Intraspinal Injection of Platelet-Rich Plasma Promotes Regeneration of the Spinal Cord and Improves Locomotor Function by Modulating the Apoptosis and the Wnt Signaling Pathways

Zahra Behroozi
Iran University of Medical Sciences

Fatemeh Ramezani
Iran University of Medical Sciences

farinaz Nasirinezhad (nasirinezhadfarinaz@yahoo.com)
Iran University of Medical Sciences

Research

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Abstract

Background: There are complex mechanisms for reducing intrinsic repair ability and neuronal regeneration following spinal cord injury (SCI). Platelet-rich plasma (PRP) is a rich source of growth factors and has been used to stimulate regeneration of peripheral nerves in degenerative diseases. However, only a few studies have investigated the effects of PRP on the SCI models. We examined whether PRP derived from human umbilical cord blood (HUCB-PRP) could recover motor function in animals with spinal cord injury. We also investigate the role of Wnt signaling pathway.

Methods: Ault male Wistar rats were randomly divided into 6 groups (n=60) as control, sham, SCI, vehicle (SCI+platelet-poor plasma), PRP2day (SCI+injection 2 days after SCI) and PRP14day (SCI+injection 14 days after SCI). SCI was performed at the T12-T13 level. BBB tests were done weekly after injury for six weeks. caspase3 expression was determined using the Immunohistochemistry technique. The expression of GSK3β, Tau and MAG were determined using the Western blot technique. Data were analyzed by PRISM & SPSS software.

Results: PRP injected animals showed a higher locomotor function recovery than those in the SCI group (p<0.0001). The level of caspase3, GSK3β and CSF- Tau reduced and MAG level in the spinal cord increased by injection of HUCB-PRP in animals with spinal cord injury.

Conclusions: Injection of HUCB-PRP enhanced hind limb locomotor performance by modulation of caspase3, GSK3β, tau and MAG expression. Using HUCB-PRP could be a new therapeutic option for recovering the motor function and axonal regeneration after spinal cord injury.

Highlights

1-SCI induces motor dysfunction and apoptosis in rat spinal cord tissue.

2- Intraspinal injection of HUCB-PRP improves motor function recovery.

3- HUCB-PRP exert their neuroprotective effects by inhibiting apoptosis and GSK3β enzymes.

Introduction

Spinal cord injury (SCI) is one of the very complicated and devastating clinical problems with constant neurological weakness, which causes due to inhibitory natural barriers of axonal growth cones and intrinsic insufficient growth capacity of the central nervous system (CNS) (1). Nowadays, the estimation of the lifetime cost of an SCI patient is $2.35 million per patient (2). Therefore, finding efficient and new strategies to treat SCI is still one of the most important clinical challenges.

The pathology of the spinal cord after the injury is not yet well understood, but the primary and secondary phases after the injury have been proven. The primary phase of the injury begins immediately after the injury and is accordance with release of inflammatory factors which results to large neural cell death. But
various pathophysiological events happen in the secondary phase of injury such as apoptosis, microglial activation, demyelination, and axonal degeneration. During the second phase after injury Wallerian degeneration in the spinal tract results in the loss of sensory and motor neuron functions (3–5).

Therefore, axonal regeneration is a necessary step to recover the injury. But axons in the adult CNS regenerate hardly, and this has been the reason for the failure of many treatments to date. Basically, different therapeutic strategies were concentrated on the inhibition of the apoptotic and necrotic cell death in the injured spinal cord and preparing an appropriate condition to promote axonal regeneration. The suitability of growth factors (GF), especially the neurotrophic factors, to reach these aims has been investigated by previous studies (6–9). All of them have shown that growth factors were induced axonal regeneration, remyelination, and motor function recovery and inhibited apoptosis.

For example, Transforming growth factor-β (TGF-β) improves the synthesis of extracellular matrix components and promotes neuron survival (10). Insulin like growth factor-I (IGF1) specifically enhances the outgrowth of corticospinal motor neuron's axons in vitro (9). platelet-derived growth factor (PDGF) protects neurons by suppressing N-methyl-D-aspartic acid current and translocating the glutamate transporter to the cell membrane (11).

