

Study the Role of Human Umbilical Mesenchymal Stem Cells in Experimental Model of Spinal Cord Injury

Mennatallah Loutfy*, Rasha Zahran, Ashraf Elghzaly, Camelia Abdel-Malak

Abstract— plenty researches have studied the role of rodent's bone marrow- mesenchymal stem cells in spinal cord injury (SCI), but limited researches scoped the light on the use of human umbilical-mesenchymal stem cells (HUMSCs). **Aim:** we investigated two routes (intravenously, intratheically) on the recovery of SCI using HUMSCs. **Methodology:** Umbilical cords were collected. Wharton's jelly was scraped off, cultured, isolated HUMSCs was immunophenotyped. Rats (n=40) were divided into 5 groups: Control group (SCI only), Sham group (SCI + intravenous administration of physiological saline solution (PSS)), IL group (SCI + intralesional administration of HUMSCs), IV1 group (SCI + intravenous administration of HUMSCs) and IV2 group (SCI + intravenous administration of double amount of HUMSCs divide by a time interval of a week). Day after surgery, 1×10^6 HUMSCs were transplanted on the 3 treated groups, but IV2 group took another dose after one week. Basso-Beattie-Bresnahan (BBB) locomotor rating scale was measured for 6 weeks. Spinal cords were dissected and cryosections were stained. **Results:** HUMSCs were fibroblast like appearance and they were +ve for CD29, CD90 and -ve for CD45. BBB scale showed that from the first week to the six week there were significant improvement between the 3 treated groups as compared to control group ($P < 0.05$), IV2 group showed the best BBB improvement with the smallest lesion then IV1 and IL groups respectively, while there were no significant difference between control group and sham group. **Conclusion:** HUMSCs are useful in subsequent motor recovery after SCI.

Index Terms—Human Umbilical Mesenchymal Stem Cells; Wharton's jelly; Spinal Cord Injury; Immunophenotyping; Intralesional; Intravenous; Basso-Beattie-Bresnahan locomotor rating scale.

1 INTRODUCTION

A spinal cord injury is an injury to the spinal cord resulting in a change temporary or permanent, in the cord's normal motor, sensory, or autonomic function. Common causes of damage are disease (transverse myelitis, spina bifida, Friedreich's ataxia, etc.) or trauma (car accident, gunshot, falls, etc.) [18].

The human umbilical cord contains two arteries and a vein buried within a mucous or gelatinous connective tissue known as Wharton's jelly. Wharton's jelly is made from proteoglycans and various types of collagen, forming a sponge-like tissue, within which stromal cells are, embedded [2]. Wharton's jelly is a rich source of MSCs, which can be mechanically or enzymatically collected [10].

Cultured Mesenchymal Stem Cells (MSCs) have common characteristics and behavioral traits. The ability to plastic adherence is mainly used for their isolation from tissues. All MSCs have similar fibroblast-like morphology in

vitro and are capable of differentiate into cells of mesodermal lineage. MSCs have many cell surface markers as CD44, CD54, CD90, CD29, CD105, CD106. Currently, the umbilical cord MSCs include cells derived from the total umbilical cord or its different sections (perivascular, intervascular, and subamniotic zones of Wharton's jelly and subendothelial layer but not from umbilical cord lining or inner blood vessel walls) [12]. Furthermore, MSCs have unique immunomodulatory properties [2].

Compared to other types of stem cells, human umbilical-stem cells have many advantages: 1-They are easily collected without harm to the baby or mother; 2- They have plenty sources considering about 135 million births globally each year; 3- They are less ethical issues as umbilical cord has been taken as a waste [27]; 4- They demonstrate low immunogenicity in clinical applications; 5- They are associated with a low risk of viral contamination [28].

MSCs are known to have a homing effect and to be neuroprotective following SCI when they are injected in the early stage of SCI [6]. The suggested neuroprotective effects of MSCs for SCI are that they act as an inductor of neurotrophic factor, a modulator of inflammation. Moreover, they are suggested to be able to replace damaged cells through transdifferentiation. However, there is little research regarding the fate of the transplanted cells effects in different transplantation conditions [19].

