In - Vivo Conversion of Astrocytes to Neuroblasts in the Injured Spinal Cord

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Abstract

Direct astrocyte reprogramming to neural progenitor cells and promotion of neurogenesis is considered as an alternative approach to replace the lost neurons in the spinal cord injury(SCI). Herein, we used the human amniotic fluid mesenchymal stem cells (hAF-MSCs) and their conditioned medium (CM), to investigating their ability to reprogramming astrocytes to neuroblasts following SCI. 54 adult rats were randomly divided into 9 groups (n = 6), included: Control, SCI, (SCI + DMEM), (SCI + CM), (SCI + MSCs), (SCI + Astrocyte), (SCI + Astrocyte + DMEM), (SCI + Astrocyte + CM) and SCI + Astrocyte + MSCs). Following laminectomy and SCI induction, DMEM, CM, MSCs and Astrocytes were injected. Wester-blot was performed to explore the levels of the Sox2 protein in the MSCs-CM. The immunofluorescence staining against DCX and GFAP was done. Finally, Basso-Beattie-Brenham (BBB) locomotor test was conducted to assess the neurological outcomes. Our results showed that the MSCs through juxtacrine and paracrine mechanisms induced the promotion of the endogenous neuroblasts and the decline of astrocytes. Moreover, in the present research, MSCs and CM could convert the transplanted human astrocytes to neuroblasts in the spinal cord injury. Taken together, our data indicate the MSCs via juxtacrine and paracrine pathways could direct the spinal cord endogenous neural stem cells(NSCs) to the neuroblasts lineage rather than astrocytes as well as induce reprogramming. Ultimately, MSCs could reverse the neurobehavioral deficit in the SCI. The striking output in our study was the capability of the MSCs in the reprogramming of the astrocytes to neuroblasts via juxtacrine and paracrine pathways in the *In-vivo* condition.

Introduction

Spinal cord injury (SCI) is damage to the spinal cord and a complex neurological condition that resulted in irreversible neuronal loss, glial scar formation, axonal injury, disruption of the myelin sheath and neural tracts and long-lasting disability and in some cases leads to permanent functional deficits in mammals(Su et al. 2014b; Higuchi et al. 2019; Abolhasanpour et al. 2019). Following SCI, astrocytes proliferate and migrate into the surrounding milieu and generate glial scar and undesirable microenvironment (Wang et al. 2016). The research demonstrated that the astrocytes and NG2 (Pre-oligodrocytes), as a result of reprogramming could convert to neuroblast and neurons in the spinal cord and brain (Wang et al. 2016). It is shown that the SRY (sex- determining region Y-box 2), also known as Sox2, is a transcription factor that is essential for maintaining self-renewal, or pluripotency, of undifferentiated embryonic stem cells. Sox2 has a critical role in the maintenance of embryonic and neural stem cells and also it is crucial for directing neural differentiation (Zhang and Cui 2014; Mercurio et al. 2019; Rodriguez-Jimenez et al. 2016). Sox2 can mediated reprogramming of astrocytes to DCX + neuroblasts and in this context, Sox2 can help to treat SCI by converting glial cells to neurons finally mature neurons (Wang et al. 2016; Rodriguez-Jimenez et al. 2016). Doublecortin (DCX) is a microtubule-associated protein expressed by neuronal precursor cells and immature neurons in embryonic and adult cortical structures and considered as a marker of developing neural progenitor cells (Karimipour et al. 2019; Nasrolahi et al. 2019). Astrocytes due to nearness to radial glial cells identity, their high quantity,

