Functional Recovery & Neurogenesis after Transplantation of Stem Cells from Human Umbilical Cord Blood into Spinal Cord Injured Rats

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Abstract

Background Data: Transplantation of umbilical cord blood stem cells into damaged spinal cords was used experimentally for several years. Such transplants survive and integrate to some degree with the host tissue and may be associated with functional improvement**.**

Purpose: To find out the functional improvement, and axon regeneration in spinal cord injured rats after human umbilical cord blood stem cells (HUCBs) transplantation.

Study Design: Prospective analytic animal experimental study.

Material and Methods: Forty rats were recruited and divided into four groups, each containing 10 rats: Group (1): Control group with no lesion and intervention. Group (2): Injured animals with no treatment Group (3): Injured and injected with saline. Group (4): Injured and injected with HUCBs. Animals were subjected to behavioral assessment using two physiological tests. Histopathological sections and immunohistochemical examination were done.

Results: The results of this study have shown that the HUCBs reduced the neurological function deficit to a moderate degree.

Conclusion: Remyelination and new astrocyte formation could be established after HUCBs transplantation to the injured rats. (2012ESJ033)

Key Words: Stem cells, Functional recovery, Neurogenesis, Spinal cord injury

Introduction

Traumatic spinal cord injury (SCI) affects many people, especially young, and can result in severe damage, leading to paraplegia, tetraplegia, or quadriplegia. Many strategies, including surgical, pharmacological, neurophysiological, and technological approaches, have been used in attempts to develop new therapies that will allow patients to regain use of their paralyzed limbs. One such strategy is the transplantation of umbilical cord blood stem cells into damaged spinal cords, which has been performed in rats and cats over the past several years. Such transplants survive and integrate with the host tissue and may be associated with functional improvement.[1,12]

Human umbilical cord blood is a valuable source of cells that have the therapeutic potential to initiate and maintain tissue repair. This capability holds special promise for the treatment of neural diseases, for which no cure is currently available. In addition, therapies based on human umbilical cord blood are attractive because the cells are readily available and less immunogenic as compared to other source of stem cells, such as bone marrow. The therapeutic potentials of human umbilical cord blood may either be attributed to the inherent ability of stem cell potential of damaged tissue outright, or alternatively, to their ability to repair damaged tissue through neural protection and secretion of neurotrophic factors by various cell types within the graft.[13]

There have been many efforts to restores normal neuronal functions and thus motor functions after spinal cord injury (SCI), in which the myelin sheaths and or myelinating cells (e.g. oligodendrocytes) are destroyed. Although some spontaneous remyleination occurs, this process is not consistent enough for complete repair.[13] This phenomenon depends on molecules (e.g., growth factor), most of which are still unidentified.[42]

Perhaps more importantly, stem cells could promote axonal regeneration either by constituting a "bridge" through a lesion site capable of supporting attachment and growth or by secreting diffuse molecules, such as growth factors, to attract injured axon.[23]

The focus of this study is to evaluate functional outcome, and characterize neural differentiation after transplantation of human cord blood stem cells into a rodent model of acute spinal cord injury.

Materials and Methods

Separation and culture of HUCBs:

Human umbilical cord blood was obtained, using sterile syringes, from the umbilical veins immediately after full-term deliveries. All the samples were collected after obtaining written informed consent. The blood sample volume was 100 to 150ml. Aspirated blood was diluted 1:1 with Hank's balanced salt solution (HBSS) and centrifuged through a density gradient (Ficoll-Paque Plus; 1.077 g/l; Pharmacia, New York, NY) at 1000xg for 30 min. The mononuclear cell layer was then recovered from the gradient interface, washed with HBSS, centrifuged at 900xg for 15 min, and then re-suspended in complete culture medium [Dulbecco's modified Eagle medium (DMEM, Gibco BRL, Carlsbad, CA) supplemented by 20% fetal bovine serum (Gibco BRL, Carlsbad, CA), 100 units/ml penicillin, and 100 ug/ml streptomycin], with the cells at a concentration of $1x10^6$/ml. The cells were next incubated at 37°C for 3 days.