This work was aimed to investigate the effects of HUMSCs transplantation by two routes (intralesionally and intravenously) on the functional restoration of experimental

- **Mennatallah Loutfy:** Assistant Lecturer of Biochemistry, Mansoura University Children Hospital, Faculty of Medicine, Mansoura University, Mansoura, Egypt. E-mail: manona_87@hotmail.com
- **Rasha Zahran:** Lecturer of Biochemistry, Faculty of Science, Damietta University, New-Damietta, Egypt. E-mail: zahran@du.edu.eg
- **Ashraf Elghzaly:** Lecturer of Clinical Pathology, Faculty of Medicine, Mansoura University, Mansoura, Egypt. E-mail: drashrafantar@mans.edu.eg
- **Camelia Abdel-Malak:** Professor of Biochemistry, Faculty of Science, Damietta University, New-Damietta, Egypt. E-mail: cameliaadly@du.edu.eg

spinal cord injury. Also, investigated if increasing the number of transplanted HUMSCs would lead to better recovery.

2 Material and Methods

2.1 Preparation of HUMSCs:

Umbilical cords were collected from full-term placenta of healthy women age ranging from 20 to 35 years. The human umbilical cords were collected under sterile conditions in Hank's Balanced Salt Solution (HBSS) containing penicillin, streptomycin, gentamycin and amphotericin B and then processed within 6–12 h at 4°C. Following disinfection in 70% ethanol for 30sec, the umbilical cord was washed twice by phosphate buffered saline; umbilical cord blood vessels were removed and the remaining matrix was scraped off Wharton's jelly. Wharton's Jelly was centrifuged at 2500 rpm for 5min. Following aspiration of the supernatant fraction, the precipitate (mesenchymal tissue) was then treated with collagenase at 37°C for 18 hours, after filtration, to remove pieces; Cultures were initiated in culture flasks by Dulbecco's modified Eagle's medium (DMEM, Sigma Chemical Comp. St. Louis, MO, USA) supplemented with 10 % fetal bovine serum (FBS, Sigma) antibiotic/ antimycotic and glutamine. Cultures were incubated at 37°C, humidified atmosphere containing CO₂. Non-adherent cells were removed after 3 days by changing the medium, and adherent cells were kept in culture, while being fed with fresh medium every 3 days and cellular growth assessed daily under an inverted microscope until the outgrowth of fibroblast-like cells appear. Cultures were harvested with trypsin and transfer into a new flask for further expansion. When cells reached 70-80% confluence, they were subdivided after trypsin /EDTA addition [31] and the culture was repeated for three passages (P3).

2.2 Immunophenotyping of HUMSCs:

Human umbilical-MSCs were centrifuged at 1200 rpm for 5 min and then suspended in PBS with concentration (1×10^6 / ml). Cells were stained with different fluorescently labeled monoclonal antibodies (eBioscience): 100 µl of cell suspension was mixed with 10 µl of the fluorescently labeled mAb and incubated in the dark at room temperature for 30 min, then washed twice by PBS containing 2% BSA and the billet was re-suspended in PBS and analyzed immediately on flow cytometry. Different combinations of mAb were used against various antigens (Table.1). The immunophenotyping was performed on EPICS-XL flow cytometry (Coulter, Miami, FL). Cells were analyzed with the appropriate gate using the combination of forward and side scatters.

FITC	PE	APC
CD 29	CD 45	CD 90

Table.1. Labeled mAb combinations used for HUMSCs staining.

2.3 Spinal cord injury model:

Male Sprague Dawely rats (n=40), aging 8-12 weeks, weighing 220 - 250 gm. were obtained. Animal handling, sampling, and scarification were performed according to the guide for the care and the use of laboratory animals, Eighth Edition [5]. They were housed in cages under controlled conditions of humidity (40–70%), lighting (12 h light/dark cycle), and temperature (20–22°C), with free access to food and water.