and the potential to proliferate in the central nervous system, astrocytes could undertake therapeutic interventional approaches including reprogramming. (Sun et al. 2019). Recent advancements in the field of direct in vivo reprogramming showed the generation of functional neurons from reactive glial cells in the repair of the brain (Li and Chen 2016; Guo et al. 2014). The concept of direct reprogramming is a process in which one mature somatic cell transforms into another mature somatic cell without undergoing an intermediate pluripotent state or progenitor cell type(Ghasemi-Kasman et al. 2015). Transplantation stem cells including MSCs and olfactory ensheathing cells (OECs) in SCI could lead to increase of neuroprotective molecules and reprogramming of astrocytes into neuroblasts which could survive and develop into mature neurons (Sabapathy et al. 2017; Sun et al. 2019). In this context, MSCs by secretion of the essential materials for neuroprotection such as growth factors, cytokines, and neurotrophic factors, could induce the replacement of lost neuronal cells, remyelination of axons, angiogenesis, and decline of inflammatory responses(Oh and Jeon 2016; Shahrezaie et al. 2017). MSCs could harvest from different sources including adipose derived tissue, bone marrow, Umbilical Cord Wharton's Jelly, amniotic membrane and amniotic fluid mesenchymal stem cells (hAF-MSCs) and so on (Gabel et al. 2017; Oh and Jeon 2016). In spite of the plethora of studies and experiments regarding the evaluation of the functional efficacy of the MSCs in the SCI, the election of the effective source of the MSCs is one of the challenging issues in the scientific community (Gabel et al. 2017). But recent studies, suggest that (hAF-MSCs) could account into consideration in the SCI because of the amnion origin from the epiblast layer as a pluripotent and undifferentiated structure in the embryo(De Coppi et al. 2007; Antonucci et al. 2014). These cells routinely gained during amniocentesis which is done in the second trimester and express embryonic and pluripotency markers for instance Oct4, Nanog and Sox2 (Aziz et al. 2019; Gholizadeh-Ghaleh Aziz et al. 2019; Dziadosz et al. 2016; Maraldi et al. 2014; Saito et al. 2012). Amniotic fluid derived- MSCs relative to other sources have high proliferative and regenerative potential as well as they maintain pluripotency features, and remain without alteration after continuous passages (Maraldi et al. 2014; Aziz et al. 2016). MSCs during cell culture can secrete paracrine factors in the form of conditioned medium (CM) (Osugi et al. 2012; Cantinieaux et al. 2013). CM contains metabolites, growth factors, extracellular matrix proteins, cytokines and anti-inflammatory agents which are secreted into the medium by the cultured cells. (Cantinieaux et al. 2013; Pawitan 2014). Sox2 which is available in hAF-MSCs-CM is the essential factor for the transform endogenous spinal astrocytes to neuroblasts (Su et al. 2014b; Zhang and Cui 2014). In this Perspective, we investigated the efficiency of the hAF-MSCs and their CM in the reprogramming of the astrocytes to neuroblasts through juxtacrine and paracrine mechanisms as well as functional behavior following the SCI.

Materials And Methods

Ethical issue and study design

In the current study, 54 adult male Wister rats (weight: 270-300g) were purchased from the animal laboratory and maintained according to the guide line of ethics committee of Tabriz University of Medical Sciences (registered number 95/5-10/7). All animals were housed in a standard condition under a 12h light/dark schedule with enough food and water. The rats were randomly divided in to 9 groups (six rats

per group), these nine groups included:Control**,** SCI, (SCI + DMEM, IP), (SCI + CM, IP), (SCI + MSCs, Focally), (SCI + Astrocyte), (SCI + Astrocyte+ DMEM, IP), (SCI + Astrocyte+ CM, IP) and SCI + Astrocyte+ MSCs). In all groups, laminectomy was performed at the T9–T10 vertebral level in the dorsal surface of the spinal cord using the Infinite Horizons Impactor with an impact force of 150 (moderate SCI) kdyn (1 dyn=1g·cm/s 2=10-5kg·m/s 2=10-5 N) by impactor device.

