of 300 ng pertussis toxins (List Biological Lab, USA) on days 0 and 2 post immunization. Then, EAE mice were divided into three groups (six mice per group) as follows: EAE group; EAE+ PBS group (the vehicle group); EAE + PRP group (the PRP group). When EAE score of 1 was observed in the mice, PBS or PRP was injected intrathecally according to the previously published protocol (Bakshi et al., 2004). In brief, the mice were anesthetized with a single intraperitoneal injection of ketamine (50 mg/kg) and xylazine (5 mg/kg) and placed in a prone position with flexion in lumbar vertebrae. After that, 1 cm longitudinal incision was performed over the L3 to L5 spinous processes. Then, a volume of 10 μl PRP or PBS was extremely slowly injected into the lumbar cistern by a Hamilton syringe (volume: 10 μl, Bonaduz, Switzerland) through the L3-L4 or L4-L5 space.

2.4. Body weight and behavioral assessments

Body weight and behavioral measurements were performed every two days from day one to day 28 after EAE induction. For EAE scoring (Stromnes and Goverman, 2006), the mice were monitored, and scored according to the following scale: 0 = normal, 0.5 = the tail paralyzed partially, 1 = the tail paralyzed completely, 2 = fore limbs paralyzed, 3 = hind limbs paralyzed, 4 = both hind limbs and forelimbs paralyzed completely, and 5 = death. In addition, the mNSS test was conducted

Table 1
List of primers.

Gene	Forward sequence	Reverse sequence	Base pair
GFAP	TGGTATCGGTCTAAGTTTGC	GATAGTCGTTAGCTTCGTGC	91
Iba-1	GGATTTGCAGGGAGGAAAAGC	CTTCAAGTTTGGACGGCAGA	131
MBP	CAGAGTCCGACGAGCTTCAG	CTAAAGAAGCGCCCGATGGA	199
IL-1β	GCACTACAGGCTCCGAGATGAAC	TTGTCGTTGCTTGGTTCTCCTTGT	147
IL-6	CAACGATGATGCACTTGCAGA	TGTGACTCCAGCTTATCTCTTGG	116
β-actin	GGACTCCTATGTGGGTGACG	CTTCTCCATGTCGTCCCAGT	103

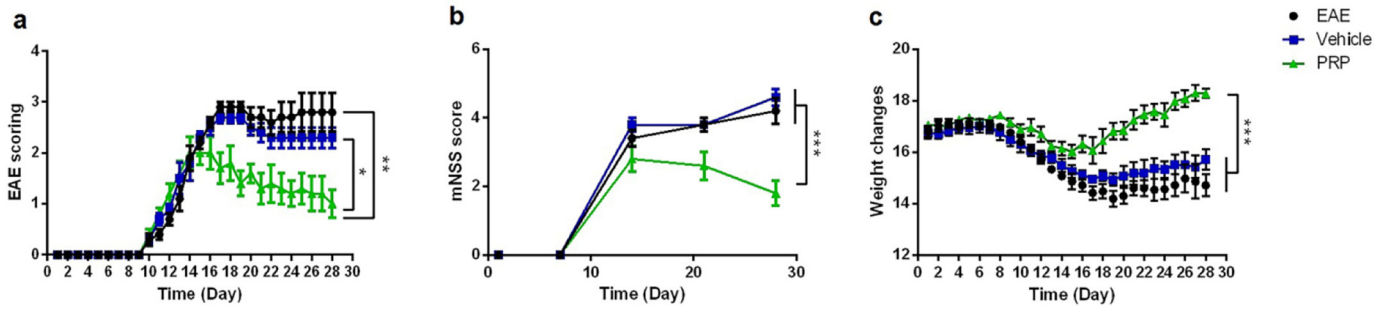

Fig. 2. the effect of platelet-rich plasma (PRP) on the clinical score (a), modified neurological severity score (mNSS) (b) and body weight (c) was evaluated in experimental autoimmune encephalomyelitis (EAE) mice between the day 0 and day 28. Data show the mean ± SEM. $^{*}p < .05$, $^{**}p < .01$, $^{***}p < .001$.