Spinal cord injury was made. Briefly, their backs were shaved and sterilized with betadine. A laminectomy was performed on the entire spinous process on vertebral plate of T9 and part of vertebral plate of T8 and T10, to expose the dorsal (posterior) surface of the spinal cord. The exposed spinal cord at the T9 level was injured by introduced a dropping rod (1g) from a height of 50mm. Since the animals were incapable of emptying their bladder after injury induction, their bladder was emptied at least two times a day until they were able to do so themselves.

Rats were divided into five groups: Control group (n=8, SCI without treatment), Sham group (n = 8, SCI + administration of physiological saline solution), IL group (n = 8, SCI + intralesional administration of HUMSCs), IV1 group (n = 8, SCI + intravenous administration of HUMSCs), and IV2 group (n = 8, SCI + intravenous administration of double amount of HUMSCs divided by a time interval of a week). After allocation, the rats were anesthetized with ketamine (50 mg/kg) and xylazine (2 mg/kg, intraperitoneal).

2.4 Preparation and implantation:

Implantation was done after one day of SCI. For Sham group, a volume of 0.5 ml Phosphate saline solution (PSS) was injected through the tail vein. For the IV1 and IV2 groups' transplantation, 1×10^6 HUMSCs in a 0.5 ml of culture medium were injected through the tail vein. For the IL transplantation, the injured sites were re-exposed and a concentration of 1×10^6 HUMSCs in 10 µL of culture medium was injected using a Hamilton needle. After one week of HUMSCs transplantation, IV2 group was injected by the second dose of 1×10^6 HUMSCs.

2.5 Behavior studies:

Basso-Beattie-Bresnahan (BBB) locomotor rating scale [3] used to evaluate the neurological outcomes over the time course of 6 weeks after SCI. Scores ranging from 0 to 21 were recorded every week after injury.

2.6 Preparation of the SCI Sections:

Half of the rats in each group were randomly selected and sacrificed. After killing; the spinal cords were dissected and fixed in 4% paraformaldehyde for 24 hours. Then, tissue blocks were prepared. We performed hematoxylin and eosin staining to analyze the extent of the lesion.

2.7 Statistical analysis:

Statistical analyses were performed using SPSS test. In all statistical analyses $p < 0.05$ consider significant.

2 Results

3.1 Harvest of HUMSCs:

We harvested the HUMSCs, Contaminated hematopoietic cells were depleted during passage 1 and HUMSCs were morphologically defined by a fibroblast-like appearance. (Figure.1).

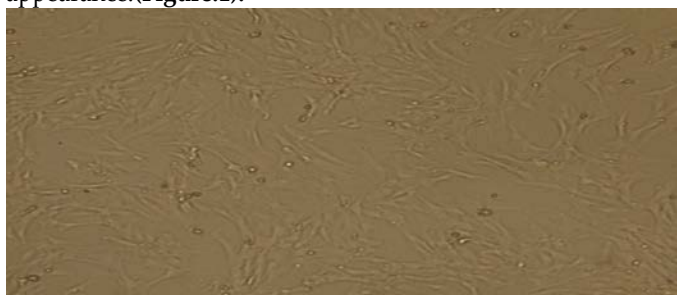


Figure.1. Under an inverse microscopy, cultured human umbilical mesenchymal stem cells at (P3) were morphologically defined by a fibroblast-like appearance (original magnification $\times 200$).

3.2 Viability Test:

The viability% of HUMSCs was 95.7 ± 8.7 . (Figure.2).



Figure.2. Light photograph of human umbilical mesenchymal stem cells (\uparrow). The MSCs didn't accepted trypan blue stain (Viable stem cells), X40.

3.3 Flow cytometry for cell surface expression assay:

Immunophenotyping of HUMSCs surface markers were tested by flow cytometry analysis and illustrated in (Figure.3). The concentrations% of MSCs were 85.79 ± 2.23 for CD29, 92.89 ± 2.93 for CD90 and 1.04 ± 0.3 for CD45.