The problem is the short half-life of growth factors. To combat this problem intravenous injection of recombinant proteins, transplantation of cells secreting GF and viral gene delivery methods were used. These methods successfully improve motor function and increase axonal regeneration post-SCI in animal models. However, they do not have the necessary safety for clinical use because the immunogenicity (12–14). Today, researchers pay special attention to platelet-rich plasma (PRP) for the treatment of the injured CNS. The autologous PRP is non-toxic with easy-to-collect that does not cause an immune response. PRP contains millions of platelets per microliter which is more than the platelet density of normal blood. α-granules of platelets have abundant growth factors including vascular endothelial cell growth factor (VEGF), brain-derived neurotrophic factor (BDNF), glioblastoma-derived neurotrophic factor (GDNF), nerve growth factor (NGF), hepatocyte growth factor, PDGF, IGF, and TGF-β (14). On the other hand, PRP is a rich source of GFs that can synergistically amplify each other's effects, and make each other more sustainable (7, 8, 15). PRP shows beneficial properties in different fields of medicine such as oral and maxillofacial bone grafting procedures, healing wounds (16, 17), plastic surgery (18), oral surgery, dentistry and ophthalmology (19). It has also been indicated to have beneficial effects on the growth, neuroprotection, anti-apoptosis and regeneration of peripheral nerves such as the sciatic nerve (20–22), facial nerve (23) and the cavernous nerve (24, 25). The studies have examined the effect of PRP in peripheral blood on axonal regeneration and remyelination of the SCI models (14, 26–30). Studies indicated that the existence of numerous growth factors such as FGF, EGF, VEGF, IGF-1, interleukins and interferons in PRP is responsible for inducing many of its beneficial effects (31).

Human umbilical cord blood (HUCB) is also abundant and can be obtained by non-invasive methods and has none of the ethical issues related to other adult human resources (32). These clinical benefits included easy access to transplantation, relatively low incidence of GVHD (Graft-Versus-Host-Disease).
The platelets count in HUCB is higher than in peripheral blood (34). HUCB Platelet-rich plasma (HUCB-PRP) has a higher amount of GFs such as vascular fibroblast growth factor (FGF), VEGF, IGF-1 and epidermal growth factor (EGF), interleukins and interferons than peripheral blood, which are used for cell growth, proliferation and differentiation in fetal blood (31). Given our knowledge of related articles, there are few studies that have investigated the effect of PRP injections on injured spinal cords and activation of intrinsic mechanisms (14, 26, 35). In this study, we investigated the effect of intraspinal injection of HUCB-PRP on the improvement of motor function and the expression of proteins affecting decreases wallerian degeneration and axonal stability.

Methods

animals

Adult male Wistar rats (weighing 150-185 g; n=10 per group) were randomly allocated into the experimental groups (Table 1). Experimental research made in accordance with the guidelines on laboratory animals of the Iran university of medical sciences and approved by the ethics committee (IR.IUMS.FMD.PEC.1398.037). The animals were kept under standard housing conditions (21±1 °C and 12 hours' light-dark cycle) with pellet food and water provided ad libitum. Also, animal weight was measured before induction of injury and at the end of the sixth week (Table 1). The injured animals were kept separately and were sprayed by OTC to prevent infection after the surgery.

Table 1: The experimental groups. HUCB-PPP: Human umbilical cord blood Platelet-poor plasma, HUCB-PRP: Human umbilical cord blood Platelet-rich plasma; SCI: spinal cord injury

| Study Groups | Treatment                                                                 | Weight of animals before SCI (mean ± SEM; n=10) | Weight of animals in the end study (mean ± SEM; n=10) |
|--------------|---------------------------------------------------------------------------|--------------------------------------------------|-----------------------------------------------------|
| Control      | Healthy animals                                                           | 170.61±4.06                                      | 329.21±6.01                                         |
| Sham injection | Removing the lamina with no injury to the spinal cord + inserting the micropipette intraspinally at 4 points | 175.88±4.35                                      | 334.9±8.94                                          |
| SCI          | Removing the lamina + SCI induction                                       | 171.93±3.8                                       | 276.19±14.13                                        |
| Vehicle      | SCI induction + injection of HUCB-PPP intraspinally at 4 points on the second day after SCI | 169.65±3.41                                      | 300.51±12.07                                        |
| PRP 2 day    | SCI induction + injection of HUCB-PRP intraspinally at 4 points on the second day after SCI | 172.88±3.29                                      | 307±1259                                             |
| PRP 14 day   | SCI induction + the injection of HUCB-PRP at 4 points Intraspinally on the fourteen day after SCI | 172.29±2.84                                      | 318±15.49                                            |
HUCB-PRP preparation

The HUCB-PRP obtained from a Blood Transfusion Organization (Tehran, Iran). The protocol used for platelet isolation was as follows:

PRP was separated from the blood by two-stage centrifugations at 1500 rpm for 10 minutes in 4 °C and then 2200 rpm for 10 minutes. After this stage, Platelets precipitate and the upper phase used as platelet-poor plasma (PPP) for injection to the vehicle group. Then the precipitate of platelets was suspended in 0.5 ml of plasma and freshly used for injection in treatment groups. The platelet concentration was calculated by an automatic counter (Model Sysmex X-S 800i; Kobe, Japan).

