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Placental Extract and Exosomes Derived from Pregnant Mice Attenuate the Development of Experimental Autoimmune Encephalomyelitis

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ABSTRACT

Placental extract (PE) and exosomes from pregnant mice appear to have immunomodulatory and neuroprotective effects. In this study, we assessed the potential therapeutic effects of PE and exosomes obtained from pregnant mice in experimental autoimmune encephalomyelitis (EAE) mouse models.

C57BL/6 mice, 8 to 12 weeks of age, were prepared and administered PE, exosomes, and glatiramer acetate (GA), as an FDA-approved treatment for multiple sclerosis (MS), after EAE induction. Thereafter, the therapeutic effects of treatment were evaluated by measuring the clinical courses of the mice as well as determining the number of regulatory T (Treg) cells using flow cytometry, cytokine levels, and microRNA-326 expression via real-time PCR.

GA, PE, and exosomes reduced clinical severity, the extent of spinal cord demyelination, and the infiltration of inflammatory cells into the spinal cord. The frequency of $CD4^+CD25^+FoxP3^+$ Treg cells increased after treatment of EAE mice with GA, PE, and exosomes. The mRNA expression of the inflammatory cytokines (interleukin-17 and interferon-gamma), as well as miR-326 expression, decreased significantly in the EAE mice after treatment with GA and exosomes.

PE and exosomes from pregnant mice are involved in the modulation of Treg/Th17 balance and provide a therapeutic approach for MS. Further clinical studies will hopefully confirm the safety and efficacy of such treatments in MS patients.

Keywords: Exosome; Experimental autoimmune encephalomyelitis; Glatiramer acetate; Multiple sclerosis; Placental extract

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INTRODUCTION

Multiple sclerosis (MS) is a chronic inflammatory neurological disorder in young people, characterized by

demyelination of neurons in the central nervous system (CNS), which affects about 2.5 million individuals worldwide.[1,2] Although the pathogenesis of MS has not yet been fully understood, it has been proposed that dysregulation of the immune system is involved in the myelin degradation, leading to the disruption of neuronal signaling.[2,3] Evidence from several studies on MS and its animal model, namely experimental autoimmune encephalomyelitis (EAE), indicates that the balance between regulatory T (Treg) cells and effector T cells is disrupted.[4] Autoreactive T helper (Th) 1 and Th17 cells are involved in the etiopathogenesis of MS and EAE through the production of several inflammatory mediators, such as interleukin (IL)-17, IL-12, IL-22, interferon-gamma (IFN-γ), and tumor necrosis factor-alpha (TNF-α). On the contrary, Treg cells have essential roles in the establishment of immune homeostasis and the amelioration of inflammatory conditions.[5-7] Thus, impaired function or decreased number of Treg cells and aberrant response of autoreactive T cells are the major underlying immunopathogenic mechanisms of MS.

MicroRNAs (miRNAs), which are small noncoding RNA fragments, are crucially involved in the pathogenesis of autoimmune diseases through the regulation of the immune system.[8] Recent studies have demonstrated that miRNA-326 (miR-326) plays a role in the induction of Th17 differentiation and that its overexpression is related to the severity of MS.[9,10] Studies on EAE mice revealed that, by targeting Ets-1, a negative regulator for the differentiation of Th17 cells, miR-326 was able to modulate Th17 differentiation and were correlated with disease severity in MS patients.[11]

Despite the development of more than 15 therapeutic strategies for MS treatment, there is currently no definitive therapy for this disease. Currently available therapies, such as immunotherapies, only slow down but do not stop disease progression and are not effective in remyelination and regeneration. In addition, they have several side effects.[12] Therefore, it seems necessary to develop novel, safe, and effective therapies for the management and treatment of MS.

During pregnancy, MS and EAE relapses are suppressed, which has been associated with the immunosuppressive and neuroprotective state of the pregnancy.[13,14] Interestingly, a flare of disease activity commonly occurs after delivery in both mice and humans.[13] Langer-Gould et al. demonstrated that serum factors derived from pregnant mice could regulate T cell response and are responsible for disease amelioration in EAE models.[15] In another study, Gaston et al. showed that induction of pregnancy in EAE mice could improve clinical signs of EAE by pregnancy-specific serum factors.[16] In addition, suppression of disease activity in pregnant women with MS is associated with decreased number and size of white matter plaques.[17]

Exosomes are naturally derived extracellular nanovesicles (30 to 100 nm in diameter) containing lipid bilayer membranes involved in intercellular communication and abundantly found in pregnant women's serum.[17,18] Previous studies have shown that exosomes derived from pregnant mice have immunosuppressive properties.[17] Recently, Clark et al. reported that placenta-derived mesenchymal stem cells (MSCs) were able to regenerate the myelin in EAE mice through the secretion of exosomes.[19] On the other hand, placental extract (PE) has been used in several Asian countries as a traditional medicinal agent to treat inflammatory diseases and heal wounds.[20,21] The immunosuppressive effects of PE have been demonstrated in various diseases, including allergic rhinitis, graft-versus-host disease, allergic dermatitis, and arthritis.[22-24] In addition, the neuroprotective and regenerative effects of PE have been reported in several studies.[25,26] Park et al. assessed the neuroprotective effects of PE n brain injury induced by hypoxic-ischemic conditions in neonatal rats. They reported that PE treatment had a protective effect on cognitive function and neuronal integrity.[25] In line with these observations, proteins purified from PE have neuroprotective effects in animal models and in patients with neurological injury caused by ischemic stroke.[27]

Despite prior efforts to treat MS patients, the results have been unsatisfactory, and the need for innovative therapies remains. PE and pregnancy-associated exosomes have promising immunoregulatory, regenerative, and neuroprotective characteristics, making them candidates for MS therapy. In this study, we evaluated the therapeutic properties of PE and exosomes derived from pregnant mice in comparison to glatiramer acetate (GA) as an FDA-approved treatment for MS in EAE mouse models through clinical presentations and immunological and histopathological assessments (Figure 1).