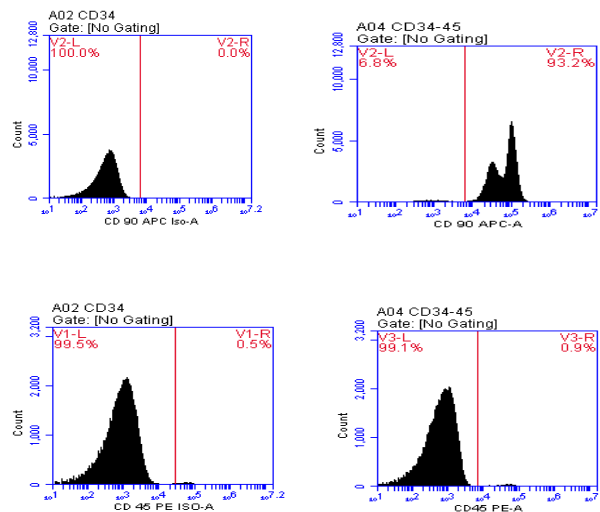
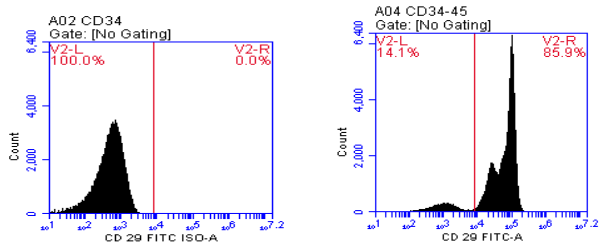


Figure.3. Phenotypic characteristics of HUMSCs, they were positive for CD29 (85.9%) and CD90 (93.2%) while they were negative for CD45 (0.9%).

3.4 Motor Activity:

Motor activity of the rats was measured by the Basso-Beattie-Bresnahan (BBB) Locomotor Rating Scale. Normal rats were graded on 21-point scale. Following surgery and transplantation, the rats in the five groups were graded at various time points. At day 1 after the induction of SCI, the rats scored 0 points among the five groups. We have observed among the first week that there were no significant differences between control and sham groups ($P > 0.05$). But the other three treated groups (IL, IV1 and IV2 groups) were slightly higher and significant as compared to the control group ($P < 0.05$). From week 2 to week 4, the quick improvement was greatly observed. The IV2 group was having the best improvement then, IV1 group and IL group, while there were slightly improvement in both control and sham groups. From week 5 to week 6, the improvement was tends to be constant, there were no significant difference ($P > 0.05$) between control and sham groups but there were a highly significant difference ($P < 0.05$) between the control group and the last 3 groups. As in Table.2. Chart illustrates the BBB score scale (Figure.4).

Groups	Week1	Week2	Week3	Week4	Week5	Week6
a)Control	0.3 \pm 0.1 5	0.66 \pm 0.19	1.06 \pm 0.25	2.31 \pm 0.387	2.51 \pm 0.39	2.71 \pm 0.45
b)Sham	0.51 \pm 0.2	0.75 \pm 0.26	1.11 \pm 0.33	2.43 \pm 0.37	2.85 \pm 0.26	3.07 \pm 0.28
c)IL	1.41 \pm 0.27	1.83 \pm 0.2	4.82 \pm 0.12	7.36 \pm 0.26	8.03 \pm 0.27	8.5 \pm 0.23*
d)IV1	2.13 \pm 0.19	3.41 \pm 0.19	6.85 \pm 0.2	9.06 \pm 0.31	9.92 \pm 0.26	10.56 \pm 0.29

e)IV2	1.9±0.4 1	4.16±0.22	8.7±0.28	10.45±0.33	12±0.29*	12.9±0.21
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Table2. BBB scoring (means±SE) of animals in each group among a period of six weeks after spinal cord injury, (*) significant as compared to SCI group within the same week.