Surgical procedure and HUCB-PRP injection

Surgery was done under intraperitoneal injection of ketamine (100 mg/kg) and xylazine (10 mg/kg) anaesthesia. After induction of incision in the skin, the back musculature was pushed aside at the midline, and a laminectomy was performed at the junction between the T12 and T13 vertebrae (in line with L2-L3 spinal segment). The last rib was as a guideline for confirming injury site. Because it is at the junction with the T13 vertebra (36). Then moderate spinal cord injury was created by calibrated aneurysm clip (force=20gr/cm2; for the 90s; FST company). The muscles and skin were sutured. Rats received tetracycline spray on the injured site to avoid infection and %70 alcohol spray in the cage daily for one week and bladder emptying was performed until the return of the bladder control reflex. The Basso, Beattie, and Bresnahan (BBB) locomotor scale were performed 48 h after the surgery to confirm the loss of motor function in the SCI rats. Tumor or bone formation was not observed in any of the animals in our experiment.

The intraspinal injection of HUCB-PRP on the second and fourteen days after induction of SCI were done on the total volume of 6 μL/4 locations in the spinal cord with coordinates 0.5 mm lateral from the midline in the depth of 1 mm from the surface. The rate of injection was 1 μl/min. At the time of injection, the animals were anesthetized with Ketamine and Xylasin. The skin, muscle and adherent tissue were carefully separated. After determining the spinal cord levels, the animal was fixed in a stereotaxic device. Approximately, 6095426.58±266778.13 platelets were drawn by Hamilton's syringe. Then the needle was inserted into a glass micropipette (with the tip diameter of about 30 μm) and fixed in the stereotaxic device. The micropipette inserted to the spinal cord. After injection of PRP, the micropipette remained in its place for two minutes for preventing PRP leakage. The above method was also used for vehicle animals, which received HUCB-PPP (351489.92±88185.41 platelets) on the second day post SCI.

The evaluation of open-field locomotor scale

The 21-point Basso-Beattie-Bresnahan (BBB) locomotion scale was used to assess the locomotor behavior recovery after SCI. Based on the movement of joints, weight-bearing, placement of paws and coordination of paw and hind limbs (37), the hind-limb motor function was assess every week until the end of the study (six weeks follow-up). For this reason, each animal was monitored for 4 min in a cylinder
with the 24 cm height and 90 cm diameter. The BBB scores were calculated by independent examiners who were not aware of the experimental groups and the mean scores were used in the analysis (38). The BBB assessment was performed before and after SCI induction. The BBB assessment was performed before SCI induction and conducted weekly after injury for six weeks.

Cerebrospinal fluid (CSF) collection

Obtaining CSF through cisterna magna is a routine method in our Center for experimental and comparative studies. Animals were anaesthetized with the same dose of ketamine and xylazine and their head skin was shaved. The rats were fixed by the ear bars of the stereotaxic frame and were placed the head at an angle of 90° (n>5). After the incision of the skin, the muscles were pushed aside to expose the cisterna magna membrane, Then the plunger of the insulin syringe was pulled slightly and inserted into the device. The needle was perpendicularly entered to cisterna magna until the beveled edge (between 0.5 and 1 mm). The pressure difference pulled CSF to the syringe and then the plunger of the syringe was gently pulled. The maximum volume of CSF collected was 210 microliters. Then the clear CSF is transferred into Eppendorf tubes and stored at −80 °C.

Tissue evaluation

For tissue assessment, the animals were deeply anaesthetized with ketamine (100 mg/kg, IP) and xylazine (10 mg/kg, IP) 6 weeks after surgery (n=3 per group).

The spinal cord was cleaned of blood by transcardial perfusion of normal saline and then was fixed by 4% Paraformaldehyde (pH=7.2-7.4). Then vertebral columns were dissected and post fixed in 4% paraformaldehyde for 24 hours. The dissection was made in the larger area of T12-T13 vertebra levels (site of injury) to prevent extra injury to the level of the spinal cord. The L2-L3 level of spinal cord was held by paraffin wax (36). The sections with 5 μm diameter were prepared for the immunohistochemically technique.

Antibodies and reagents

MAG and Tau antibody were obtained from the biorbyt Company (Cambs, UK)). GSK3β, β-actin antibody, Anti-rabbit IgG HRP-linked Antibody were purchased from Santa Cruz Biotechnology Company (Santa Cruz, CA, United States). Caspase3 antibody was purchased from the Cell Signaling Technology company. Rabbit SpecificHRP/DAB (ABC) was prepared by the Abcam Company (Cambridge, UK).