hAF-MSCs isolation, cultivation and characterization

Briefly, isolation of hAF-MSCs was done undergoing amniocentesis for the routine karyotype screening of about 5 ml of amniotic fluid samples from eight mothers in Al-Zahra hospital (Tabriz, Iran). Prior to the amniocentesis, patients written informed consent for donating amniotic fluid samples voluntarily for this research. Amniotic fluid extraction carried out under supervision the gynecologist with using a 22G Needle. After sending the samples to the desired laboratory, samples were centrifuged at 450 g for 10 minutes, next the settled pellet was washed twice by PBS (Gibco; Thermo Fisher Scientific, Darmstadt, Germany). Then, the cells transferred in 6 well plates with AmnioMAX II Complete Medium (Gibco, cat# 11269) for 1-2 weeks in condition 37°C and 5% CO2. In the cultivation period, the medium was changed twice a week, and in the 90% confluence, the cells trypsinized with trypsin-EDTA [0.25%] (Gibco) and centrifuged, and finally, cells pellet re-seeded in DMEM-low glucose media with 15% FBS, 1% penicillin/streptomycin and10 ng/mL bFGF in the optimized condition. The phenotype profile of the hAF-MSCs was examined by flow cytometry analysis. For this purpose, the cells at passage 3-5 were trypsinzed and two times washed with PBS and centrifuge at 1500 RPM for 3 min, then, the cell pellets were stained with antibodies including CD 105 (Catalog No. 1p-298-To25 Exbio), CD 73 (Catalog No. 561260 BD Biosciences) antibodies as mesenchymal stem cell markers and CD 45 (Catalog No. 1F-222-T025 Exbio), CD 14 (Catalog No. 12-0149-42 eBioscience) as hematopoietic stem cell markers) with dilution 1/30 in the PBS for 20 min on ice.

Preparation of CM

The MSCs at the 3rd-5rd passage and 90% confluent, were trypsinized and washed with PBS three times, and re-fed with DMEM-low glucose culture medium in serum-free condition for 48 h. Then, CM was harvested and centrifuged at 450g for 10 min up to eliminate free-floating cells. Finally, CM was sterilized through 0.22 µm filters and concentrated by freeze-drying processes and was stored at -80 ℃ until use.

Western blot

CM was collected from MSCs culture and was sterilized by 0.22 mm filters, then CM was concentrated 2, 4, 8, 16 and 32 folds by freeze dryer device. Sox2 (Sex determining region Y-box 2) secreted by HAF-MSCs into MSCs-CM, was measured using western blot analysis.

Human astrocyte culture

Human astrocytes (line 1321N1) (Ghasemi-Kasman et al. 2015) were purchased from Pasteur Institute of Iran and cultured in DMEM low glucose medium with 10% FBS and 1% penicillin/streptomycin. This

medium was changed twice in a week.

Spinal Cord Surgery

The rats were anesthetized by inhalation of 5% isoflurane and oxygen (1 L/min) in a closed space. After deep anesthesia, an adequate level of anesthesia was determined by checking withdrawal to painful stimuli applied to the hind limb. The rats were shifted to the surgical location and via the mask received an isoflurane vapor inhalation (3-5%) and oxygen (0.8-1 L/min) to the end of surgical procedure. Briefly, animals under anesthesia conditions, their back was shaved and in the midline, the skin s incision was performed around 2cm, and in order to laminectomy, Paravertebral muscles were cut up on the T9-T11 spinal processes, and with the dental drill, a hole of 1.5 mm diameter was made in the vertebral arch of T10 as far as dura mater could be seen. Then, using the Horizons Impactor, animals received a force of 150-kilodyne (moderate SCI) on the targeted spinal cord segments, subsequently, the muscles and skin were closed. Also for bilateral injury, transverse process of the rats throughout the surgery and injury fixed by a clamp. To prevent infection following SCI, ciprofloxacin (350 ml units/days) was injected via IP for one week. After SCI, the bladder sac was discharged manually twice a day for one week.

Infusion of MSCs- CM

In order to explore the effect of the CM on the rate expression of the endogenous neuroblasts and astrocytes, 500 µl of CM, following SCI was infused through intraperitoneal (IP) per day for 7 days.