MATERIALS AND METHODS

The Mice

Male and female C57BL/6 mice (8 to 12 weeks of age) were provided by the Pasteur Institute of Iran, Tehran, Iran, and kept under a 12-hour light/dark cycle with optimized access to food and water at room temperature. The protocols of this study were reviewed and approved by the Animal Care and Use Committee of Iran University of Medical Science (IR.IUMS.FMD.REC.1398.434).

Isolation and Characterization of Exosomes

For the induction of pregnancy, female mice were caged with male mice. Peripheral blood was collected from pregnant mice (n=10) in the late phase (16 to 18 days) of pregnancy, and serum was separated by centrifugation (10 min, 3000 rpm). Then, exosomes were extracted from serum using an EXOCIB kit (Cat. No. 3604-100, Cib Biotech) based on the company's manuals. In brief, serum centrifugation (20 min, 3000 rpm) was conducted to separate particles and debris. Then, serum samples were mixed with reagent A (the exosome precipitation solution) and incubated overnight at 4°C. Then, the samples were centrifuged (40 min, 3000 rpm) to remove the supernatant. The plate of exosomes was resuspended in phosphate-buffered saline (PBS) and stored at −70°C. The amount of protein in exosomes was measured with a bicinchoninic acid (BCA) protein assay kit (DNAbiotech Co., Tehran, Iran). Using dynamic light scattering (DLS) analysis (Zetasizer Nano-ZS, Malvern Instruments, UK), the distribution of exosome size was assessed. The morphological characteristics of exosomes were determined by transmission electron microscopy (TEM; Zeiss-EM10C-100KV). Additionally, Western blotting was performed for the evaluation of CD63 expression in exosomes by a mouse anti-CD63 antibody (Abcam, USA) and a goat anti-mouse IgG horseradish peroxidase (HRP)-linked antibody (Santa Cruz, USA).

Preparation and Characterization of PE

Placentas were obtained from pregnant mice (n = 10) in the late phase of pregnancy (16 – 18 days) and washed with antibiotic-containing PBS and red blood cell lysis buffer. The placentas were then minced and homogenized in a homogenizer buffer, followed by sonication (amplitude 60, pulse 4, 45s, repeat 8) on ice. Then, samples were centrifuged (4000 rpm, 20 min, 4°C), and the supernatant was collected and stored at −70°C (28). A BCA protein assay kit (Cat. No. DB9684, DNAbiotech Co., Tehran, Iran) was used for the evaluation of the protein content of extracts.

Induction of EAE and Evaluation of Mice

Induction of EAE in female C57BL/6 mice (n=24) was performed by Salari Institute of Cognitive and Behavioral Disorders, as already described elsewhere.[3,29] Briefly, after anesthesia with ketamine/xylazine (Merck, Germany), mice were subcutaneously injected in four body parts (one behind the cervical region, one near the base of the tail, and two injections in the hind flanks) with 300 μg of mouse myelin oligodendrocyte glycoprotein peptide (MOG35-55, SICBD, Iran) in complete Freund's adjuvant having 5 mg/mL heat–inactivated *Mycobacterium tuberculosis* (Sigma Co.). Also, 400 ng pertussis toxin (Sigma-Aldrich, P7208) was administered intraperitoneally in mice both immediately and 48 hours after immunization. The weight and clinical symptoms of mice were assessed on a daily basis. Scoring was performed as follows:[1,3] no symptoms (score 0), partial loss of tail tonicity (score 1), complete loss of tail tonicity (score 2), flaccid tail and abnormal gait (score 3), hindleg paralysis (score 4), hindleg paralysis along with hind body paresis (score 5), paralysis in the hindleg and foreleg (score 6), and deceased (score 7).

Treatment Groups

Thirty female C57BL/6 mice were categorized into 5 groups (each group containing 6 mice) as follows; Group 1: healthy control (healthy mice) treated with PBS; Group 2: EAE control (EAE mice) receiving PBS; Group 3: EAE mice receiving GA; Group 4: EAE mice receiving PE; Group 5: EAE mice receiving exosomes. PE (400 μg/mouse), exosomes (350 μg/mouse), and PBS were injected intraperitoneally every other day from day 3 to 24 post-immunization. GA-treated mice received GA as previously described.[30,31] The mice were sacrificed on day 25 post-immunization and their spleens and spinal cords were collected for further analysis.

Histopathological Assessment

The spinal cords were first fixed in 10% formalin. Subsequently, paraffin-embedded sections (5 μm thick) were prepared and stained with luxol fast blue (LFB) and hematoxylin and eosin (H&E) for the evaluation of the

extent of demyelination and the presence of inflammatory cells, respectively. Sections were examined with a light microscope, and histopathological scoring was performed as described in our previous study.[3]