Immunohistochemistry

At the day of experiment, deparaffinized and hydrated longitudinal sections were boiled in 0.1 M sodium citrate buffer for 10 min to perform the heating antigen retrieval, then the sections were washed three times with PBS, Triton X-100/10% and subsequently blocked with 10% normal goat serum or with 1% BSA in TBS for 2 h at room temperature. After washing, The primary Rabbit monoclonal antibodies capases3 (cat. no. 14220) were diluted (1/100) in blocking solution overnight at 4 °C and the next day were
incubated with goat antirabbit diluted in PBS/0.3% BSA for 1h. Then they were incubated with enough Streptavidin–HRP (pink) for 10 minutes. Then enough DAB was added to the washed slides. After optional counterstain and dehydration, cover slips were done. Finally, for calculating the density of caspase3, three sections from the beginning, middle and the end of the injured location of the spinal cord (N=3 each group) were selected. Then five fields of the dorsal surface in each section were randomly selected, the density of caspase3 expression in each section was calculated as follows.

The density at each area = the density of DAB stain at an area / The area of the section

The final density of caspase3 expression = the total density of DAB substrate (caspase3 expression) in five areas /5

The mean of density in all three sections is calculated for each animal.

Protein extraction of spinal cord tissue and CSF for western blotting

To assay the effect of HUCB-PRP injection on proteins the western blot (WB) technique at 6 weeks after injury were performed. For this purpose, the animals were anesthetized with the same dose of ketamine and xylazine (n=4 per group). Fresh spinal cord containing the injury location (5 mm in length) quickly dissected carefully and immediately transferred to ice and stored at − 80 °C before performing the Western blotting. For the extraction of protein, 300 µl of RIPA buffer was used for every 100 mg of tissue. RIPA buffer was contained 10 µl of all three enzymes: PMSF, Protease inhibitor cocktail, Sodium orthovanadate. Tissues were homogenized on the ice and were centrifuged (13,000 g, 30 min, 4°C). The supernatants were collected and protein concentrations assayed by using the nanodrop equipment (Thermo Science, USA).

The protein concentrations of CSF were directly assayed by using the Nano drop equipment. The total concentrations of proteins in the CSF and in the supernatants of the spinal cord tissue are expressed in the table 2.

Table 2: The total concentrations of proteins in the CSF and supernatants of the spinal cord tissue
| Study Groups         | Protein concentration in spinal cord tissue (mean ± SEM; n=4) | Protein concentration in CSF (mean ± SEM) |
|---------------------|---------------------------------------------------------------|-----------------------------------------|
|                     | mg/ml                                                         | mg/ml                                   |
| Control             | 18.83± 3.08                                                  | 3.08± 0.179                             |
| **Sham injection**  | 25.56± 3.7                                                   | 3.32±0.165                              |
| SCI                 | 18±1                                                          | 3.18±0.185                              |
| Vehicle             | 12.83± 4.28                                                  | 3.49±0.382                              |
| PRP 2 day           | 12.48±1.63                                                   | 3.35±0.103                              |
| PRP 14 day          | 14.33± 4.86                                                  | 3.69±0.118                              |

**Western blotting:**

Equal amounts of supernatant tissue and CSF proteins (50μg) were electrophoresed in 10 SDS-polyacrylamide gel electrophoresis, and then the proteins were transferred to polyvinylidene difluoride membranes (PVDF) (Time: 25 min, voltage: 15 V). The membranes were blocked with a fresh blocking buffer (containing 5% skim milk and 0.1% Tween-20 in Tris-buffered saline, pH 7.4) for 2 h, and then the membranes incubated with specific primary antibodies GSK3β antibody (1:200, sc-81462), Tau antibody (1/1000, orb158145, MW: 52/79 kDa), MAG antibody (1/1000, orb536682, MW: 63 kDa), β-actin antibody (1:500, sc-47778, MW:45KD) in blocking buffer overnight at 4 °C overnight at 4. After three washes with TBST, the blots were incubated with horseradish peroxidase conjugated IgG (1/1000, sc-516102) for 2h. Protein bands on the membranes were detected by enhanced chemiluminescence (ECL) and exposed by the ChemiDoc Imaging System. The β-actin antibody was used to normalize the western blot bands. Band intensity was measured quantitatively and analyzed by Image J software.

**Statistical analysis**

All the evaluations have been performed blindly without aware of animals grouping. SPSS 22.0 and Graph Pad Prism 8.0 was used for statistical assessment of the data (mean ±SEM). The one-way ANOVA followed by a Tukey post-hoc test was used for analysis the data of western blot and immunohistochemistry methods, and the repeated-measure ANOVA with a Bonferroni post hoc test was used for comparison of the BBB scores between different groups. p<0.05 was considered as significant.

**Results**

In this study, dead animals or animal with rotational motion were excluded from the study. These rats were replaced and the data from 60 animals were used in the analysis.
The intraspinal injection of HUCB-PRP improves locomotion recovery function.

Initial evaluation of SCI locomotor recovery was carried out using the BBB scale which has been shown to be useful in evaluating post-SCI function in rats. The score (0–21) represents different combinations of joint movements, coordination, paw placement, and toe clearance where a higher score represents a better functional motor recovery. Significant differences in the mean score between the HUCB-PRP-treated groups and no-HUCB-PRP group (SCI and vehicle groups) represents improvement in the movement of injured animals (df= 30.324; F=59.63; p<0.0001).