(Chen et al., 2001) for assessment of sensory, motor, balance, and reflex evaluations on days 7, 14, 21, and 28 after immunization. The test was graded on a scale of 0 (normal score) to 18 (maximum deficit score).

2.5. Gene expression analysis

Real-time PCR was used to analyze the gene expression of myelin basic protein (MBP), glial fibrillary acidic protein (GFAP), (ionizing calcium-binding adaptor molecule 1 (Iba1), interleukin 1 beta (IL-1β), and IL-6. The lumbar parts of spinal cords ($n = 4$ per group) were removed and immediately frozen in liquid nitrogen. The samples kept at −80 °C until the experiment. Total RNA was isolated from the specimens using the QIAGEN RNeasy Kit (Qiagen, Tokyo, Japan). After that, the cDNA was synthesized with the cDNA Reverse Transcription kit (Applied Biosystems, USA) according to the manufacturer's instruction. RealQ Plus 2× Master Mix Green (Ampliqon, Denmark) was used for real-time PCR reaction. The sequence of primers is shown in Table 1. Relative fold change of expression of all mRNAs was calculated after normalizing to the β-actin gene expression.

2.6. Western blot

Tissues ($n = 3$ per group) were lysed in lysis buffer (0.1 M NaCl, 0.01 M Tris, 0.1 mM EDTA) and centrifuged (15 min at 4 °C). The total protein concentration of the supernatant was determined by the standard Bradford method. Gel electrophoresis step was followed by protein transfer to polyvinylidene difluoride (PVDF) membrane. Nonspecific binding sites of the membrane were blocked with 5% skim milk for 2 h at room temperature. Then, the membrane was incubated with antibodies against MBP, GFAP, Iba1, IL-1β, and IL-6 overnight at 4 °C. After washing with TBST (Tris-buffered saline, 0.1% Tween 20), the membrane was incubated for 2 h with appropriate secondary antibodies (1:500, Santa Cruz Biotechnology, Germany) at room temperature. After all, the chemiluminescence kit (Fermentase, Germany) was used to detecting the bands. The expression level of each protein was normalized to β-actin protein expression by ImageJ software.

2.7. Myelination assessment and inflammatory cell infiltration study

For histopathology analysis, 30 days after immunization mice were sacrificed and the spinal cords were collected and fixed with 10% formalin for one week. Afterward, the lumbar part of the spinal cord was embedded in paraffin, cut into 5 μm sections and stained with luxol fast blue (LFB) and hematoxylin and eosin (H&E). Light microscopic photos were taken from 10 random sections from each mouse ($n = 3$ per group) and then the percentage of the demyelinated area and inflammatory cell infiltration were analyzed with ImageJ software (Noorzehi et al., 2018).

2.8. Statistical analysis

Statistical analyses were performed using the GraphPad Prism 5 software. The behavioral and weight changes were statistically compared between the groups using two-way ANOVA repeated-measures. Gene and protein expression and histopathological changes were analyzed using one-way ANOVA followed by Tukey post hoc tests. All the data are presented as the mean ± SEM and P value < .05 is considered statistically significant.

3. Results

3.1. PRP reversed neurological impairments and loss weight

The results showed that PBS did not have a significant effect on the behavior or weight of the EAE mice ($p > .05$). As demonstrated in Fig. 2a, administration of PRP significantly attenuated the clinical severity of EAE mice compared to the other groups ($p < .05$ vs. vehicle group; $p < .01$ vs. EAE group) by reducing the EAE scoring. Similarly, the intrathecal administration of PRP significantly reduced the mNSS score in the treated animals compared with the animals in the other groups ($p < .001$, Fig. 2b). In addition, the EAE mice lost weight considerably but PRP administration reversed their weight ($p < .001$, Fig. 2c).

3.2. PRP protected oligodendrocytes and reduced gliosis and pro-inflammatory cytokines secretion

Based on the results, the expression of GFAP, Iba1, and pro-inflammatory cytokine IL-1β genes was significantly decreased in the PRP treated mice compared to the EAE and vehicle groups ($p < .05$).