Transplantation of hAF-MSCs

The next, to examine the effect of the hAF-MSCs on endogenous expression of neuroblasts and astrocytes, the MSCs were detached and harvested using 0.25% Trypsin-EDTA. Prior to the transplantation, the number of cells was estimated by counting in a neobar lam and washed by PBS three times. Following SCI, 5×105 cells per 5µl PBS were transplanted to the proximal, central, and distal parts of the lesion site using a capillary glass needle through a Hamilton syringe. For immunosuppression, the rats received cyclosporine (1 mg/100 g body weight) two days before cell transplantation until the end of the experiment(Springer et al. 2018).

Injection of the exogenous human astrocytes

In the next series of experiments, we decided to investigate the effect of the MSCs and their CM on astrocyte reprogramming and converting the human astrocytes to neuroblasts. To this end, the human astrocytes (Cell line 1321N1) were injected focally into the lesion site of the spinal cord concomitantly with transplantation of MSCs and infusion of CM.

Tissue preparation and immunofluorescence staining

For immunofluorescence examinations, animals were sacrificed two weeks after SCI by ketamine (100mg/kg) and xylazine (5mg/kg) overdose. The rats were transcardially perfused with normal saline

(NaCl 9%) and 4% paraformaldehyde, respectively. The animal's spinal cord was carefully removed and post-fixed overnight with 4% paraformaldehyde solution. Then, samples of the spinal cord were processed and embedded in paraffin and 5-µm thick histological sections prepared by microtome and mounted on poly-lysine-coated slides. After overnight incubation at 4 °C temperature, the sections were deparaffinized and rehydrated in decreasing ethanol and washed in tap water, and finally stored at 4 ◦C until use. Following the washing in PBS (0.1M, pH 7.4, and 0.9% NaCl), antigen retrieval was done by incubating the sections in preheated 10mM sodium citrate buffer for 15 min at 100 °C and blocking endogenous peroxidase step was performed using incubation the sections in 0.6% H2O2 in PBS for 30 min. Then the Sections were exposed to the primary antibodies, Anti- Doublecortin antibody (ab18723), Anti-GFAP antibody [2A5] (ab 4648), Human Anti- Doublecortin antibody (A83146) and Human GFAP antibody MAB2594, for overnight at 4 °C. After three times PBS wash, the sections were incubated with secondary antibodies including Goat Anti-Mouse IgG H&L (Phycoerythrin) (ab 97024), Goat Anti-Rabbit IgG H&L (FITC) (ab 6717), Donkey Anti-Goat IgG H&L (FITC) (ab6881) and Goat Anti-Mouse IgG H&L (Texas Red ®) (ab6787) at room temperature for 1 hour. Also, nuclei were counterstained with 4', 6-diamidino-2-phenylindole (DAPI) (ab 104139). Images were taken and observed by an Olympus fluorescence microscope and data was analyzed using the Image J program software plugin.

Neurobehavioral examination

Locomotor performance on 1, 7 and 21 days after SCI was assessed with the use of the 21 point (a score from 0 (complete paralysis) to 21 (normal gait)) BBB (Basso, Beattie and Bresnahan) score by two examiners blinded.

Statistical analysis

The results were presented as mean±SEM. One-way analysis of variance (ANOVA) and post hoc Tukey tests were performed to detect the statistically significant difference between groups. $P<0.05$ was considered as statistically significant. All the statistics presented in the article were analyzed and drawn using Graph Pad Prism (version 6.01; Graph Pad Software, CA, USA).

Results

Flow cytometry analysis and phenotype acquisition

In this research, isolated and cultivated of MSCs from human amniotic fluid showed spindle-like morphology and fibroblast-like under bright-field microscope. Evaluation of cell surface markers of the MSCs at Passages (3-5) by flow cytometry analysis revealed that the MSCs express CD105 (95.80%) and CD73 (92.2%) as the MSCs markers while they didn't express, CD14 (9.90%) and CD45 (9.16%) as the hematopoietic markers (Figure 1).