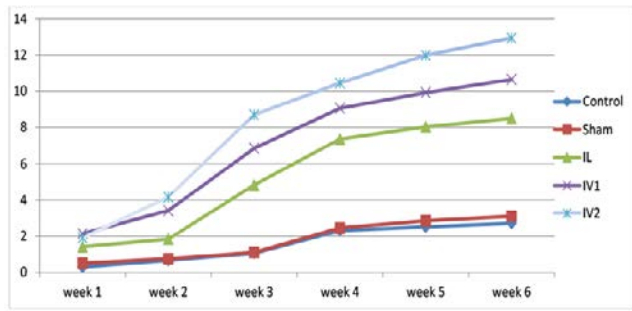


Figure4: chart illustrates the BBB score scale among the five groups within 6 weeks.

3.5 Spinal cord lesion cavity:

The cavity ratio in the control group was bigger than those in the sham, IL, IV1, and IV2 groups, respectively. As shown in (Figure.5).

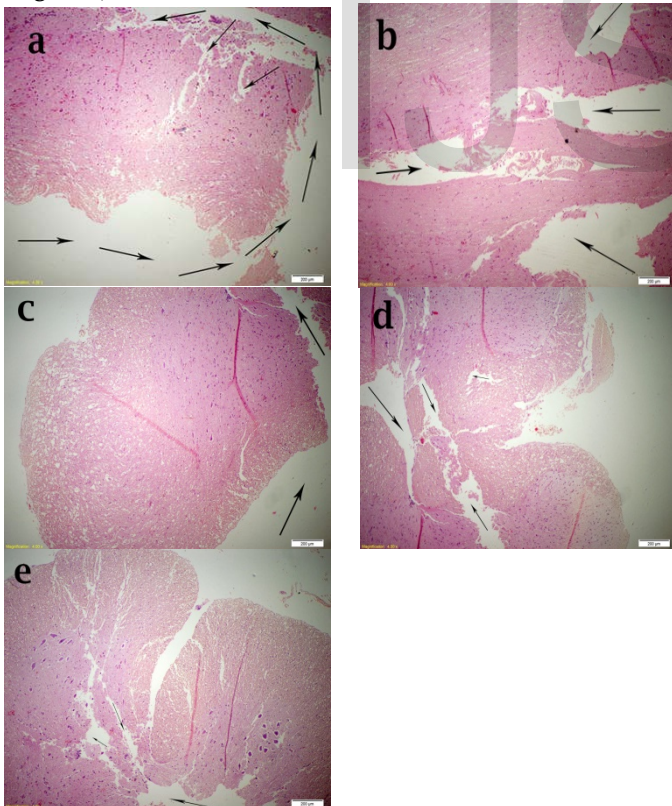


Figure.5. A–E: Representative H & E-stained sections of the lesion epicenter showing the cystic cavities in the 5 groups: a) Control group b) Sham group c) IL group d) IV1 group e) IV2 group. Original magnification×4.00. (→)indicated the cavities.



3 Discussion

Different types of stem cells have been transplanted into experimental models of spinal cord injury (SCI) with promising results but minimal functional benefit [29], [30]. Ideal donor cells for neurological disease therapy should be (i) easily available; (ii) capable of rapid expansion in culture; (iii) immunologically compatible; (iv) capable of long-term survival and integration in the host tissue [4]. So, MSCs from umbilical cord were considered as the most ideal donor cells with less ethical issues [27].

In the presented work, we have harvested the mesenchymal stem cells from Wharton’s jelly by treating it with collagenase [10] and like most stem cells they were isolated by plastic adherence and were morphologically defined by a fibroblast-like appearance [2]. According to previous studies, CD29 and CD90 were regarded as positive cell-surface markers for MSCs, while CD 34 and CD45 was regard as negative surface markers [2], [8]. The cells used in this study were positive for CD29 and CD90 while they were negative for CD45, indicated that the isolated HUMSCs showed typical MSCs characteristics.