Animals:

Forty adult (300-350 g) Sprague-Dawley rats from the Center of experimental animals in Zagazig University were used in all experimental groups. All the experiments were performed in compliance with relevant laws and institutional guidelines. The procedures followed were in accordance with the standards set forth in the Guide for the Care and Use of Laboratory Animals (published by the National Academy of Science, National Academy Press, Washington, D.C.). Animals were acclimatized for one week and kept with free access to standard pellet animal diet and tap water under controlled conditions of room temperature.

Spinal cord injury:

Acute SCI was induced using chemical spinal cord injury by using single intracisternal injection of 0.4 ml 2.5 % Gentamicin sulfate according to the method of Hodges (Hodges and Watanabe, 1980). Rats were anesthetized with pentobarbital sodium (50mg/kg, i.p.). Laminectomy was performed between T8 and T10 after midline vertical incision over the thoracic spine then, the paraspinal muscles were retracted laterally. The dura mater was not opened. The spinous process was clamped to stabilize the spine. Injury was induced chemically by using single intracisternal injection of 0.4 ml 2.5 % Gentamicin sulfate. After injury, the muscles were closed and haemeostasis was ensured. Postoperative nursing care included bladder expression twice a day. Prophylactic kanamycin (1mg/kg) was regularly administered for a week.

Study Plan:

Animals were equally divided into four groups, each containing 10 rats**:** Group (1): Control group with no lesion and no intervention. Group (2): Injured animals with no treatment. Group (3): Injured animals and injected with saline. Group (4): Injured animals and injected with stem cells.

Transplantation:

One week after surgery; group 3 animals were

injected with 5µl normal saline by using insulin syringe and group 4 animals were injected with total amount of 1×10^6 cells dissolved 5µl saline at the site of the injured spinal cord.

Behavioral assessment after spinal cord injury (SCI):

1- A behavioral test was performed to measure functional recovery of the hind limb. The open field testing procedures used in this study was described by Basso et al. This scale measures hind limb movements with a score of 0 indicating no spontaneous movement, with an increasing score being given for the use of individual joints, coordinated joint movement, coordinated limb movement, weight-bearing and so on to a maximum score of 21. Behavioral testing was performed weekly upon each hind limb from the first postoperative day to 8 weeks after SCI for all animals using the Basso, Beattie, and Bresnahan locomotor rating scale (BBB) scoring system, by an independent examiner who was kept blind regarding the rat's treatment status.[2,37]

2- Inclined plane test (modified Tarlov test).[15,39] The device consists of a hinged board raised and lowered to different angle. The object is for the rate to maintain itself on the board for 5 seconds as the angle is gradually increased at 5 degrees intervals. Uninjured rats achieve approximately 80 degrees.

Histology:

Eight weeks after the induction of injury, the rats were anesthetized with diethyl ether to sacrifice. Spinal cords were immediately removed and the injured region dissected. Segments 20mm rostral and caudal to the injury site were then embedded in paraffin. Each block was serially sectioned to prepare 5 µm thick sections, which were stained with hematoxylin & eosin (H & E) and Luxol fast blue/ Cresyl violet (LFB/CV) stain. The slides were viewed under a light microscope to study the structural changes.

Immunohistochemistry:

For the immunological studies, deparaffinized spinal cord sections were boiled in citrate buffer (pH 6) for 10 minutes in a microwave oven. Following blocking in normal serum, the sections were incubated with monoclonal antibodies Anti-glial fibrillary acidic protein (GFAP) which is specific for astrocytes in the central nervous system.

Statistical analysis:

Data was analyzed with the SPSS statistical software program version 15.0 (SPSS Inc.) and all summary statistics for numerical data (quantitative continuous data) were presented as means± standard deviation (SD). Result of BBB locomotor and modified Tarlov inclined plane test were compared between the studied group with analysis of variance (ANOVA), followed by post-hoc test. The level of significance was at ($p<0.05$).

Results

BBB score:

All rats were evaluated before induction of injury and all had normal motor function and the score was 21 for all rats (maximum score). After the induction of injury, rats were evaluated every week to check for progress (Table 1). There was statistically significant difference between group 4 and both group 2 and 3. Data was analyzed by post hoc test (scheffe test) after ANOVA.