The BBB score for animals that received HUCB-PRP on the second days after SCI had the best locomotor function recovery (with a mean initial BBB score of 8.7 ± .597 in the first week and a mean BBB score of 15.15 ± 0.5377 in week 6) than the SCI group (p<0.0001) and vehicle group (P = 0.0042).

Improvement of the BBB score for animals which received platelets after 14 days (PRP 14 day), started two weeks after injection of PRP with a mean initial score of 5.7 ±0.27 and was significantly different versus the SCI group at the end study (p<0.0001). The recovery process in PRP 14-day group showed a slower recovery rate than in the PRP2 day group until the end of study. There was a significant difference between the PRP2 day and PRP 14-day group. It is noteworthy that the motor function score in the both groups injected with PRP was still lower than healthy animals up to the end of the study (p<0.0001) (Figure 1).

HUCB-PRP decreased caspase3 expression, as an apoptosis marker in injured animals

The statistical result showed a significant difference in caspase3 expression between groups at the end of the study (dF=5; F =18.852; p<0.0001). At 6 weeks postoperatively, immunohistochemical technique showed an obvious increase in the caspase3 expression in the SCI (58.947±1.01; p<0.0001) and vehicle (52.537±5.72; p<0.0001) groups versus the control animals. Indicating that the injury increased apoptosis process and plasma without sufficient platelet concentration has no improving effect on the caspase3 expression (vehicle group). Significant decrease in caspase3 expression observed in animals which received PRP on the second and fourteenth day after injury (43.426±1.28; p=0.02 and 45.26±0.584; p=0.043 respectively) compared to the SCI groups. Also there was a significant difference in the caspase 3 expression between both HUCB-PRP-treated groups and the control group at 6 weeks postoperatively (p=0.004 and p=0.002 respectively) (Figure 2).

HUCB-PRP decreased glycogen synthase kinase-3β (GSK3β) expression as an inducer of apoptosis and axonal degeneration in injured animals

The statistical result showed a significant difference in GSK3β expression between groups at 6 weeks post-SCI (df=5; F =4.704; p=0.013). At the end of the study, an obvious increase in GSK3β expression in the SCI group (1.35±0.116; p=0.027) versus the control group (0.822±0.049) was observed which indicated that the SCI increased the inducer of apoptosis and axonal degeneration. The expression of
GSK3β in animals which received HUCB-PRP on the second and fourteenth day after injury (0.792±0.119; p=0.018 and 0.83±0.075; p=0.029 respectively) was significantly decreased compared with the SCI groups. There was no significant difference between both PRP-treatment groups and the control group at 6 weeks postoperatively (Figure 3)

**HUCB-PRP decreased the tau expression in CSF as a biomarker of axonal degeneration in injured animals**

The statistical analysis showed a significant difference in the tau expression in CSF between groups at the end study (df=5; F=7.655; p=0.002). At the sixth week after injury the tau expression significantly increased in the SCI animals (3.89±0.372; p=0.001) compared to the control animals (0.372±0.117) indicating that the axons severely degenerated in the injured group and the tau protein separated from the microtubules and leaked into the CSF.

The total tau expression in animals which received HUCB-PRP on the second and fourteenth day after injury (1.128±0.227; p=0.009 and 1.044±0.315; p=0.008 respectively) significantly decreased compared with the SCI groups. There was no significant difference between both PRP-treatment groups and the control group on sixth week postoperatively (Figure 4).

**HUCB-PRP increased the MAG expression of spinal cord in injured animals**

The statistical result showed a significant difference in MAG expression between groups at 6 weeks post-SCI (df=5; F=9.334; p<0.0001). Six weeks after injury a significant decrease observed in MAG expression in the SCI (0.8±0.358; p=0.009) and the vehicle group (1.09±0.282; p=0.037) versus the control group (2.55±0.281). This decrease in the level of MAG expression was compensated by injection of HUCB-PRP and in animal which received HUCB-PRP on the second and fourteenth day after injury (2.57±0.227; p=0.008 and 3.24±0.399; p<0.0001 respectively) the expression level increased compared with the SCI groups. There was no significant difference between both PRP-treatment groups and the control group at 6 weeks postoperatively (Figure 5).

**Discussion**

In this experiment, the clip compression injury model was chosen since the symptoms of injury in the acute stage are quite similar to spinal cord injury at the time of the accident, and in chronic conditions, glial scars form in the spinal cord, which was quite similar to patients with spinal cord injury (39). Our study reported for the first time the effect of PRP in human umbilical cord blood on spinal cord injury. HUCB-PRP was chosen because it provides easier access, lower sensitivity, longer durability, low immunogenicity and most importantly, richer growth factors such as PDGF, FGF-2, VEGF than adult PRP (31, 40–43). Umbilical cord blood is less exposed to environmental pathogens and vaccines compared with adults' blood and it has a high ratio of T cells (44) and a lesser amount of inflammatory factors (45).