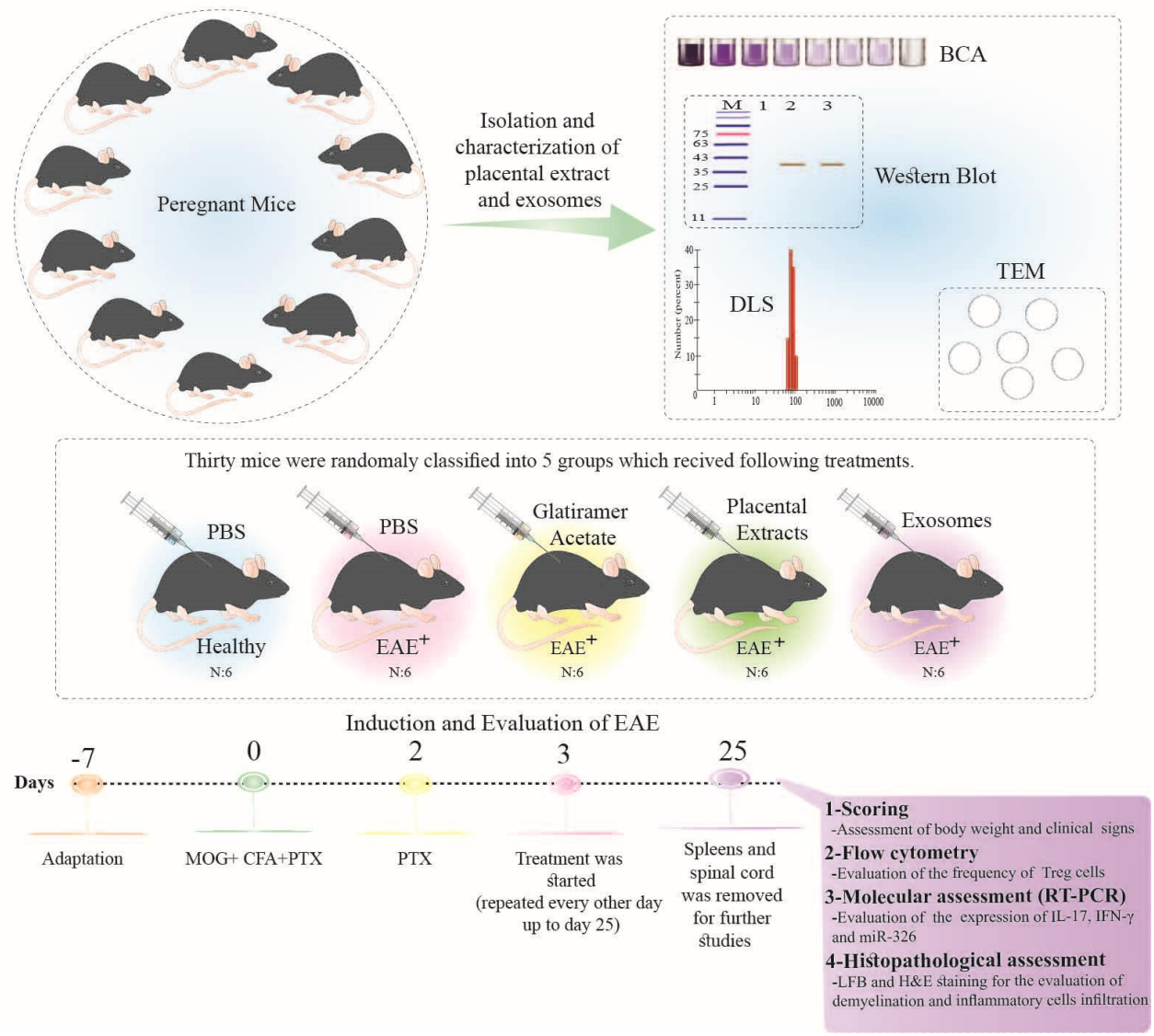

Figure 1. Study design. Female mice were caged with male mice for developing pregnant mice. Peripheral blood was collected from pregnant mice (n = 10) in the late phase of pregnancy (16–18 days) and serum was separated by centrifugation. Subsequently, exosomes were extracted from serum. Placenta samples were also isolated to prepare PE. EAE was induced in 24 female C57BL/6 mice. Mice (n=6 in each group) were treated with PBS (non-EAE mice), PBS (EAE mice), GA (EAE mice), PE (EAE mice), and exosome (EAE mice). Treatment was started on day 3 after EAE induction, and spleen as well as spinal cord samples were collected on day 25 to evaluate the number of Treg cells (flow cytometry), the transcript levels of IL-17, IFN-γ, and miR-326 (real-time PCR), and histopathological evaluations for assessing demyelination (LFB staining), and inflammatory cell (H&E staining) infiltration. The clinical course of the disease and body weight were assessed on a daily basis. BCA, bicinchoninic acid; DLS, dynamic light scattering; TEM, transmission electron microscopy; EAE, experimental autoimmune encephalomyelitis; MOG, myelin oligodendrocyte glycoprotein peptide; CFA, complete Freund's adjuvant; PTX, pertussis toxin.

Flow Cytometry Analysis

Single-cell suspension of splenocytes was prepared, and the frequency of Treg cells was assessed by flow cytometry using a mouse Treg Cell Staining Kit (Cat. No. 88-8118, eBioscience, San Diego, CA, USA), according to the company's instructions. Briefly, splenocytes (1×10^6) were stained on the surface for CD25 and CD4 with phycoerythrin (PE)-labeled rat anti-mouse CD25 (clone PC61.5) and fluorescein isothiocyanate (FITC)-labeled rat anti-mouse CD4 (clone RM4-5) antibodies for 30 min at 4°C. Fixation, permeabilization, and intracellular staining for FoxP3 molecule with allophycocyanin-labeled anti-mouse/rat FoxP3 (clone FJK-16s) antibody was performed for 30 min at 4°C. Finally, the number of Treg cells was analyzed using flow cytometry (Mindray, BriCyte E6, China) and FlowJo software version 10.

