Dedifferentiation-reprogrammed human mesenchymal stem cells for treating ischaemic stroke: abridged secondary publication

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KEY MESSAGES

1. Dedifferentiated-reprogrammed human mesenchymal stem cells (De-neu-hMSCs) reveal distinguished stem cell phenotype such as enhanced neuronal differentiation potential, cell migration, and cell survival, compared with naïve hMSCs.
2. Systemic administration of hMSCs/De-neu-hMSCs significantly improves stroke recovery, with De-neu-hMSCs exhibiting stronger repair function.
3 De-neu-hMSC treatment results in more improved motor function recovery and less brain damage, compared with hMSC treatment.
4 The enhanced therapeutic effects of De-neu-hMSCs might be attributed to suppression of endogenous Bax-induced apoptosis pathway.

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Introduction

Mesenchymal stem cell (MSC)–based therapy is a promising strategy in the treatment of stroke. However, low levels of MSC recruitment, cell survival, and directed differentiation in vivo largely limit their overall effectiveness and clinical use. Dedifferentiation reverts differentiated cells to an earlier, more primitive phenotype. Previous studies have demonstrated that dedifferentiation is a prerequisite for MSCs to change their cell fate and re-differentiate into a different lineage.[1] By manipulating cell fates of MSCs in vitro, we have found that after in vitro induction of neuronal differentiation and dedifferentiation, rat MSCs that have already committed to neuronal lineage revert to primitive cells distinct from naïve rat MSCs. The dedifferentiated rat MSCs exhibit enhanced cell survival and differentiation compared to unmanipulated rat MSCs in vivo, with significantly improved cognition function in a neonatal hypoxic-ischemic brain damage rat model.[2] These results indicate that dedifferentiated MSCs have potential to enhance the therapeutic effects in ischaemic brain disease. Thus, we hypothesise that dedifferentiation-reprogrammed human MSCs may exhibit enhanced therapeutic potential in treating stroke.

Methods

We tested our hypothesis in both cell culture and rats with middle cerebral artery occlusion (MCAo) model. We aim to (1) characterise the dedifferentiation-reprogrammed human bone marrow-derived MSCs (De-neu-hMSCs), (2) compare the phenotypic properties (proliferation, cell survival, differentiation, migratory abilities) of De-neu-hMSCs with unmanipulated human MSCs in vitro, and (3) evaluate the therapeutic efficacy of De-neu-hMSCs and determine the mechanisms underlying the beneficial effects of De-neu-hMSCs in MCAo rat model.

Results

After 24 hours of neural induction, >95% cells presented with neuron-like morphology (Fig. 1a). In line with the morphological changes, immunofluorescence analysis showed that the expression of neural markers Nestin and MAP2 were barely detectable in uncommitted hMSCs but significantly increased after 24 hours of neuronal induction (Fig. 1b). However, withdrawal of MNM rapidly reverted hMSC-derived neuron-like cells to characteristic mesenchymal morphology, and suppressed Nestin and MAP2 expression within 24 hours (Fig. 1b). These De-neu-hMSCs from differentiated neuronal cultures could be re-induced into neuronal phenotype upon re-exposure in MNM without pre-induction with ATRA and bFGF. We then further characterised immunophenotypic