Our statistical analysis indicated that injury to the spinal cord decreased locomotor function recovery and the expression of MAG protein in spinal cord tissue, and increased expression of capase3, GSK3β and
CSF-tau proteins and treatment with HUCB-PRP compensated these alterations. Spontaneous recovery happened at a slow-rate until the end of the study in the SCI group which confirms the correctness of our model. Spontaneous recovery is mostly happen due to local structural rearrangements, such as collateral sprouting from remaining axons in gray matter than the regeneration of axons in white matter (46). In our study, the induction of apoptosis and axonal degradation may be the cause of motor disability in the SCI animals.

Under normal conditions, there is a balance between the pro-apoptotic factors and the anti-apoptotic factors which disrupted during injury and promotes the cell death. Caspase3 is a definite factor in promoting apoptosis. Apoptosis exerts its effects via two main apoptotic pathways depended on caspase 3: the extrinsic or death receptor pathway, the intrinsic or mitochondrial pathway. Since caspase3 production, is the starting point of the executive phase of apoptosis, it is considered the principal indicator of apoptosis (47). It is believed that apoptosis is the main factor in the loss of motor neuron functions after injury (3–5, 48). In our experiment the existence of big cavity size in the SCI and vehicle groups confirm degeneration in the site of injury (Fig. 2). Oligodendrocytes and Schwan cells are the source of storage and release of Myelin-associated glycoprotein (MAG) protein and play an important role in the myelination of motor fibers axons (49). Its function is the maintenance of myelinated axons (49), inducing axon durability and entirety, and protection of axons under pathological conditions (50, 51). MAG stimulates axonal growth of young neurons and in the opposite role, suppresses the growth of older neurons in the adult CNS. This shift of function depends on age, developmental stage and type of the neurons (52, 53). Genetically deleting MAG decreased the axonal sprouting in the corticospinal tract (54, 55). Apoptosis induced oligodendrocyte destruction at 2 to 3 weeks post-SCI (56, 57). Oligodendrocytes are particularly susceptible to environment toxicity after SCI. They stand both necrosis and apoptosis in the acute phase, and their destruction continues with apoptosis at the chronic phase. Since oligodendrocytes are the main source of MAG in CNS, apoptosis of oligodendrocyte reduces MAG protein.

Also, in our study, the level of GSK3β increased following injury to the spinal cord. GSK-3β is a major member of the Wnt pathway. The Wnt pathway is the one of most important cascade in the nervous system. Evidence has shown that dis-regulation of the Wnt pathway causes pathogenic signals in neurodegenerative diseases (58). It has been shown that injury to the spinal cord causes the dysfunction of motor system in three ways: 1) the induction of the apoptotic process with caspase 3 expression (59, 60). 2) the activation of GSK3B–MCL1 pathway as a basic factor for Wallerian degeneration which results in axonal degeneration (61) and 3) hyperphosphorylation of Tau and neurodegeneration (62).

Phosphorylated tau is observed in the primary stages of neurodegenerative diseases (58). Tau is a neuronal microtubule-associated protein and in normal condition has a role in microtubule stabilization or the regulation of axonal transport. In many cases, tau phosphorylation results in isolation from microtubules and induce tau toxicity (63). Also, The presence of phosphorylated tau in the cerebrospinal fluid is a biomarker for the severity of CNS injuries such as Multiple Sclerosis (64), SCI (65) and Alzheimer's disease (66). The dysfunction of Tau protein could play a vital role in the development of the
injury in SCI situation (67). It has been reported that the hyperphosphorylation of tau by GSK3 in dendritic spines damages the synaptic activity (68).