Real-time PCR Analysis

Total RNA (both small and large-size RNA) was isolated from splenocytes with RiboEx total RNA isolation kit (Cat. No. 325-150, GeneAll Biotechnology, Seoul, South Korea) based on the producer's protocols. RNA quality and quantity were determined using NanoDrop (Thermo Fisher Scientific, USA). Complementary DNA (cDNA) synthesis from total RNA was performed using a cDNA synthesis kit (Cat. No. A101161, Parstoos Com, Tehran, Iran) and a Mastercycler gradient instrument (Eppendorf, Germany). Primers (Supplementary Table 1) were designed by the National Center for Biotechnology Information (NCBI) primer BLAST tool (https://www.ncbi.nlm.nih.gov/tools/primer-blast/). Transcript levels of IFN-γ and IL-17 were measured by real-time PCR exerting the SYBR Green Master Mix (Cat. No. DQ383-40h, BioFACT, Korea) and Rotor-Gene Q equipment (Qiagen). The relative mRNA expression levels of IFN-γ and IL-17 were normalized to hypoxanthine-guanine phosphoribosyl transferase (HGPRT) mRNA as the housekeeping gene. Additionally, the expression of miR-326 was assessed by the PARSGENOME MiR-Amp kit (Parsgenome, Tehran, Iran) according to the company's guidelines. Briefly, poly-A polymerase was used to add poly-A tails to miRNAs and then cDNA was synthesized with a specific primer designed by Parsgenome (Tehran, Iran). Finally, the expression level of miR-326 was evaluated using specific primers designed by Parsgenome (Tehran, Iran) and SYBR Green Master Mix using real-time PCR. The results were normalized using RNU6 as an internal control. To calculate the relative transcript levels for IL-17, IFN-γ, and miR-326, the comparative Ct method was employed using the $2^{-\Delta\Delta ct}$ formula.[32]

Statistical Analysis

Data were assessed for normal distribution using the Kolmogorov-Smirnov test. Analysis of variance (ANOVA) was employed to analyze data with multiple comparisons versus control groups by Tukey's method. Pearson's correlation analysis was performed to assess for potential correlation between the molecular/cellular measurements and clinicopathological presentations of mice. p values<0.05 were considered statistically significant. GraphPad Prism version 8.3.0 (GraphPad Software, USA) was used for statistical analysis, and data were presented as mean±standard error of the mean (SEM).

RESULTS

Exosomes Characterization

TEM was performed to confirm the morphology of isolated exosomes (Figure 2c), and their size distribution was determined by DLS (Figures 2a and b). Western blot ascertained the expression of CD63, an exosome-specific marker, in the isolated exosomes (Figure 2d).

GA, PE, and Exosomes Improved Body Weight and Clinical Scores of EAE Mice

To investigate the therapeutic effects of GA, PE, and exosome administration, mice were weighed and examined daily for clinical presentations. It was observed that treatment of EAE mice with GA, PE, and exosome postponed the onset of clinical scores and significantly declined clinical scores compared to the PBS-treated EAE mice (p<0.001, p<0.01, and p<0.01, respectively; Figure 3a). According to Figure 3b, body weight loss was observed in the EAE control group (EAE+PBS), however, administration of GA, PE, and exosome significantly improved body weight compared to the EAE control group (p<0.001, p<0.01, and p<0.01, respectively).

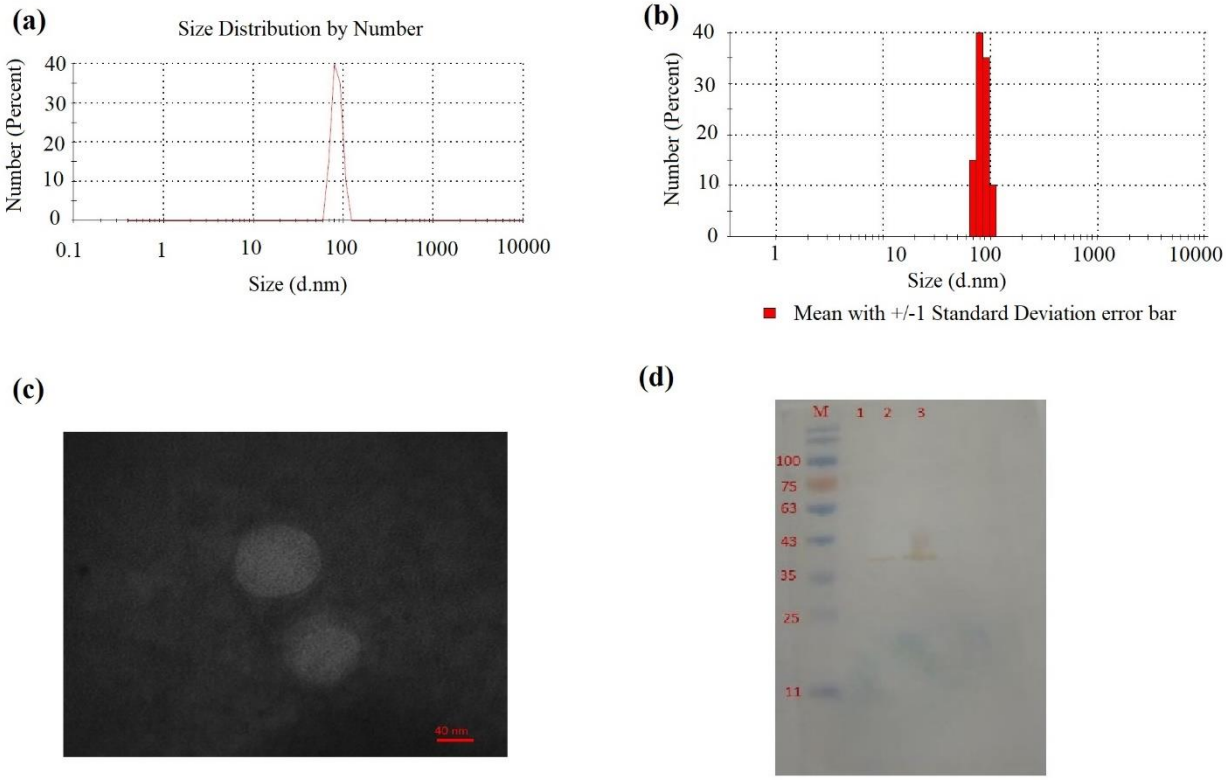

Figure 2. Characterization of exosomes derived from pregnant mice. The size distribution of isolated exosomes was monitored by DLS (a and b). Their mean size was in a single peak at 84.27 nm. TEM evaluation showed that exosomes have a lipid bilayer membrane and are round (c). Western blotting revealed that the exosomes were positive for CD69, a commonly used exosome marker (d). DLS, dynamic light scattering; TEM, transmission electron microscopy.