The score of group 1 was the same value through the 8 weeks and this score was used as the baseline during the observation period (Figure 1). In the first week, the three injured groups' score was zero, indicating a gait characterized by no hind limb weight bearing and no coordinated hind limb movement, whereas group 4 score showed consistent plantar stepping and consistent forelimb-hind limb (FL–HL) coordination. Toe clearance occurred frequently during forward limb advancement and predominant paw position was parallel at initial contact, lift-off was 16.58± 0.88 which as seen at 8th week. Thus, the HUCBs transplantation group showed an early and dramatic improvement in neurological functions compared with the other control groups ($P<0.05$).

Inclined Plane Test Score:

All animals were evaluated before injury and the score was normal (4) for all groups. Progress was observed every week (Table 2). Stem cells transplanted group (group 4) showed also early and dramatic improvement in motor functions compared with other groups ($P<0.05$).

Histological Results:

1-Haematoxylin and Eosin Results:

The injured groups (groups 2, 3) sections showed alterations of white and gray matter which indicated demylination. It showed spongiosis of white matter as a result of myelin damage and vacuolation around axons. It also showed cellular infiltration. In the gray matter, most neurons showed degenerative or

necrotic changes, as many neurons were small and rounded, with pale and/or eosinophilic cytoplasm. Others showed pyknotic nuclei. Perineuronal vacuolation and cavitations of the gray matter were also detected (Figure 3). The condition was more severe in the saline injected group which showed extensive vacuolation and necrosis in both gray matter and white matter, with huge cavitations of the gray matter (Figure 4). Sections of stem cells treated group (group 4) also revealed alterations indicating demylination, but less than detected in the injured group, as spongiosis of white matter was less marked and many normally appearing myelinated fibers were detected. In the gray matter, normal neurons were seen side by side to small degenerated neurons with vacuolated cytoplasm (Figure 5).

2- Luxol Fast Blue/ Cresyl Violet (LFB/CV) Stain Results:

LFB/CV sections of the injured group revealed extensive demylination, while sections of stem cells injected group showed less demylination of the white matter compared with other groups (Figure 6, 7, 8).

Immunohistochemical results (Anti-GFAP Immunohistochemical staining):

The injured group showed remarkably increased anti-GFAP positively stained processes of astrocytes compared to the control group; with a huge glial scar extending through the gray matter (Figure 9, 10). Sections of the stem cells treated group showed less intensely stained cytoplasmic processes of astrocytes compared to the injured non-treated group, more or less similar to the control (Figure 11).

Table (1): The Mean Score ± SDV of BBB Sale Rate of all Groups in Every Week

Week	Group1	Group2	Group3	Group4	P value (ANOVA)
1	21±0.000	0.00±0.000	0.22±0.411	0.33±0.500	0.550
2	21±0.000	1.13±0.354	1.11±0.333	4.00±1.118*	0.020*
3	21±0.000	1.29±0.488	1.44±0.527	5.11±1.453*	0.001*
4	21±0.000	1.57±0.537	1.78±0.441	6.89±1.616*	0.000*
5	21±0.000	1.43±0.535	2.25±0.463	8.67±1.732*	0.0008
6	21±0.000	1.71±0.756	2.75±0.463	10.78±1.716*	0.000*
7	21±0.000	2.14±0.690	3.13±0.354	13.33±1.414*	0.000*
8	21±0.000	2.33±0.516	3.50±0.535	16.56±0.882*	0.000*

*P<0.05 statistically significant

Table (2): The Inclined Plane Mean Score ± SDV Results

Weeks	Group1	Group2	Group3	Group4	P-value (ANOVA)
1	4±0.00	0.00±0.000	0.00±0.00	0.56±0.520	0.611
2	4±0.00	0.022±0.441	0.33±0.500	1.22±0.441	0.556
3	4±0.00	0.5±0.535	0.56±0.527	1.78±0.441*	0.015*
4	4±0.00	0.86±0.378	0.67±0.500	2.11±0.601*	0.000*
5	4±0.00	0.86±0.378	1±0.000	2.67±0.500*	0.000*
6	4±0.00	1±0.000	1±0.000	2.86±0.500*	0.000*
7	4±0.00	1±0.000	1±0.000	2.97±0.500*	0.000*
8	4±0.00	1±0.000	1±0.000	3.10±0.441*	0.000*