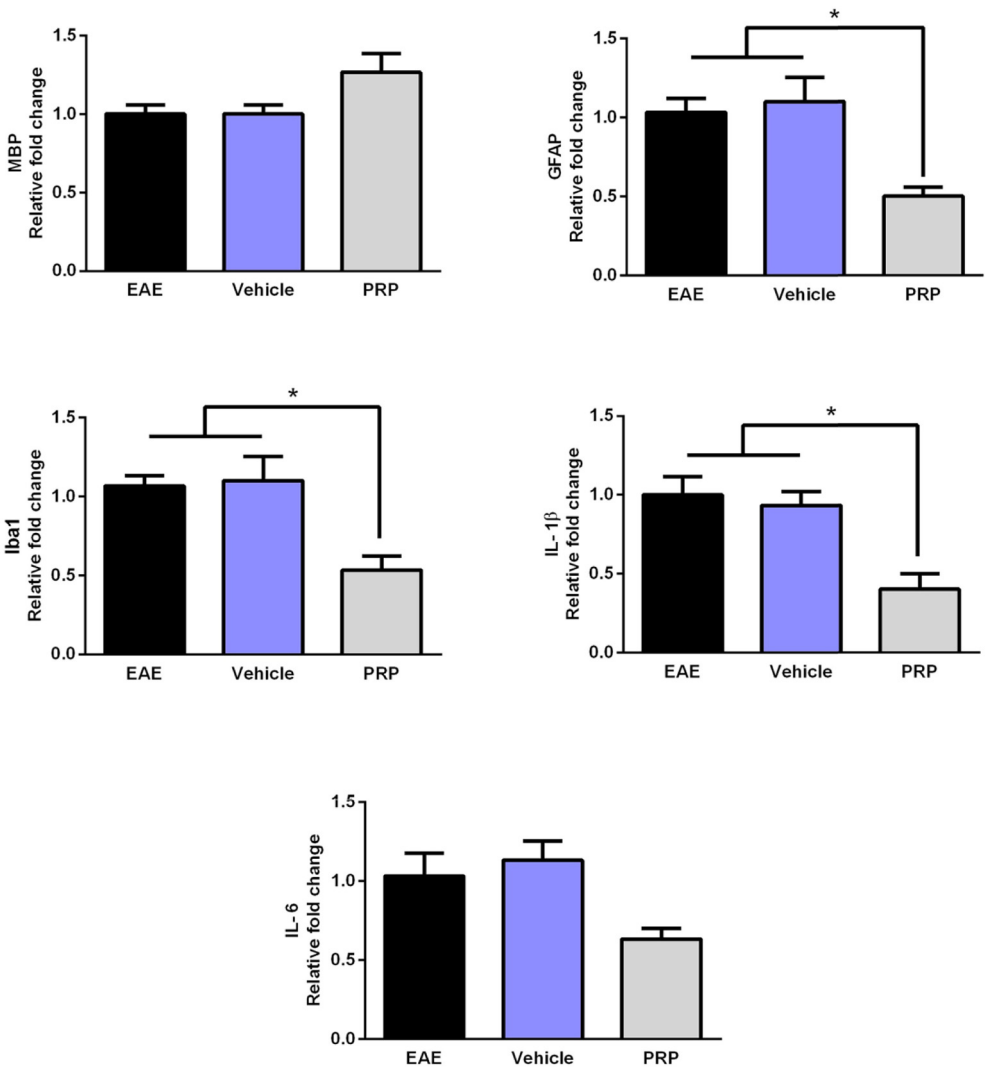

Fig. 3. real-time PCR was used to reveal the relative expression of MBP (myelin basic protein), GFAP (glial fibrillary acidic protein), Iba1 (ionizing calcium-binding adaptor molecule 1), IL-1β (interleukin-1β) and IL-6 in the EAE (experimental autoimmune encephalomyelitis) mice after intrathecal injection of platelet-rich plasma (PRP). Data show the mean ± SEM (four samples per group). $^{*}p < .05$.

However, PRP did not change the transcriptional level of MBP and IL-6 compared to the EAE and vehicle groups ($p > .05$). In contrast, PBS did not show an effect on the expression of genes compared to the EAE group ($p > .05$) (Fig. 3).