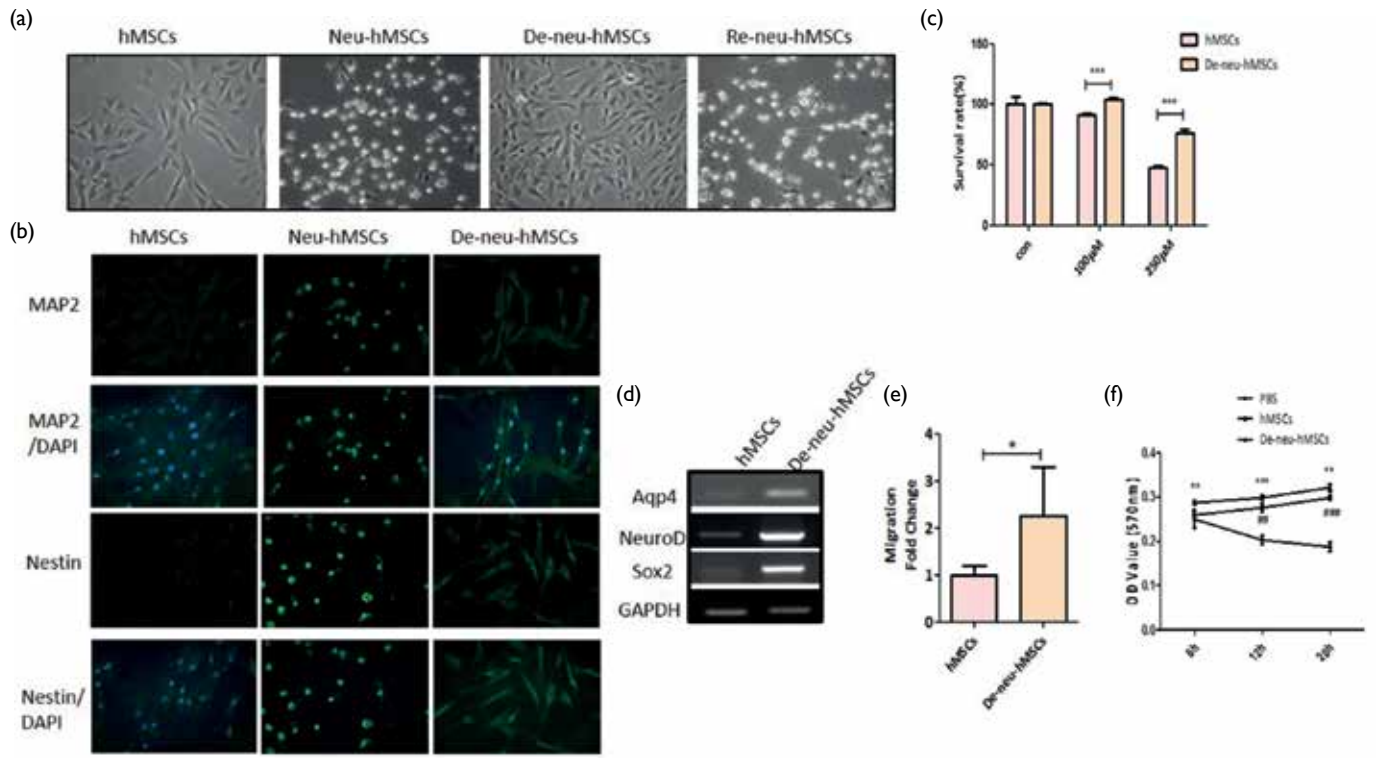

FIG 1. Neuronal differentiation, dedifferentiation, and re-differentiation of human bone marrow mesenchymal stem cells (hMSCs). (a) Phase contrast photographs of neuronal differentiation, dedifferentiation, and re-differentiation of hMSCs. (b) Immunostaining of the neuronal markers: MAP-2 and Nestin. (c) Dedifferentiation-reprogrammed hMSCs (De-neu-hMSCs) exhibit survival advantage over untreated hMSCs. The untreated hMSCs and De-neu-hMSCs are plated in 96-well plates and challenged with 0-250 μM H_2O_2 for 24 hours. (d) RT-PCR analysis of Aqp4, Sox-2, and NeuroD in hMSCs and De-neu-hMSCs. (e) De-neu-hMSCs exhibit advantage in migration over untreated hMSCs. (f) The condition media derived from either hMSCs or De-neu-hMSCs are added into serum-deprived and low glucose treated PC-12 cells for 24 hours. Cell proliferation is assessed using MTT assay. The absorbance at 450 nm is measured.

marker in hMSCs/De-neu-hMSCs. Fluorescence-activated cell sorting showed that De-neu-hMSCs retained their immunophenotype similar to that of undifferentiated hMSCs. These results indicate that dedifferentiation can be achieved in human MSCs.