*P < 0.5

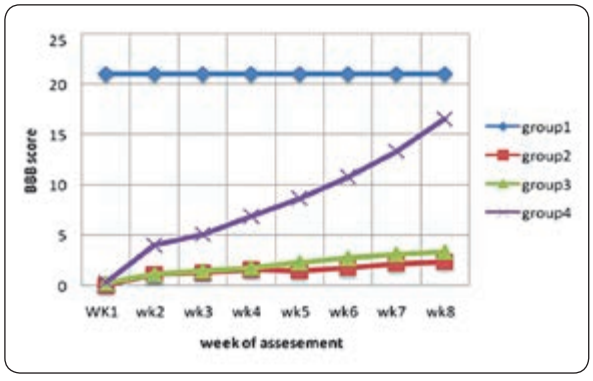

Figure (1). Hind limb function recovery after spinal cord injury

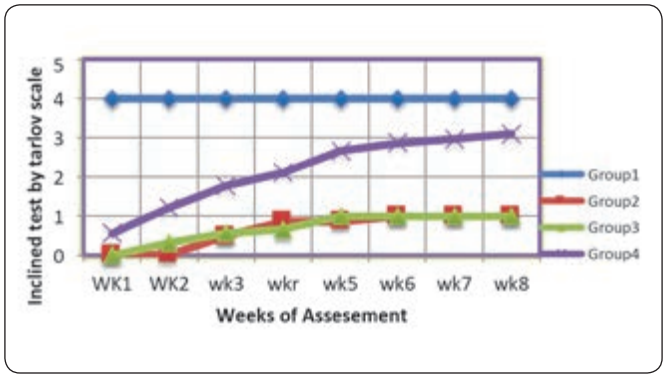

Figure (2). inclined plane test result

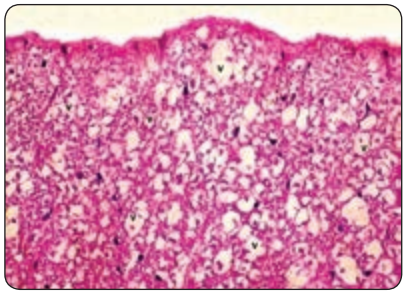

Figure (3). Spongiosis of white matter and vacuolation around axons (v) in the injured untreated group. It also shows cellular infiltration (circle) (H&E400×)

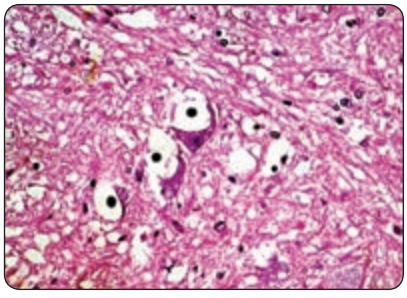

Figure (4). Huge cavitations of the gray matter (circles) saline injected group, with abnormally shaped neurons & large perineuronal vacuolation (asterisk) (H&E400×).

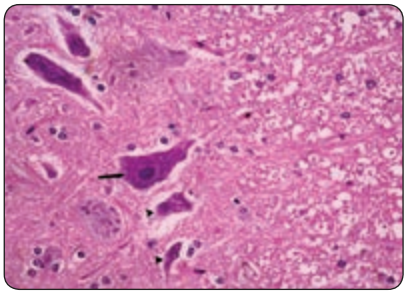

Figure (5). Vacuolation, spongiosis, and cellular infiltration are less marked in stem cells treated group. It shows normally appearing myelinated fibers (circles). It also shows a normal neuron (arrow) side by side to small degenerated neurons with vacuolated cytoplasm (arrow heads) (H&E400×).