Western blotting results (Fig. 4a,b) revealed that the protein level of MBP was significantly ($p < .05$) increased in the PRP group compared to the EAE and vehicle groups. In contrast, EAE-associated high levels of Iba1, IL-1β, and IL-6 were significantly reversed after PRP injection

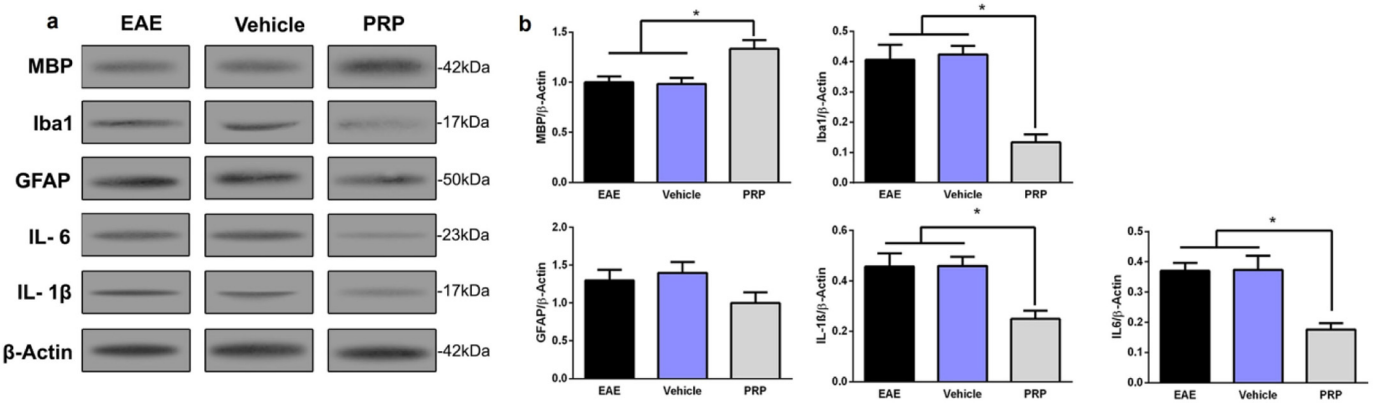

Fig. 4. a: western blots represent protein synthesis of MBP (myelin basic protein), GFAP (glial fibrillary acidic protein), Iba1 (ionizing calcium-binding adaptor molecule 1), IL-1β (interleukin-1β) and IL-6 in the EAE (experimental autoimmune encephalomyelitis), vehicle and PRP (platelet-rich plasma) groups. b: Comparison of quantified blots between the groups. However, GFAP synthesis did not change by PRP. Data show the mean ± SEM (three samples per group). $^{*}p < .05$.