We then used various cell functional analyses to characterise the De-neu-hMSCs. De-neu-hMSCs exhibited a survival advantage over undifferentiated hMSCs when challenged with hydrogen peroxide (Fig. 1c). In addition, the expression level of neural markers decreased upon dedifferentiation, with the level higher in De-neu-hMSCs than in undifferentiated hMSCs (Fig. 1b). RT-PCR analysis revealed that the expression of neural progenitor markers such as NeuroD1, Sox-2, and Aquaporin 4 was much higher in De-neu-hMSCs than in hMSCs (Fig. 1d). These results are consistent with our previous study that De-neu-rMSCs have enhanced cell survival and higher potential to re-differentiate into neurons.[2]

We then proceeded to evaluate the effect of dedifferentiation on the migratory ability of hMSCs. De-neu-hMSCs exhibited enhanced migratory ability as demonstrated by transwell assay (Fig. 1e). Oxygen glucose–deprived PC-12 cells were co-cultured with hMSCs or De-neu-hMSCs for 24 hours. Co-culture dramatically increased the number of viable cells after oxygen and glucose deprivation, with a significantly larger number of cells observed with De-neu-hMSCs than with hMSCs (Fig. 1f), indicating enhanced cell survival. Collectively, these results indicate that the enhanced stemness observed in the De-neu-MSCs represents a general property among MSCs derived from different species.

The therapeutic efficacy of De-hMSCs was evaluated using the middle cerebral artery occlusion (MCAo) rat model. A total of 140 adult Sprague Dawley rats were used. In the preliminary experiment, 20 rats were used to establish focal cerebral ischaemia and 15 rats were used to establish intracarotid stem cell delivery. In the first batch of experiments, 45 rats were divided into PBS, hMSC, and De-neu-hMSC groups (15 per group). At day 1 and day 10 after stem cell transplantation, the severity of MCAo-induced motor and sensory deficits were assessed by the modified Neurologic