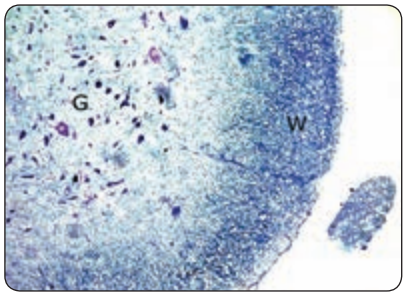

Figure (6). Section in the spinal cord of a control rat. It shows the white matter (W) sharply demarcated from the gray matter (G) (LFB/CV100×).

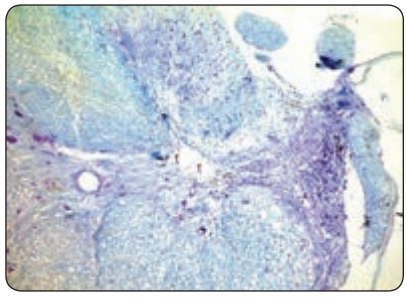

Figure (7). Section in the spinal cord of a rat from the injured untreated group. It shows extensive demylination (LFB/CV100×).

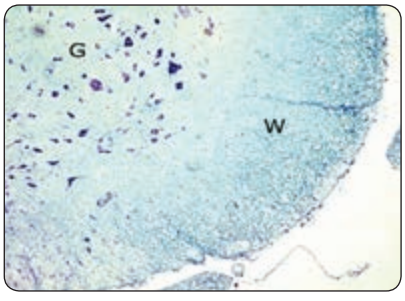

Figure (8). Section in the spinal cord of a rat from stem cells treated group. It shows demylination of the white matter (W) which is not sharply demarcated from the gray matter (G) compared to the control group (LFB/CV100×).

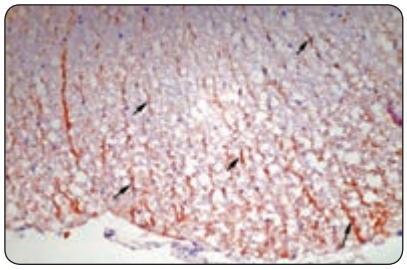

Figure (9). Section in the spinal cord of a rat from the control group. It shows the positively and intensely stained processes of astrocytes which appeared brown in color (arrows) (Anti-GFAP immunostained400×).

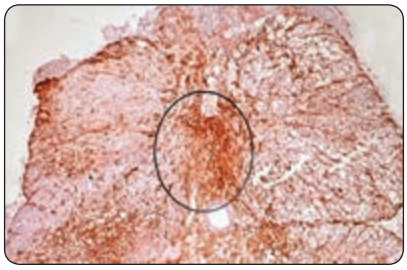

Figure (10). Remarkably increased anti-GFAP positively stained processes in the injured group with a huge glial scar extending through the gray matter (circle) (Anti-GFAP immunostained100×).

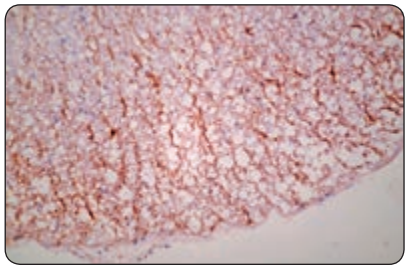

Figure (11). Section in the spinal cord of a rat from the stem cells treated group. It shows a picture more or less similar to that of the control (Anti-GFAP immunostained400×).

Discussion

Cell therapy treatment of cord injury includes cell substitution for the destroyed spinal cord to enhance axon regeneration with or without application of neurotrophic factors to recover the neural tissue. The neural stem cell has pluripotency to differentiate into various neural cell types. Human umbilical cord blood cells (HUCBs) are more pluripotent and genetically flexible than bone marrow neural stem cells. They can also be obtained more easily. It has been reported that stem cells transplanted into the injured lesion were able to differentiate into oligodendrocytes and astrocytes and then integrate into axonal pathways and regenerate and remyelinate the injured axons.[14,21,24,26,27]

It has also been reported that HUCBs can be differentiated into hematological cells and bone marrow stem cells, and can be replicated and differentiated into muscle, myocardium, skeletal cells, hepatocytes, oligocytes, and neurons. [17, 35, 38, 41] For in vitro cultures of HUCBs, there was differentiation into cells positive for the markers of NeuN, Neurofilament, MAP-2, GFAP, betatubulin III, and Gal-C.[17,3,6,18,43]