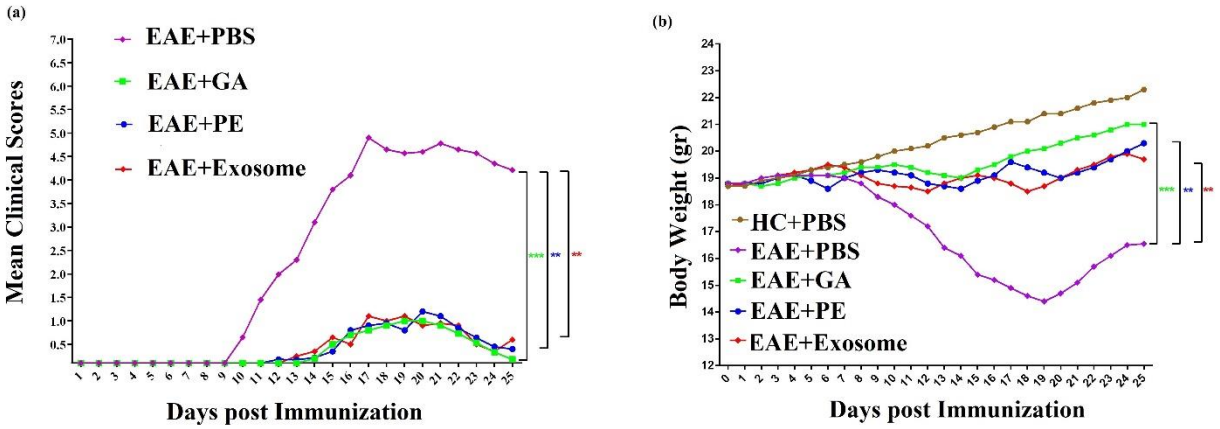

Figure 3. Therapeutic effects of GA, PE, and exosome administration on clinical signs and body weight of EAE-induced mice. Clinical scores in GA, PE, and exosome-treated EAE mice were significantly lower than in PBS-treated EAE mice (a). Body weight was significantly higher in GA, PE, and exosome-treated groups compared to the EAE group treated with PBS (b). *****p*<0.01, ******p*<0.001. HC, healthy control; EAE, experimental autoimmune encephalomyelitis; GA, glatiramer acetate; PE, placental extract.

GA, PE, and Exosomes Reduced the Extent of Spinal Cord Demyelination and Inflammatory Cell Infiltration to Spinal Cord

LFB and H&E staining of spinal cords were performed to evaluate the histopathological effects of GA, PE, and exosome administration. According to the LFB staining analysis (Figure 4a and b), the extent of spinal cord demyelination in EAE mice treated with GA, PE, and exosome was significantly less than in the EAE control group ($p<0.001$, $p<0.01$, and $p<0.01$, respectively). In addition, H&E staining assessment (Figure 4a and c) showed that administration of GA, PE, and exosome significantly decreased infiltration of inflammatory cells to the spinal cord compared to the control mice ($p<0.01$, $p<0.05$, and $p<0.05$, respectively).

Effects of GA, PE, and Exosomes on Treg Cell Frequency

To examine the effects of GA, PE, and exosome treatment on $CD4^+CD25^+FoxP3^+$ Treg cell frequency, splenocytes were prepared, and the frequency of Treg cells was assessed by flow cytometry. The frequency of Treg cells was significantly increased in GA, PE, and exosome-treated groups compared to the EAE mice treated with PBS ($p<0.001$, $p<0.05$, and $p<0.05$, respectively) (Figure 5).