MSCs secreted the SOX2 protein in the CM

To evaluate of the Sox2 protein existence as a pluripotency marker in the CM by MSCs, Western blot experiment was performed. This analysis showed that the Sox2 protein was observed in the 32 folds concentrated CM. This finding indicated that the MSCs release the Sox 2 protein in their condition media. (Figure 2).

hAF-MSCs increased endogenous neuroblasts and decreased astrocytes via juxtacrine and paracrine mechanisms.

For this purpose, immunofluorescence staining was conducted (Figure 3. A). The results from this panel showed that the MSCs after two weeks of transplantation promoted the level of DCX expression and suppressed the rate of GFAP marker. Data analysis showed significant differences among the groups (Figure 3.A, B). Our experiment revealed that the SCI significantly decreased the number of neuroblasts relative to the control group, but and increased the quantification of astrocytes compared to the control group ($p<.001$) (Figure 3.A, B). MSCs transplantation significantly increased the number of neuroblasts relative to the SCI and SCI+ DMEM groups ($p<.001$) meanwhile the expression level of astrocytes at the protein level in the MSCs group was significantly decline relative to the SCI and SCI+ DMEM groups ($p<.001$) (Figure 3.A, B). In our study, the increase of the number of neuroblasts and decline astrocytes under influence of MSCs was significant relative to the CM ($p<.05$). Moreover, CM could increase the number of neuroblasts and decreased the number of astrocytes compared to the DMEM group ($p<.01$) (Figure 3.A, B). In general, these data indicate the MSCs through juxtacrine and paracrine pathways could promote the neuroblasts and induce neurogenesis, as well as, diminish the astrocytes in the SCI and finally suppress gliosis and glial scar formation. Ultimately, it is concluded that the MSCS could direct the fate of endogenous neural stem cells into neural lineage and neurogenesis by involving the juxtacrine and paracrine pathways in the SCI.

hAF-MSCs converted the injected astrocytes to neuroblasts through juxtacrine and paracrine pathways in the *in-vivo condition.*

Our main aim from this panel was whether the MSCs and their CM could convert the transplanted human astrocytes to neuroblasts in the *in-vivo*? To this end, immunofluorescence staining against GFAP and DCX markers was performed (Figure 4. A). The results showed that there was a significant difference among groups after two weeks of cell transplantation (Figure 4.A, B). The number of neuroblasts was significantly increased in the (SCI +Astrocytes+ MSCs, $p<.001$) and (SCI+ Astrocytes and CM, $p<.05$) versus the (SCI +Astrocytes) and (SCI +Astrocytes + DMEM) (Figure 4.A, B). In this study, MSCs and CM could increase the number of neuroblasts relative to the (SCI +Astrocytes + DMEM) group ($p<.001$, $p<.05$) respectively (Figure 4.A, B). In this context, the raise of neuroblasts in the (SCI +Astrocytes+ MSCs) group was significantly more than (SCI+ Astrocytes and CM) ($p<.01$) (Figure 4.A, B).

The other aim in the current research was the evaluation of the effect of the MSCs and CM on the number of the transplanted human astrocytes in the *in-vivo* condition. Our data showed that the MSCs and CM significantly reduced the number of the transplanted human astrocytes in the (SCI +Astrocytes+ MSCs $p<.001$) and (SCI+ Astrocytes + CM, $p<.05$) groups relative to the (SCI+ Astrocytes) group as well as the

(SCI +Astrocytes+ MSCs p<.001) and (SCI+ Astrocytes + CM, p<.05) groups compared to the (SCI+ Astrocytes+ DMEM) group (Figure 4.A, B). It should be considered that the number of astrocytes in the (SCI +Astrocytes+ MSCs) group was significantly less than those in the (SCI+ Astrocytes + CM) group (p<.05) (Figure 4.A, B). Taken together, these striking findings indicate that the MSCs through secretion of the important and essential molecules for example SOX 2 and other materials by the mediation of juxtacrine and paracrine pathways could convert the astrocytes to neuroblasts and overall contributed to neuroblasts maturation, neurogenesis, and ultimately neural neuroregeneration in the SCI.

hAF-MSCs promoted the behavioral performance by the mediating of juxtacrine and paracrine mechanisms in the SCI injury.