Efficacy of MSCs transplantation on neuropathic pain depends on numerous factors, such as source (donor species) and number of the cells [23], route of administration (at injury site or intravascular), type of injury (central or peripheral), time between injury and cell transplantation, and follow-up duration [1]. At the present study, we tried different transplantation routes of stem cells for SCI. The efficacy and fate of the transplanted cells were observed according to different transplantation routes. So, we have made a spinal cord injury for five groups: a) Control group (SCI only), b) Sham group (SCI+PSS), c) IL group (SCI + intralesional administration of HUMSCs), d) IV1 group (SCI + intravenous administration of HUMSCs) e) IV2 group (SCI + intravenous administration of double amount of HUMSCs divided by a time interval of a week).

In most of studies, the differentiation of transplanted MSCs could be observed and to a limited extent, neuron differentiation were reported [21], [15]. However, these studies did not present the differences depending on the transplantation route. A few researches that concentrate on comparing the efficacy following MSCs transplantation for SCI have showed that more efficient engrafting of transplanted cells into lesion site when grafting by the intralesional routes [6], [11]. These studies demonstrated this difference of efficacy through examining the engrafting MSCs by histological examination. As suggested in many studies, intravenous delivery has deep concerns of its efficacy; IV delivery is probably the most convenient way of cells delivery both in experiments and clinical trials. This method is minimally invasive and allows repeated injections of multiple doses of cells at certain time intervals [24], [13]. Though the IV route has the advantages of safe and easy delivery, trapping of the

transplanted cells in the other organs and the high chance of exposure to an immune reaction limits its clinical utility. In the present study, as predicted and suggested by other previous studies, IV delivery showed a lesser number of engrafted MSCs as compared to that of IL delivery. However, IV delivery showed more effective clinical improvement as compared to that of IL group and this might be explained from findings of other studies, that homing of the MSCs to the disrupted blood-spinal cord barrier tissue and avoidance of additional injury that can be caused by intralesional delivery could have better results [25], [26]. By comparing IV1 and IV2 groups, we have observed that IV2 group has the better result but the improvement was not significant between them despite having a double amount of MSCs. Yousefifard et al, study revealed that transplantation of 1 million mesenchymal cells derived from bone marrow, umbilical cord, and adipose tissue were sufficient for clinical improvement and neuropathic pain symptom relief after transplantation [22]. Also many studies related to the treatment of SCI by MSCs were used to inject 1 million MSCs [16], [9]. This study was the initial in trying to transplant a double amount of HUMSCs (2 million cells) intravenously in case of spinal cord injury treatments, and we obtain this idea from [17] study as it injected 2 million mesenchymal stem cells intravenously to improve anal pressures after anal sphincter injury and it showed better results.

It is difficult to conclude that IV delivery could result in better clinical improvement in the early stage of transplantation. However, as other studies have reported, IV delivery could be an effective route for early MSCs transplantation following SCI [25], [26]. Control group and sham group were having very less improvement and thus insure the importance of HUMSCs in neurological disorders.

Most studies showed that MSCs from umbilical cord have a protective role in the harm tissue and may reduce inflammation by secretion of cytokines and growth factors [14]. HUMSCs can also play a role in neural regeneration by differentiation into neural cells [7], [20]. Thus, it seems that they provide a favorable environment for endogenous regeneration. In the present study, hematoxylin and eosin staining showed that HUMSCs transplantation reduce the healing in the 3 treated groups, IV1 and IV2 groups were showed less cavity size compared to IL group, as it have an additional injury when supplemented with MSCs that may cause more tissue harmful.

Further work defining the dose, timing, and effective therapeutic Strategies may enhance the successful response rate of SCI patients for this challenging-to-treat population that may represent a valuable addition to the assisted neurological technology therapeutic. Also, there is a need for investigating possible use of triple doses of HUMSCs; we believe that it may not give same results, as it has been showed that double doses of HUMSCs give better results on SCI models.

4 Conclusion

From above, we have concluded that HUMSCs have useful results in subsequent motor recovery after SCI. Also, intravenous route is the suitable way for MSCs delivery to the injury site and enhance more improvement. Increasing the supplemented amount of HUMSCs more than 1 million may lead to slightly improvement.

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