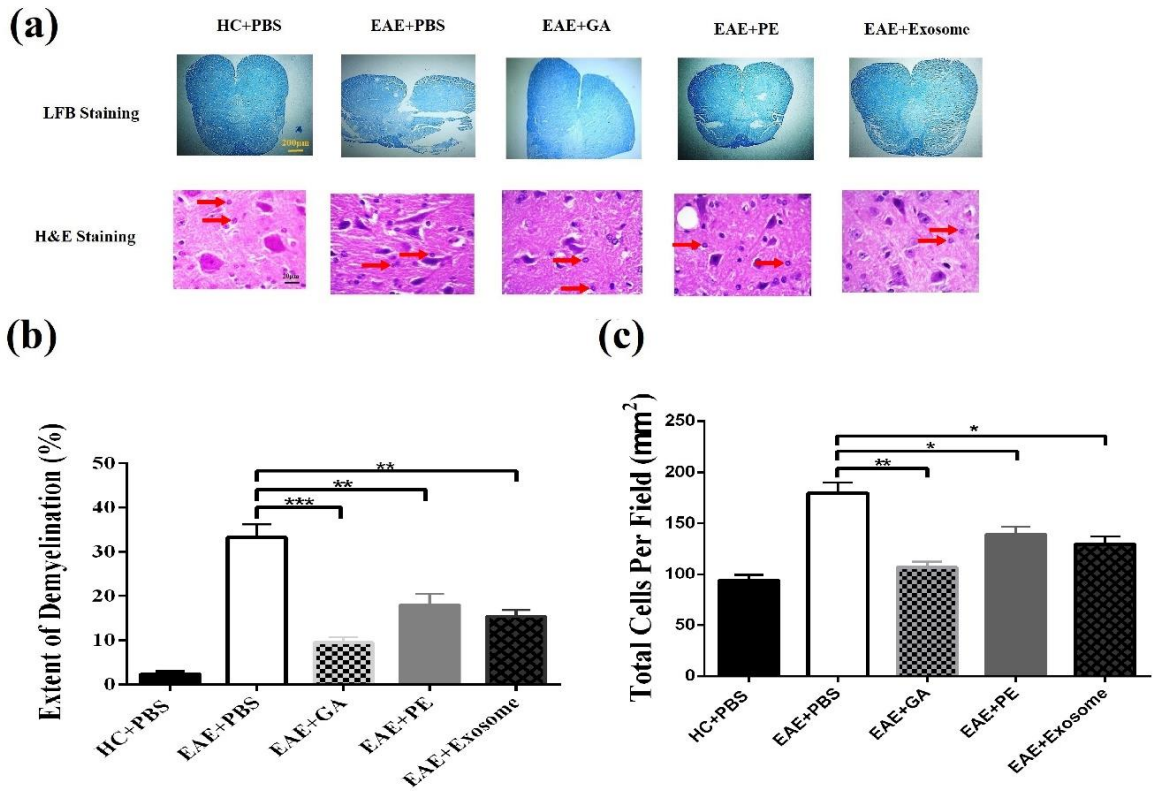

Figure 4. The histopathological effects of GA, PE, and exosome administration on the spinal cord. (a) LFB and H&E staining of spinal cords were performed to evaluate the extent of demyelination and the presence of inflammatory cells, respectively. Arrows show inflammatory cells in the H&E-stained tissues. (b) The extent of spinal cord demyelination in GA, PE, and exosome-treated groups was significantly less than in PBS-treated EAE mice. (c) Administration of GA, PE, and exosome significantly reduced the infiltration of inflammatory cells to the spinal cord compared to the EAE control group. Data are shown as mean±SEM. *$p<0.05$, **$p<0.01$, ***$p<0.001$. LFB, luxol fast blue; H&E, hematoxylin and eosin; HC, healthy control; EAE, experimental autoimmune encephalomyelitis; GA, glatiramer acetate; PE, placental extract.

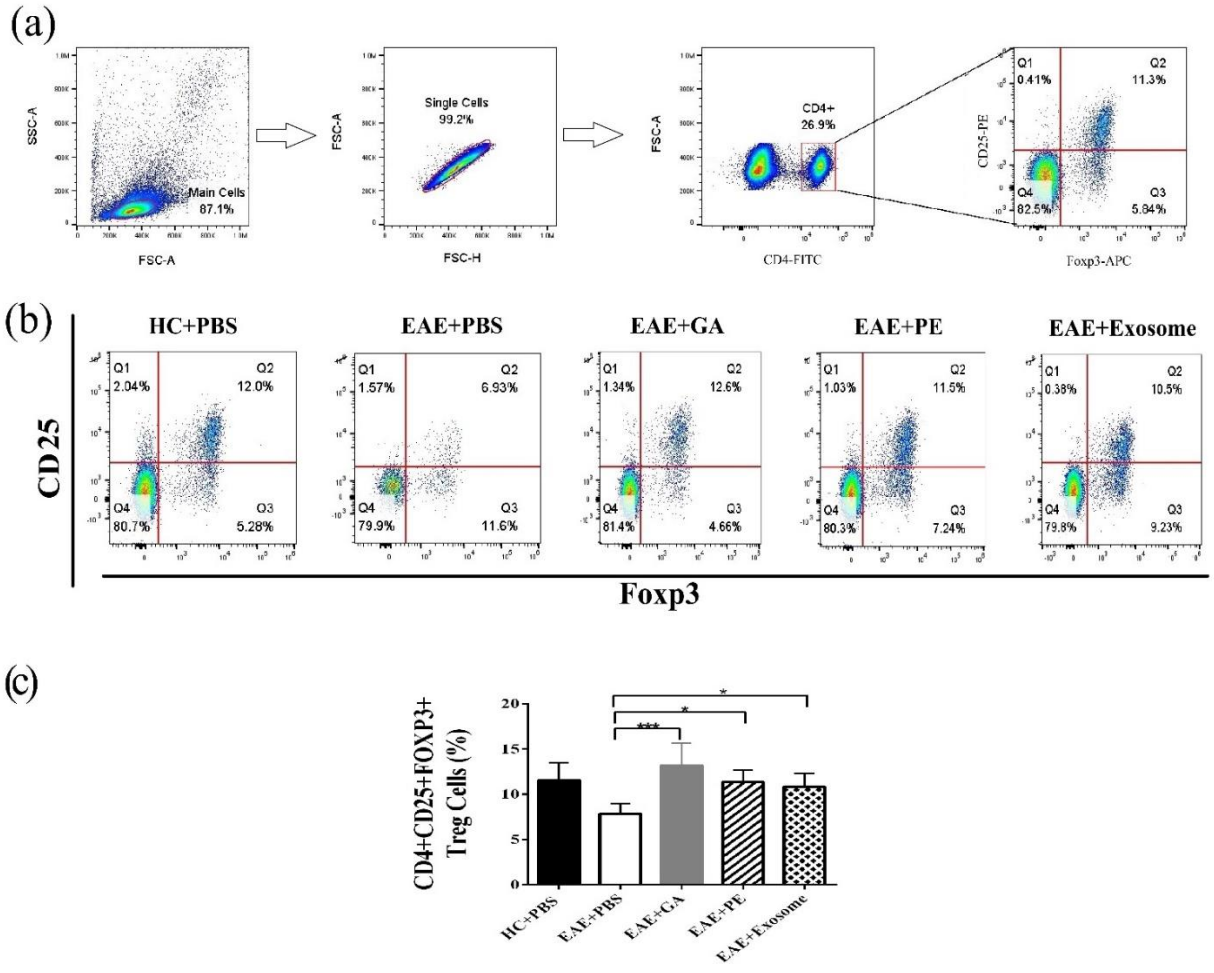

Figure 5. GA, PE, and exosome treatment i the percentage of Treg cell frequency. At the end of the experiment (25 days postimmunization), mice were sacrificed, their spleens were collected to prepare a single-cell suspension of splenocytes, and the frequency of Treg cells was assessed by flow cytometry. (a) After lymphocyte gating based on forward-scatter and side-scatter properties, the lymphocytes were gated for $CD4^+$ T cells and sorted based on CD25 and FoxP3 expression. (b and c) The frequency of $CD4^+CD25^+FoxP3^+$ Treg cells in GA, PE, and exosome-treated EAE mice was significantly higher than PBS-treated EAE mice. Data are shown as mean±SEM. *p < 0.05, ***p < 0.001. HC, healthy control; EAE, experimental autoimmune encephalomyelitis; GA, glatiramer acetate; PE, placental extract. FSC-A, forward scatter area; SSC-A, side scatter-area; Tregs, regulatory T cells.