In this study, we used HUCB stem cells, since it provides a rich source and is safe to use, easy to obtain, and almost not associated with any ethical issues. Many other cells have been used for the same purpose such as neural stem cells, embryonic stem cells and bone marrow stromal cells. However, neural stem cells are obtained from human fetal tissue, which raises critical ethical issues. The use of embryonic stem cells may entail genetic problems, including the possibility of tumor formation. With regard to bone marrow stromal cells, it is difficult to obtain a large number of cells from bone marrow because the cells have to be amplified in vitro to meet the needs of clinical use.[31,23,7]

In this study, chemically induced cord injury method described by Hodges et al,[20] in 1980 was used as it causes reproducible and quantified injury. It requires minimal soft tissue dissection and bone removal. The procedure can be performed rapidly. Behavioral and histological data supported that the animals used in our study developed complete spinal cord injury. Mechanically induced injury model was not used as the posterior (dorsal) surface of the spinal cord receives the traumatic insult, which may not fully simulate all aspects of pathology of spinal cord injury. Most patients suffering from SCI experience a circumferential compression of the cord, being that forces are acting on both posterior and anterior aspects.

Motor function was assessed by the BBB scoring scale described by Basso et al,[2] in 1995 and the Tarlov modified inclined test.[15,39] These methods are easy and the materials can be made locally in comparison to other methods such as paw compression test and tail flick tests.

So far; stem cells of human sources grafted into the injured spinal cord mostly included barely defined heterogeneous mesenchymal stem cell populations derived from bone marrow or umbilical cord blood. Still, reports on functional recovery are rather inconsistent. While improvement of

sensory and motor activity was reported in some studies[8,9,19], no recovery was observed in others. [28, 36] Park SI et la,[32] (2012) reported that endogenous cell proliferation and oligogenesis contribute to functional recovery following spinal cord injury, after injection of rats with human umbilical cord blood-derived mesenchymal stem cells.

This study demonstrated that stem cell derived from HUCB improved functional recovery in rats after SCI by assessing hind limb motor function score on the BBB Locomotor Scale[2]. A significant recovery of hind limb function was observed in rats of the stem cells treated group. The BBB scores improved continuously after 1 week, and this might be due to continuous axon regeneration effects of the various neurotrophic factors that were secreted automatically in combination with HUCBs transplantation. The neurological motor function of spinal cord injured rats improved due to remyelination and regeneration effects of stem cells on the injured axons, and the neural differentiation of the transplanted HUCBs.

In this study the mean BBB score was 16.58 ± 0.8 at the end of eight weeks period, which corresponds to consistent plantar stepping and consistent FL–HL coordination. Toe clearance occurred frequently during forward limb advancement; predominant paw position was parallel at initial contact and rotated at lift-off. Nishio[33] and his group in 2006 reported a score of 9.8 following laminectomy and after complete spinal transaction. This difference can be explained by the shorter follow up (5 weeks) in their study compared to 8 weeks in ours. Similar results were also obtained by Dasari et al,[10] who found that the HUCB transplanted group improved 8 weeks after injection (BBB score about 15.78 ± 0.5) that may confirm the great role of immediate treatment after injury.

Inclined plane performance data showed significant improvement in stem cells treated group compared with other groups with a score of 3.1±0.41. Teng et al,[40] reported a score of 2.99± 0.91 after they used HUCB in hemi-sectioned SCI model. Overall the inclined plane result mirrored the BBB scoring confirming that the stem cells involvement is associated with improvement of motor function.

The histopathological results of the injured untreated group (group 2) showed that chemical injury induced myelin damage and vacuolation in the areas of white matter as well as extensive necrotic changes and irregular cavitations of grey matter, which appeared atrophic due to neuronal loss. The remaining neurons were necrotic and abnormally shaped, with large perineuronal vacuolation. This was in accordance with Dasari et al,[10] (2007) who stated that spinal cord injury resulted in loss of tissue, including important myelinated fiber tracts carrying descending motor and ascending sensory information. They added that reduced myelination could result from loss of myelinating cells and/or reduced myelin synthesis by surviving oligodendrocytes. The gray matter in the injured groups of this study showed many neurons with features characteristic of ischemic cell death, including cytoplasmic eosinophilia with disintegration of cytoarchitecture and nuclear pyknosis.