In the present study, SCI was resulted in complete hindlimb motor function paralysis, as shown in (Figure 5). The BBB score as an indicator of functional performance slowly increased between 1 and 5 score at 1 week in all groups. In this section, we evaluated the functional recovery in two panels based on our groups (Figure 5.A, B). In Panel A (Figure 5.A), we would like to assess the effects of the transplantation of MSCs and CM on the score of the BBB test and functionality and in panel B (Figure 5.B), the functional efficiency of the reprogrammed neuroblasts from the grafted exogenous astrocytes was examined.

In panel A, MSCs and CM could promote functional recovery after two weeks' cell and CM infusion. Regarding this, our data showed that the MSCs significantly increased the score of the functional behavior relative to the (SCI and, SCI+ DMEM, (p<.001)) groups as well as compared to the (SCI+ CM) group (p<.05) (Figure 5.A). In the present research, CM could increase the functional recovery in the (SCI + CM group) relative to the (SCI+ DMEM) group(p<.01) (Figure 5.A).

In panel B, the results from the BBB test evaluation indicated that the score of the functional recovery under influence of reprogrammed neuroblasts significantly increased in the (SCI+ Astrocytes + MSCs) group relative to the (SCI+ Astrocytes) and (SCI+ Astrocytes+ DMEM) groups(p<.001) (Figure 5.B). Also, in our experiments, the functional recovery in the (SCI+ Astrocytes + CM) group under influence of the reprogrammed neuroblasts significantly promoted relative to the (SCI+ Astrocytes + DMEM) group (p<.05) (Figure 5.B). Moreover, presented data showed that MSCs in comparison to CM could raise the functional recovery score more than the CM(p<.05) (Figure 5.B). Altogether, MSCs by the mediating of juxtacrine and paracrine mechanisms contributed to increasing the endogenous neuroblasts and exogenous astrocytes reprogramming to neuroblasts and finally could rescue the functional deficit in the SCI.

Discussion

Astrocytes in large quantities have been distributed throughout the CNS and provide essential factors and desire microenvironment for optimal neural tissue structure and function in a healthy condition(Sofroniew and Vinters 2010). In response to any CNS damage, they are activated, proliferated, migrated to the lesion site, and participate in glial scar formation (Sofroniew 2009; Yiu and He 2006; Buffo et al. 2008). In the early phase of neuronal injury, these reactive astrocytes exert the lesion sealing effects, which followed by creating a mechanical and biochemical obstacle in a later stage to axonal