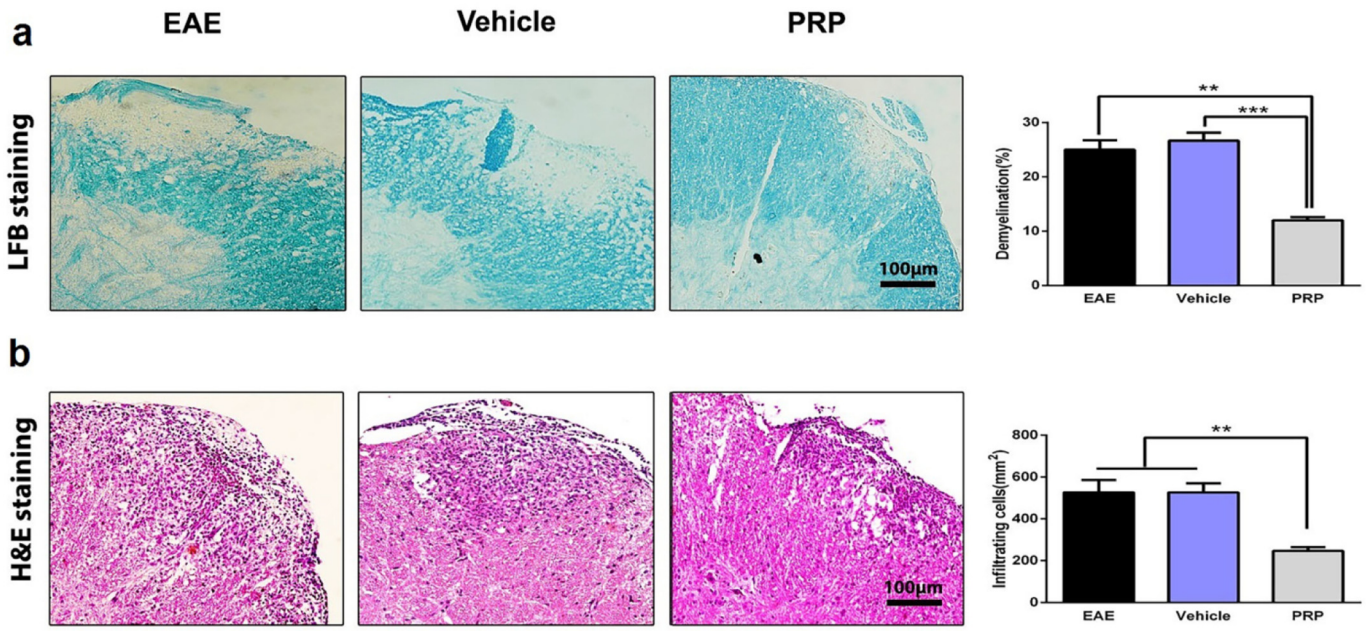

Fig. 5. for histological assessment, the lumbar part of the spinal cord was stained by luxol fast blue (LFB) or H&E. a: demyelination of white mater is detectable in the LFB-stained sections. b: infiltration of the inflammatory cells into the demyelinated area was examined in the H&E photos, quantified and compared between the groups. Data show the mean ± SEM (three samples per group). PRP: platelet-rich plasma; EAE: experimental autoimmune encephalomyelitis. $^{**}p < .01$; $^{***}p < .001$.

($p < .05$), but not after PBS injection ($p > .05$). However, the mean production of GFAP was not significantly different between the groups ($p > .05$).

3.3. PRP decreased demyelination and inflammatory cell infiltration

Data from the LFB staining demonstrated that the percentage of demyelination area significantly declined in the PRP group (12 ± 1%) in comparison to the EAE (25 ± 2%; $p < .01$) and vehicle (26 ± 2%; $p < .001$) groups (Fig. 5a). Quantification of the infiltrating inflammatory cells in the lumbar spinal cord sections stained with H&E showed that PRP application considerably reduced the number of infiltrated inflammatory cells (230 ± 25 cells per mm^2) compared to the EAE (515 ± 80 cells per mm^2) and vehicle (510 ± 70 cells per mm^2) groups (for both $p < .01$; Fig. 5b). No significant difference was seen between the percentage of the demyelinated area or the cell infiltration in the vehicle group compared to the EAE group.

4. Discussion

Here, we demonstrated that intrathecal injection of PRP in the EAE mice significantly ameliorated the neurological function, reduced the demyelination, astrogliosis, microgliosis, and inflammatory cell infiltration and also downregulated the expression of proinflammatory cytokines.