Anti-GFAP immunostaining of the injured untreated groups (group 2) showed remarkably increased anti-GFAP positively stained processes with a huge glial scar extending through the gray matter. Upregulation of GFAP expression and accumulation of glial fibers is the histological landmark of the astrocyte response to CNS lesion; appropriately named reactive gliosis.[4,16] There is considerable evidence that implicates the role of scar-forming astrocytes as inhibitors of axon regeneration.[5,25,33]

Animals treated with HUCB cells showed changes indicating demylination, however, these changes was less compared to injured non-treated group. Spongiosis of white matter was less marked and many normally appearing myelinated fibers were detected. These findings may indicate partial regenerative and remyelinating effects of stem cells. John et al., and McDonald et al,[22,24] stated that restoring myelin through the transplantation of cell therapy may offer a logical approach to recover optimal neurological functions. They further explained that in addition to replacing lost cells, transplantation appears to modify the host environment to promote endogenous remyelination. Ning G et al,[29] (2013) found that early transplantation of HUCBs (day 1) could promote the functional recovery better than during the subacute phase (day 6). This could be a further point to explore in following studies.

Conclusion

We have shown that the HUCBs reduced the neurological function deficit to a moderate degree for spinal cord injured rats. Remyelination and new astrocyte formation could be established after HUCBs transplantation to the injured rats.

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Acknowledgement:
This work was funded by MUNDO NPT 107 Ethiopia from Netherlands.

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الملخص العربي

التجدد العصبي والاستشفاء الوظيفي بزراعة الخلايا الجذعية من الحبل السرى البشرى في الحبل الشوكي المصاب في فئران التجارب

المقدمة: تعتبر إصابات الحبل الشوكي إصابة قد لا تكون فيها رجعة من حيث الضرر الناجم عن كدمات الأنسجة بسبب قوة خارجية حادة. وقد تم استخدام زرع الخلايا الجذعية المستخرجة من دم الحبل السري البشري بحقنها في الحبل الشوكي التالف على سبيل التجربة لسنوات عدة. ويمكن لهذه الخلايا المزروعة البقاء على قيد الحياة والاندماج مع النسيج المضيف وقد يترافق ذلك مع التحسن الوظيفي للحبل الشوكي.

الهدف: تهدف هذه الدراسة إلى معرفة مدى التحسن الوظيفي الناجم عن حقن الخلايا الجذعية المستخرجة من الحبل السري البشري بعد حقنها في الحبل الشوكي التالف في فئران التجارب.

الطرق: تم تقسيم الفئران بالتساوي إلى أربع مجموعات، كل مجموعة تحتوي على ١٠ فئران: مجموعة (١): المجموعة الحاكمة بدون إصابة أو تدخل. مجموعة (٢): مجموعة تم إجراء إصابة لها ولم يتم أي تدخل علاجي بحقن الخلايا الجزعية. مجموعة (٣): تم إجراء الإصابة بالحبل الشوكي ثم حقن محلول ملحي. مجموعة (٤): تم إجراء الإصابة وحقن الخلايا البشرية الجذعية لدم الحبل السري. وتعرضت الحيوانات للتقييم السلوكي باستخدام طريقتين من الاختبارات الفسيولوجية بعد الإصابة، ثم القيام بفحص الأنسجة هستولوجياً باستخدام الهيماتوكسيلين، يوزين، واللكسول ذو البقع الزرقاء سريع، وتم الفحص المناعي استخدام الأجسام المضادة للبروتين الحمضية الدبقية ييفي (GFAP).

النتائج: أوضحت الدراسة أن الخلايا الجذعية من الحبل السرى البشرى يخفض العجز الوظيفي إلى درجة معتدلة بالنسبة للفئران المصابين في النخاع الشوكي.

الاستنتاج: يمكن بذلك إثبات إمكانية تكوين خلايا نجمية جديدة فعالة بعد زرع الخلايا الجذعية في الفئران المصابة.