Effects of GA, PE, and Exosomes on miR-326 and mRNA Expression of Inflammatory Cytokines

To verify the anti-inflammatory effects of GA, PE, and exosomes, we evaluated the mRNA expression of IFN-γ and IL-17. As shown in Figures 6a and b, administration of GA, PE, and exosome significantly decreased IL-17 and IFN-γ expression compared to EAE mice treated with PBS (p<0.05). Additionally, our results showed that treatment with GA and exosomes (but not PE) significantly decreased miR-326 expression compared to EAE mice treated with PBS (p<0.001 and p<0.01, respectively). Although the transcript level of miR-326 was reduced in the PE-treated group compared to the PBS-treated EAE mice, this decrement was not significant (Figure 6c).

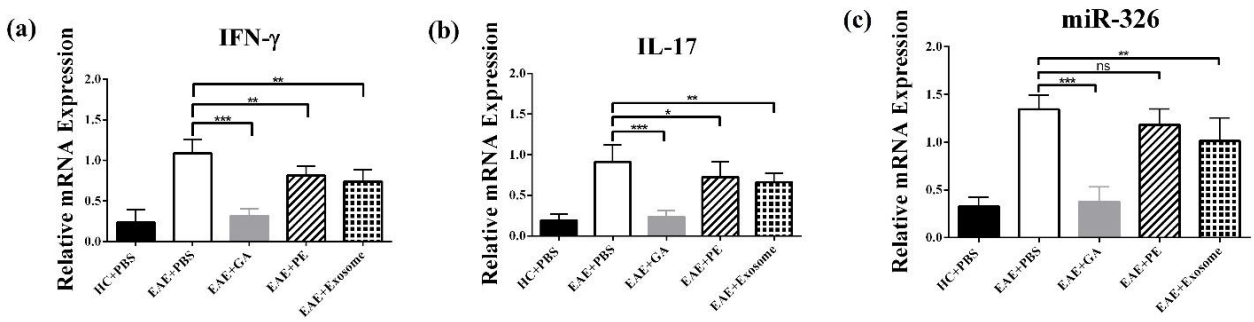

Figure 6. Effects of GA, PE, and exosomes on miR-326 and the mRNA expression of inflammatory cytokines in the splenocytes of EAE mice. (a and b) Administration of GA, PE, and exosome significantly decreased the expression levels of IFN-γ and IL-17 compared with the EAE control group (EAE+PBS). (c) The expression levels of miR-326 significantly decreased in EAE mice treated with GA and exosome compared to EAE control mice (EAE+PBS). However, there was no significant difference in the expression levels of miR-326 in PE-treated EAE mice compared to EAE control mice (EAE+PBS). Data are shown as mean ± SEM. *p < 0.05, **p < 0.01, ***p < 0.001. ns, not significant; HC, healthy control; EAE, experimental autoimmune encephalomyelitis; GA, glatiramer acetate; PE, placental extract; IL-17, interleukin-17; IFN-γ, interferon-gamma; miR-326, microRNA-326.

Table 1. Correlation analysis of the molecular and cellular measurements with clinicopathological presentations of the mice treated with GA, PE, and exosome.

Treatment groups	Clinicopathological presentations	Treg number r* (p value)	IFN-γ expression r (p value)	IL-17 expression r (p value)	miR-326 expression r (p value)
GA	Clinical score	r = −0.23 p = 0.041	r = 0.31 p = 0.019	r = 0.28 p = 0.030	r = 0.33 p = 0.019
	Body weight	r = 0. 23 p = 0.048	r = -0.16 p = 0.064	r = −0.20 p = 0.088	r = −0.17 p = 0.188
	Extent of spinal cord demyelination	r =−0.34 p = 0.017	r = 0.25 p = 0.022	r = 0.39 p = 0.012	r = 0.44 p = 0.002
PE	Infiltration of inflammatory cells	r = −0.44 p = 0.003	r = 0.31 p = 0.0.10	r = 0.59 p = 0.001	r = 0.55 p = 0.001
	Clinical score	r = −0. 21 p = 0.039	r = 0.28 p = 0.0.37	r = 0.24 p = 0.021	r = 0.15 p = 0.080
	Body weight	r = 0.25 p = 0.044	r = −0.22 p = 0.110	r = −0.19 p = 0.073	r = −0.09 p = 0.207
	Extent of spinal cord demyelination	r = −0.30 p = 0.027	r = 0.33 p = 0.017	r = 0.35 p = 0.004	r = 0.10 p = 0.066
	Infiltration of inflammatory cells	r = −0.40 p = 0.011	r = 0.34 p = 0.026	r = 0.53 p = 0.002	r = 0.13 p = 0.058
Exosome	Clinical score	r = −0.25 p = 0.033	r = 0.30 p = 0.014	r = 0.20 p = 0.016	r = 0.24 p = 0.040
	Body weight	r = 0.29 p = 0.042	r = −0.18 p = 0.071	r = −0.11 p = 0.223	r =-0.20 p = 0.055
	Extent of spinal cord demyelination	r = −0.33 p = 0.029	r = 0.36 p = 0.036	r = 0.38 p = 0.014	r = 0.48 p = 0.009
	Infiltration of inflammatory cells	r = −0.38 p = 0.018	r = 0.41 p = 0.002	r = 0.40 p = 0.007	r = 0.56 p = 0.001

Treg, regulatory T cell; GA, glatiramer acetate; PE, placental extract, IL-17, interleukin- 17; IFN-γ, interferon-gamma.
* r indicates Pearson's correlation coefficient.