MS is mainly characterized by inflammation and demyelination (Jahan-Abad et al., 2019). There is currently no definitive therapeutic strategy for MS in its progressive phase. Current research on MS is focused on reducing the extent of demyelination (Liu et al., 2018). Given that the growth factors could regulate the survival, proliferation, and differentiation of the oligodendrocytes, researchers have taken a keen interest in the growth factor-based therapy in MS with the aim of protecting oligodendrocytes as myelin-forming cells (Armstrong, 2007). Growth factors, therefore, may be the keystone for MS treatment (Rajendran et al., 2018). Other sources of growth factors like stem cells (Borhani-Haghighi et al., 2018; Caplan and Dennis, 2006) are able to inhibit inflammation and improve nervous system diseases (Mohamadi et al., 2019). PRP as a biomaterial containing growth factors has been examined in numerous clinical settings (Y. Zhang et al., 2015). The receptors related to PRP growth factors are extensively localized on the surface of glial and neural cells (Sariguney et al., 2008). The therapeutic effects of PRP were reported in the injury of peripheral nerves such as sciatic nerve, cavernous nerve and facial nerve (Anjayani et al., 2014; Cho et al., 2010; Wu et al., 2016). The therapeutic effect of PRP has been also reported in the animal model of SCI (Chen et al., 2018; Kojima and Tator, 2002) but, to our literature review, no study has ever evaluated the effect of PRP administration in MS. The data from our study that revealed a beneficial effect of intrathecal administration of PRP in EAE-induced MS model were in accordance with previous studies. As Chen et al. (2018) reported that PRP raised the locomotor recovery in an animal model of SCI, the results of mNSS and EAE scoring in the current study showed that PRP enhanced the functional recovery in the EAE mice. Our results demonstrated the protective effect of PRP on the oligodendrocytes, manifested by the overexpression of MBP at the protein level after the PRP administration. In addition, LFB staining revealed that the PRP alleviated the demyelination. In line with our results, Chen et al. showed that PRP can enhance remyelination in an animal model of SCI (Chen et al., 2018). In addition, another study has also reported that PRP enhanced myelination in an acute nerve injury model (Cho et al., 2010). It has been shown that upregulation of the growth factors protects the oligodendrocytes and enhances remyelination consequently (Armstrong, 2007; Ye et al., 2007). In most studies, the administration of a recombinant protein is considered a way of upregulation of growth factors (Karimi-Abdolrezaee et al., 2012; McMorris and McKinnon, 1996). A large body of research has shown the role of EGF, one of the growth factors of PRP, in the survival and proliferation of oligodendrocytes (Kojima and Tator, 2002; Vinukonda et al., 2016). Besides, EGF enhances oligodendrocyte progenitor cell migration and oligodendrogenesis (Xia et al., 2018; Yang et al., 2017). In addition, it has been suggested that EGF preserves myelin (Vinukonda et al., 2016). Moreover, PRP contains IGF-1 which its role in MS treatment has been revealed previously. It has been reported that IGF-1 attenuates demyelination and increases the production of myelin proteins in EAE mice (Yao et al., 1996; Ye et al., 2007). PDGF is another

growth factor in PRP which is able to improve remyelination (Jean et al., 2002). Taken these studies to consider, we attribute the beneficial effects of PRP on the oligodendrocytes survival and remyelination in our study to its high growth factors content.

Besides the growth factors, PRP also contains other factors such as hepatocyte growth factor (HGF) and VEGF which their anti-inflammatory effects have been shown in the previous reports (Kruger et al., 2013). It has been demonstrated that HGF can protect tissue from inflammatory injuries. Bendinelli et al. reported that PRP exerts its anti-inflammatory effect by regulating NF-kB-transactivation using HGF (Zhang et al., 2013). In addition, HGF upregulates the expression of anti-inflammatory cytokines and downregulates the expression of pro-inflammatory cytokines (Zhang et al., 2013). HGF can also modulate the infiltration of inflammatory cells (Giannopoulou et al., 2008). Besides, the administration of TGF-β, another growth factor in PRP, moderates the histological and clinical severity after demyelination and inflammation (Ishikawa et al., 1999). In confirmation of the anti-inflammatory properties of PRP, our data revealed that PRP administration decreased inflammatory cell infiltration in the PRP group compared to the EAE groups. Furthermore, the expression of pro-inflammatory cytokines and the microglia/macrophage marker Iba1 were decreased in the spinal cord after PRP injection. In keeping with our data, Zhang et al. indicated that PRP has anti-inflammatory effects on the injured tendons (Zhang et al., 2013). Based on the results of the previous and the present studies, it could be suggested that PRP may have superior advantages over the previously recognized therapeutic growth factors. Economically, there is a very important benefit for PRP application as a therapy. It is much less costly to prepare PRP than to synthesize the commercial growth factors. Furthermore, the set of growth factors available in PRP may have a synergistic effect in treating diseases than using a specific growth factor (Boswell et al., 2012).

In conclusion, the present study revealed the potential of PRP as a promising treatment option for MS. Attenuating the clinical severity of EAE using the PRP could be attributed to the oligoprotection, the promotion of remyelination, the modulation of proinflammatory cytokines, and the reduction of the inflammatory cell infiltration and gliosis. Additional research is recommended to support the current results and to investigate the mechanisms of beneficial neuroprotective effects of PRP in MS.

Conflict of interest

None.

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