Our data showed that HUCB-PRP treatment improved the motor function, but did not recover it completely. The reason for this difference could be due to inhibitory natural barriers for axonal growth cones in mature neurons and intrinsic insufficient growth capacity of CNS (1). Also, HUCB-PRP dramatically increased MAG expression and decreased GSK3β, caspase3 and CSF-tau levels compared to the injury group. Previous studies showed that PRP promoted or/and help axonal regeneration and sprouting of axons in the SCI models (14, 26–30, 69). Similar to the results from Salarinia's study (29) we showed that PRP injection reduced apoptosis which resulted in an increased in the MAG level and reduction in the destruction of oligodendrocytes. However, some studies have not indicate any appreciable changes in the content of MAG in the early and later stages of central Wallerian degeneration (70). PRP is rich in the growth factors which each of them has beneficial effects on cell growth and survival. Some of growth factors in PRP assist in the repair processes after the injury and play a role as a catalyst for improving the repair ability of regenerating fibers. It has been shown that IGF-1 and NGF are able to increase the axonal growth of motor neurons in vitro and in vivo respectively (9). Sharma et. al showed that the use of BDNF, GDNF, and IGF-1 alone or in combination reduced blood-spinal barrier failure, edema, and cell damage in rats with spinal cord injury, although treatment is more effective when growth factors were combined (7, 8). Also, BDNF and GDNF have significantly improved motor function(8). In another study, revealed that combined PDGF and IGF-I did not recover sciatic nerve regeneration in a transaction and anastomosis model (71). Also, an NGF administration develops the motor function and induce the survival of the neurons in the SCI. NGF diminishes neuronal apoptosis by inhibition the caspase-3 cascade (72). Some study showed that NGF (73) and IGF-I has neuroprotective effects that are done through activating Akt, inhibiting GSK-3β subsequently reducing tau hyper phosphorylation in vivo (74) and invivo (75). If these factors are applied locally just once, their effects on recovery will not be exerted sufficiently. To increase their function, they must be applied several times or applied by some additional methods (used of fibrin sealant clots, foams, organic/synthetic tubes, and osmotic pumps) (76–78). However, PRP is the combination of growth factors that boosts the synergy effects (7, 8). We did not check the composition and concentration of the growth factors in the HUCB-PRP and this is the limitation of our study. It has previously mentioned that PRP delivered from umbilical cord has richer growth factors than adult PRP (31, 40). A few studies have examined the effect of PRP on axonal regeneration and remyelination of the spinal cord injury models (14, 26–30).

In 2012, Takeuchi et al, the first time used a rat organ co-culture system (brain-spinal cord co-culture) to estimate the ability of PRP to stimulate the axonal growth in spinal cord tissues and identified the growth factors in PRP that contribute to the regulation of the axonal growth. They found that PRP enhanced the axonal growth in the spinal cord tissues through mechanisms related to IGF-1 and VEGF, while TGF-β1 in PRP exerted destructive effects on the axonal growth (35). In another study, Salarinia et. al. showed that PRP injection 24 hours and one week after spinal cord injury improves motor function and increases axonal repair (26). He also showed that combined use of platelet-rich plasma and adipose tissue-derived mesenchymal stem cells has a synergistic effect on motor function recovery by inhibiting the caspase3
pathway (29). Chen et al.'s study indicated that intrathecal PRP injection caused neuronal regeneration (14). Also in 2020, a study stated that in chitosan scaffolds filled with PRP and human endometrial stem cells (hEnSCs), PRP increased the survival of hEnSCs cells and prevented the formation of scar tissue. It also was responsible for angiogenesis and regeneration of the nerve fibers (28).

The results of our experiment are in agreement with previous studies (14, 26, 29, 48).

We propose the following mechanism for the effectiveness of HUBC-PRP on motor function recovery after SCI (Fig. 6).

1) Oligodendrocyte apoptosis decreases MAG expression and induces Wallerian degeneration. PRP improves axonal regeneration and sprouting in nerves via suppressing the caspase3 enzyme and reducing apoptosis (35, 79). Sprouting is a naturally occurring repair mechanism (80). Studies have shown that during sprouting of healthy axons, MAG secretion increases to inhibit ectopic sprouting of axons and modulates the sprouting process (53, 80), and supports axons from later degeneration in the adult CNS (81). But the effect of MAG on the regeneration of injured axons is limited (80). Also, the study showed that only after damage of the myelin and axonal attempt for regeneration, MAG is released and affect the growth cones (81).

2) PRP Inhibits the expression of GSK-3β. GSK-3β participates in several signaling pathways such as Wnt signaling pathway and apoptosis. It has been shown that GSK-3 inhibitors regenerated injured neuronal fibers and functional recovery in rats with spinal cord injury (82). Reducing the GSK-3 levels promotes axonal elongation and branching in cortical and hippocampal neurons (83). Also, apoptosis was diminished by inhibiting GSK-3β in primary neurons (84). On the other hand, PRP boosts contact between Tau protein and microtubule by inhibiting GSK-3β expression. Therefore, Tau maintains the stability of microtubules in the spinal cord axons. As a result, Tau repairs axonal transport and synaptic dysfunctions.

The other possibility of beneficial effect of PRP injection could be through mTOR signaling pathway. Our previous study indicated that HUCB-PRP increases mTOR signaling (69). MTOR signaling (as an upstream protein for apoptosis and GSK-3β) regulates central neuronal myelination and oligodendrocyte differentiation (85, 86) and inhibits apoptosis (87) and GSK3β expression (88).

Therefore, it seems that PRP could act through different mechanisms to help the central nervous system to prevent cell death and induce axonal regeneration and could be considered as a new treatment strategy in a situation with spinal cord injury.