Correlation Analysis

The correlation analysis showed a statistically significant correlation between Treg cell number, IFN-γ expression, IL-17 expression, and miR-326 expression and the treatment-related presentations of mice, including clinical score, body weight, the extent of spinal cord demyelination, and infiltration of inflammatory cells into the spinal cord, based on the treatment type. Treg cell number statistically correlated with all presentations in mice treated with GA, PE, and exosome. Moreover, expression levels of IFN-γ and IL-17 showed a statistically significant correlation with the clinical score, extent of spinal cord demyelination, and infiltration of inflammatory cells to the spinal cord in mice treated with GA, PE, and exosome. Expression of miR-326 revealed a statistically significant correlation with the clinical score, extent of spinal cord demyelination, and infiltration of inflammatory cells into the spinal cord in mice treated with GA and exosome but not in the PE-treated mice (Table 1).

DISCUSSION

In this investigation, we intended to assess the therapeutic effect of PE and exosomes obtained from pregnant mice on the treatment of MS in the related animal model, namely EAE. Based on the molecular, cellular, and histopathological evaluations along with clinical assessments, we realized that PE and exosomes had promising effects in ameliorating EAE mice. Additionally, these therapeutic effects were comparable to that of GA, an FDA-approved medicine for MS.

Placental tissue and its extract have been used in traditional medicine to cure various disorders since long ago.[33] Among the factors found in PE are predominantly platelet-derived growth factor (PDGF), vessel endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), transforming growth factor (TGF)-α and TGF-β, which account for the potential of PE to trigger tissue regeneration, as well as cell proliferation,[23] which in turn describe therapeutic effects of PE inflammation,[34] tissue regeneration,[35] and age-related disorders.[36] PE seems to be involved in epithelialization and wound healing and suppresses fibrosis, oxidative stress, and inflammation.[23,37] Due to such therapeutic characteristics, several studies have investigated the potential effects of PE in treating neurodegenerative disorders. Park, et al. revealed that PE had protective effects on neuronal integrity and cognitive function after hypoxic-ischemic brain damage.[25]

Exosomes are secreted by almost all cell types and are involved in intercellular cross-talk.[17,18] Reports show that exosomes from pregnant mice possess immunosuppressive characteristics.[17] Furthermore, pregnancy has been found to have a neuroprotective effect.[38] Exosomes obtained from the serum of pregnant women have been observed to repress the activation molecules involved in T cell signaling.[38] Additionally, exosomes taken from late pregnant mice can suppress T cell proliferation more than exosomes obtained from non-pregnant controls.[39] Pregnancy-related suppression of MS relapses was attributed to a diminished frequency and area of white matter lesions in such patients.[38] The development of new oligodendrocytes (the myelin forming cells found in the CNS) and the frequency of myelinated axons increase during pregnancy.[39] Because of their small size and lipophilic properties, exosomes are easily taken up by several tissues like the brain. This feature facilitates the application of exosomes in devising therapeutic approaches for several disorders.

Here we developed the mouse models of MS and evaluated the ameliorative effects of PE and pregnant mouse-derived exosomes on the clinical course of EAE; PE and exosomes delayed the onset of clinical presentations and decreased the severity of the clinical scores in the EAE mice. The extent of spinal cord demyelination in EAE mice treated with PE and exosome was reduced. In addition, the administration of PE and exosomes diminished the infiltration of inflammatory cells to the spinal cord.

From the cellular viewpoint, the number of $CD4^+CD25^+FoxP3^+$ Treg cells increased in the PE and exosome-treated EAE mice. Additionally, to validate the anti-inflammatory effects of PE and exosomes, we observed that the mRNA expression of inflammatory mediators, including IL-17 and IFN-γ, which are involved in the pathogenesis of EAE, was significantly reduced in the EAE mice after treatment with PE and exosomes. Our results also showed that treatment with exosomes derived from pregnant mice decreased miR-326 expression in the EAE mice. Although miR-326 expression was diminished in the PE-treated group, this decrease was not significant. miR-326 is involved in the induction of differentiation of Th17 cells. As a result, PE and exosomes may impose a therapeutic

effect in EAE mice by repressing the Th17 pathway, which is the main culprit in MS etiopathogenesis.[40] Interestingly, the number of Treg cells and expression levels of IL-17, IFN-γ, and miR-326 showed a significant correlation with the clinical score, extent of spinal cord demyelination, and infiltration of inflammatory cells into the spinal cord in mice treated with PE and exosome. This issue indicates the potential of PE and exosomes in improving the Treg/Th17 profile in favor of EAE disease course amelioration.

Our study suffers from a number of limitations. First, we did not evaluate the simultaneous effect of PE and exosome on the amelioration of the EAE clinical course. This could be advantageous if the combinational approach yielded other positive results. Second, even though we did not observe adverse events in the mice, the safety of treatment was not considered systematically in this investigation.

In conclusion, this study showed the therapeutic effects of PE and pregnant mice-driven exosomes in improving the disease course of the EAE mice. This therapeutic effect was mirrored in the increased number of Treg cells and the suppressed function of Th17 cells (reduced IL-17 levels). The effects of PE and exosomes were almost comparable with that of GA. However, our approach's safety and adverse events should be evaluated in future investigations before approval in the clinical trials of the MS subjects. Additionally, strategies for simplifying the extraction and assessment of PE and exosomes could be advantageous in the inexpensive and straightforward induction of immune tolerance toward MS treatment. Using PE and exosomes alongside the other approved immunotherapeutics could open up new horizons in the therapy of MS.

STATEMENT OF ETHICS

All experimental protocols of this study were reviewed and approved by the Animal Care and Use Committee of Iran University of Medical Science (IR.IUMS.FMD.REC.1398.434)

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CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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