regeneration (Okada et al. 2006). A plethora of studies has shown that the activated astrocytes in CNS injuries could be isolated and cultivated in vitro cultivation and produce neurospheres with the capability of a generation of neurons, astrocytes, and oligodendrocytes. (Buffo et al. 2008; Nakagomi et al. 2009; Su et al. 2014b; Ghasemi-Kasman et al. 2015). Regardless of these findings, astrocyte-derived neurons have not identified around the injured area in both the brain and spinal cord (Ohori et al. 2006; Buffo et al. 2008; Buffo et al. 2005). During gliosis, is observed an elevation in the levels of GFAP and cyclooxygenase (COX)-2 in the astrocytes, as well as other factors like nitric oxide (NO), IL-6, and TNF-α increased in astrocytes and microglia. (Langley et al. 2005). A similar study demonstrated that following SCI or demyelination in the spinal cord, oligodendrocyte and ependymal cells can increase Sox 2 expression, which is followed by Sox 2 binding to regulatory regions of several genes involved in regulating reactive astrocyte action (Chen et al. 2019). The previous studies have demonstrated that the neural stem and progenitor cells after grafting into the undamaged spinal cord migrate in a rostral or caudal direction and two months later, a significant proportion of these transplanted cells differentiated into GFAP-positive astrocytes, and just a low percentage remained as undifferentiated NSCs (Falnikar et al. 2015). Therefore, finding new and effective therapeutic strategies including reprogramming and neurogenesis promotion is a challenging and debate subject in the regenerative medicine field. However, focusing on these data, it could be clear that the best targets for reprogramming of in vivo linage might be endogenous astrocytes, and in vivo reprogramming non-neuronal cells mainly astrocytes to neuroblasts has been considered at the center of attention in the scientific community (Guo et al. 2014; Torper et al. 2013; Niu et al. 2013; Grande et al. 2013). Previous studies have indicated the feasibility and efficiency of the MSC-derived CM in the models of acute brain injuries and also highlighted that the route of administration could have an impact on the degree of protection (Deng-Bryant et al. 2012; Deng-Bryant et al. 2015). As the enriched source of MSCs/CM, hAF-MSCs hold several advantages due to high cell recovery, noninvasive preparation and pluripotency characteristics (Fierabracci et al. 2015). Pischiutta et al. to investigating the effects of Ah-MSCs-Secreted Metabolites on brain injuries have shown that hAMSC and CM treatments exert protective effects on the cortical region of acute brain injury (Pischiutta et al. 2016). In the present study, we explored the juxtacrine and paracrine effects of the hAF-MSCs in the reprogramming of the endogenous and exogenous astrocytes into neuroblasts in adult male rats after SCI. According to previous evidence, transcription factor Sox2 could be sufficient to converting endogenous differentiated astrocytes into neuroblasts and also mature neurons in the adult spinal cord with different severity of the injury (Su et al. 2014a). Currently, it is well documented that the Sox2 is a single transcription factor that leads to the reprogrammed of astrocytes into active neurons (Ghasemi-Kasman et al. 2015). Also, the factor of Sox2, has a critical role in brain development, neurogenesis, and synapse-forming interneurons(Su et al. 2014b). Therefore, in the current research, Sox 2 was considered as a key factor in astrocyte reprogramming to neuroblasts. Since in the adult spinal cord there are a few newly produced neurons, researchers believe that adult spinal cord-dwelling astrocytes could generate neuroblasts and mature neurons using reprogramming methods(Su et al. 2014b; Li and Chen 2016). Several previous studies have used CM in various disorders like skin wounds (Chen et al. 2008), CNS (Teixeira et al. 2014), hepatic transplant (Du et al. 2013), acute lung injury (Ionescu et al. 2012), and chronic kidney diseases (van Koppen et al. 2012). The expression of the cell surface markers of the

isolated and cultivated MSCs in the present study was consistent with the previous researches (Markmee et al. 2017; Dominici et al. 2006), So that, the MSCs at 3–4 passage, were positive for MSCs including CD105 (95.80%) and CD73 (92.2%) markers meanwhile they were negative for CD14 (9.90%) and CD45 (9.16%) markers for hematopoietic stem cells. Western-blot analysis showed that the harvested CM from the hAF-MSCs expresses the SOX 2 protein as a main driver in astrocyte reprogramming to DCX-positive neuroblasts. DCX is a microtubule-associated protein expressed predominantly in neuroblasts, immature neurons in developmental processes, and most importantly, in the neurogenic area of the adult brain (Brown et al. 2003; Gleeson et al. 1999). Expression of DCX has a relationship with neurogenesis but didn't observed in regenerative axonal growth or reactive gliosis (Couillard-Despres et al. 2005). Consistent with these findings, in the current study, DCX was very low in spinal cord induced injuries. Our data revealed that the MSCs and CM could significantly increase the number of endogenous neuroblasts and reduced the number of endogenous astrocytes in the injured spinal cord. Although in this context, the effect of the MSCs was more in comparison to CM. It is noteworthy that the MSCs release the critical factors in the CM and thereby could play a major role in the astrocyte reprogramming to neuroblasts. In line with our research, Su et al have shown that the SOX 2 exclusively could induce the conversion of astrocytes to neuroblasts and MAP2- positive mature neurons in the adult spinal cord. (Su et al. 2014a). In cell therapy, stem cells are transplanted into the tissue to exert effects on treating a disease with or without gene therapy (Abolhasanpour et al. 2020). Regarding this issue, the MSCs have commonly used in the treatment of various diseases in the clinic, and prominently in animal studies were used in the treatment of tissue injury as well as immune disorders. (Salehi-Pourmehr et al. 2019). *In-vitro* studies have shown that the AF-MSCs can differentiate into cells that express neural lineage marker (Tsai et al. 2006). However, following the transplantation into the ischemic rat brain, it was observed that they have the ability to survive, migrate and differentiate into astrocytes and also immature neurons (Cipriani et al. 2007). Supporting these findings, our results showed that after MSCs transplantation in spinal cord injured rats, the level of GFAP decreased, and the level of DCX significantly increased in the MSCs treated group. In the next series of experiments, we decided to explore whether hA-MSCs and CM could covert the exogenous astrocytes to neuroblasts in the in-vivo condition?