**Conclusion**

Our results showed that intraspinal injection of HUCB-PRP could significantly improve motor function recovery after SCI. HUCB-PRP injection decreased caspases3, GSK3β and tau levels and increases MAG expression six weeks after injury. Our findings provide valuable information on the influence of a HUCB-
PRP on alleviating SCI symptoms. Since HUCB-PRP has long durability and richer growth factors than adult PRP and because it does not stimulate the immune system responses it might provide a suitable therapeutic opportunity for axonal regeneration and motor function recovery after injury to the nervous system in human. However, more investigation is required to translate the results to the clinics.

**Declarations**

**authors’ contributions:**

F.N: Designed the experimental and supervised the procedure. Z.B: Carried out the experimental, the statistics and wrote the manuscript under supervision of F.N.

F.R: Supervised and analyzed the western blot technique. All authors read and approved the final manuscript.

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**Availability of data and material:**

Data are however available from Zahra Behroozi upon reasonable request.

**Ethics approval and consent to participate:**

Experimental research protocol on animals was approved by the ethics committee of Iran University of Medical Sciences (IR.IUMS.FMD.PEC.1398.037). All procedures were strictly conducted in accordance with the code of ethics.

**Consent for publication:**

All authors consented for publication.

**Competing interests:**

The authors declared that they have no competing interests.

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Figures
Figure 1

The effect of intraspinal injection of HUCB-PRP on motor function recovery during six weeks after SCI; quantitative data are expressed as the mean ± SEM. *p<0.05, **p<0.01, ***p<0.001 versus SCI groups. #p <0.05, ##p<0.01, ###p<0.001 versus control group. $p<0.05, $$$p<0.001 versus group which received PRP on the second day after injury. b: ###, $$$

Figure 2

IHC images of longitudinal sections of L2-L3 levels of spinal cord. The IHC images of caspase3 expression in control (A), sham (B), spinal cord injured (C), vehicle (D), PRP 2day (E) and PRP 14day (F) groups were shown. The reduction of caspase 3 as a hallmark of apoptosis by HUCB-PRP is a result of...
remarkable antiapoptotic activity of HUCB-PRP. Red arrows showed the apoptotic cells. Normalized density of caspase 3 in different experimental groups (G). Data are expressed as mean±SEM. ### p<0.001, ##p<0.01, #p<0.05 compared to the control group and $p<0.05 compared to the groups which received PRP second and fourteen days after SCI. The images on the left showed at an original magnification ×4 and the images on the right at an original magnification ×40.

![Graph showing normalized density of caspase 3 in different experimental groups.](image)

**Figure 3**

Western blot analysis of GSK3β protein expression showed that expression of GSK3β in the spinal cord after intraspinal injection of HUCB-PPP in injured rats was decreased. The test was performed thrice on GSK3β proteins and integrated optical density of each band was normalized to corresponding β-actin as a loading control. Total GSK3β (A); western blot bands of total GSK3β protein (B). Data are expressed as the mean±SEM. # p<0.05 compared to the control group and $ p<0.05 compared to the groups which received PRP on the second and fourteenth day after injury.

![Western blot analysis showing GSK3β protein expression.](image)

**Figure 4**
Western blot analysis of tau protein expression showed that expression of tau in the spinal cord after intraspinal injection of HUCB-PPP in injured rats was decreased. The test was performed thrice on tau proteins and integrated optical density of each band was normalized to corresponding β-actin as a loading control. Total tau (A); western blot bands of proteins of total tau (B). Data are expressed as the mean±SEM. ## p<0.01 compared to the control group and $$ p<0.01 compared to the groups which received PRP on the second and fourteenth day after injury.

**Figure 5**

The increased expression of MAG in the spinal cord after intraspinal injection of HUCB-PPP in injured rats. The test was performed four times on proteins and integrated optical density of each band was normalized to the corresponding β-actin level. MAG expression (A); western blot bands of proteins (B). Data are expressed as the mean ± SEM. ## p<0.01, # p< 0.05 compared to the control group and $$ p<0.01, $ p< 0.05 compared to the groups which received HUCB-PPP on the second and fourteenth day after spinal cord injury.
Figure 6

Schematic representation of relation between apoptotic pathways and Gsk3β expression on motor function recovery. The spinal cord injury results in increasing caspase3 and GSK3β. When death signals are received by the cell, the pro-apoptotic protein such as caspase3 was activated, following this, Wallerian degeneration is enhanced in the cell through the destruction of oligodendrocyte and decrease the expression of MAG. On the other hand, the GSK3β enzyme hyper-phosphorylates tau protein and disconnects it from microtubule. Therefore, impaired connection between tau protein and microtubule, as a result, failed microtubule stability. Also, GSK3β increases caspases3. Therefore, GSK3β synergically reinforces the apoptosis process and diminishes the recovery of motor function. HUCB-PRP thereby reversed the above pathway and improve the motor function.

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