To address this key question, we transplanted the human astrocytes concomitantly with MSCs and CM into the rat injured spinal cord and evaluated the fate of the grafted human astrocytes using the immunofluorescence staining technique in the *in-vivo*. Regarding our results, the majority of the grafted human astrocytes converted to neuroblasts in those groups who have received the MSCs and CM. It should be considered that this converting rate was significantly more in the (SCI + Astrocytes + MSCs) group relative to the (SCI + Astrocytes + CM) group. In-vivo tracing showed that the grafted human astrocytes were significantly was decreased in the (SCI + Astrocytes + MSCs) group and (SCI + Astrocytes + CM) groups. But this decline in the number of the transplanted human astrocytes was significantly more in the (SCI + Astrocytes + MSCs) group in comparison to the (SCI + Astrocytes + CM) group. This is indicating that the MSCs compared to CM could better direct the astrocytes reprogramming to neuroblasts and neurogenesis provocation. Overall, this experiment revealed that the MSCs and CM could increase the DCX – positive cells and diminish the GFAP-positive cells in the in-vivo condition and MSCs

and CM, could be considered as future relevant tools in the field of regenerative medicine (Dominici et al. 2006). Neuhuber et al. [107] showed that CM of BM–MSCs, because of the presence of IL-6, BDNF, SDF-1α, VEGF, MCP-1, and SCF, can induced axon growth and functional recovery in SCI (Neuhuber et al. 2005). A plethora of studies shows that away from cell-cell interaction, the immunomodulation and regenerative capacity of MSCs in the lesion site could be the result of the secretome efficacy (Shi et al. 2010). Therefore, these protective and regenerative actions induced by MSCs secreted molecules may explain significant therapeutic effects in all of the CNS(Uccelli et al. 2011).

In conclusion, the results of the present research point out the protective and regenerative potential of MSCs in the SCI through reprogramming astrocytes to neuroblasts by mediating juxtacrine activity and paracrine effects. Astrocytes reprogramming to neuroblasts is a fundamental and intricate phenomenon in the field of regenerative medicine. Our study is preliminary research in this context and up to now cellular and molecular mechanisms underlying this cellular reprogramming are not fully understood and regarding this, more and complementary studies should be done to address all aspects of the reprogramming subject.

Declarations

Author Contributions

M.PA and P.SH designed and supervised the whole study. M.K prepared and wrote the manuscript and analyzed data. M.A performed the experiments and involved in data acquisition. H.SZ was involved in animal surgery.

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Data Availability:

Data will be available in request.

Compliance with Ethical Standards

Conflict of interest: The authors declare that they have no competing interests.

Ethical Approval

All experiments were conducted according to international principal guidelines and approved by a local ethics committee of Tabriz University of Medical Sciences.

Informed Consent

All of the patients who participated in the current research, prior to sampling donation received the information and all of the considerations regarding the project and filled the informed consent.

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