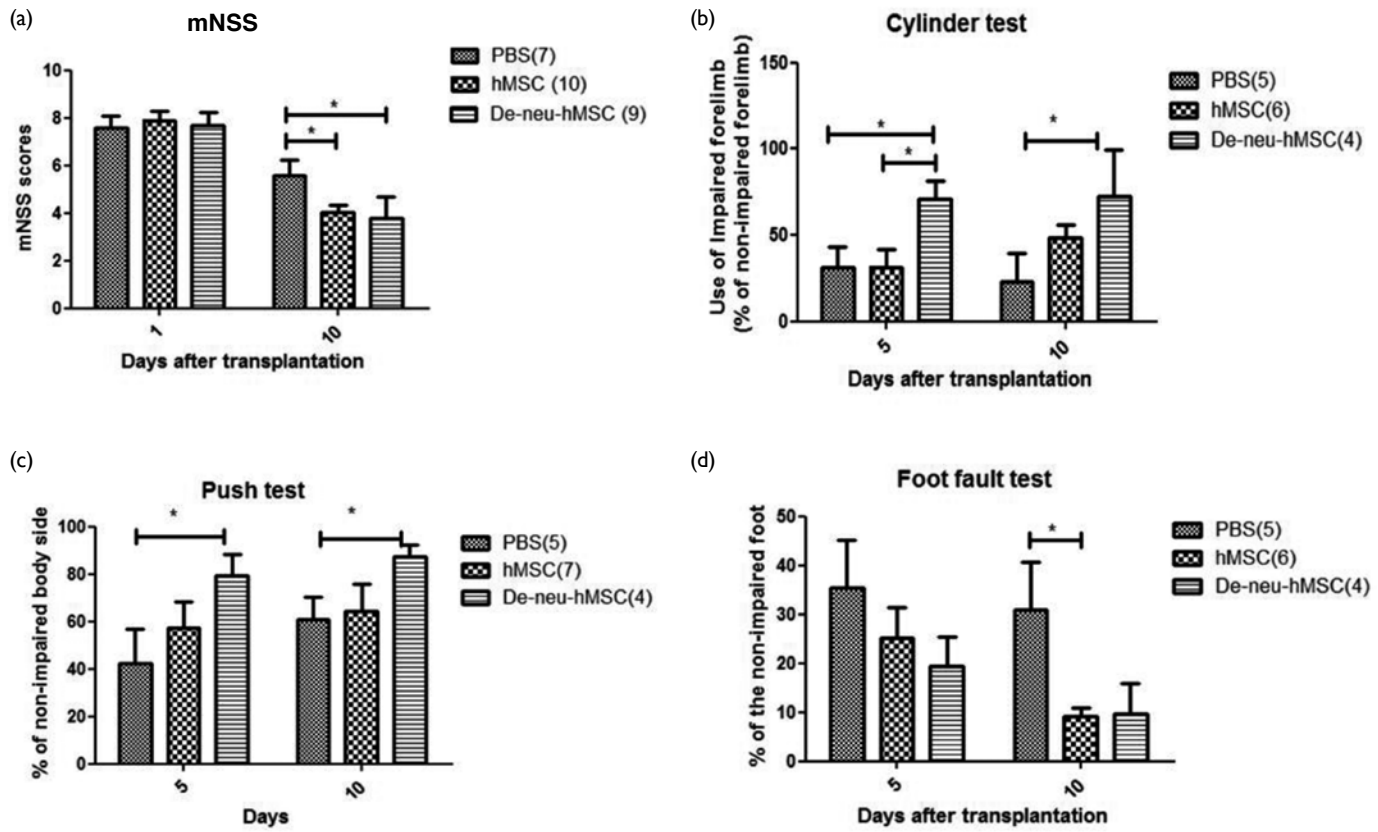

FIG 2. Dedifferentiation-reprogrammed human bone marrow-derived mesenchymal stem cells (De-neu-hMSCs) significantly improves motor function recovery in rats with middle cerebral artery occlusion. (a) hMSC and De-neu-hMSC transplantation improve modified Neurological Severity Score (mNSS). (b) De-neu-hMSC transplantation improves cylinder test performance at days 5 and 10 after transplantation. (c) De-neu-hMSC transplantation improves push test performance. (d) hMSC and De-neu-hMSC transplantation improve foot fault test performance at day 10 after transplantation.

Severity Score (mNSS). In the second batch of experiments, 30 rats were used for behaviour tests (cylinder test, push test, and foot fault test) at day 5 and day 10 after stem cell transplantation. In the third batch of experiments, 30 rats were randomly assigned to three groups. At day 7 after stem cell transplantation, rats were deeply anaesthetised and the brain was fixed by trans-cardiac perfusion of 4% PFA. The fixed brain was embedded in optimal cutting temperature, and 5-μm coronal sections were cut by cryostat.

Neurological function of rats was assessed using the mNSS at days 1 and 10 after stem cell transplantation. The mNSS is a composite test for motor (muscle status, abnormal movement), sensory (visual, tactile, and proprioceptive), and reflex,[3] with scores ranging from 0 (normal) to 18 (maximal deficit). The mNSS was close to 8 in MCAo rats on day 1 post stem cell treatment, indicating neurological functional deficits. Significant reduction in the mNSS was found in the PBS-treated animals compared with day 1 after injury (Fig. 2a), suggesting that a significant spontaneous sensorimotor functional recovery occurred after MCAo. Functional recovery was significantly higher in the hMSCs and De-neu-hMSCs groups than in the PBS group. There was no significant difference between hMSCs and De-neu-hMSCs groups at day 10 (Fig. 2a).

For the cylinder test, at day 5 after stem cell treatment, only De-neu-hMSCs–treated animals exhibited decreased forelimb asymmetry. At day 10 both hMSCs and De-neu-hMSCs groups exhibited decreased forelimb asymmetry, but this effect was significant only in rats receiving De-neu-hMSCs, compared with control rats receiving PBS alone (Fig. 2b).

For push test, De-neu-hMSCs treatment produced a significant increase in percentage resistance to lateral push after day 5 and day 10, when compared with the PBS treatment. However, the hMSC group did not have this effect (Fig. 2c).

The foot fault rate of the affected limb is the ratio of the number of fault footsteps to the total number of steps. The test was repeated three times and was performed on day 5 and day 10 after stem cell treatment. At day 5, the foot fault rate in the

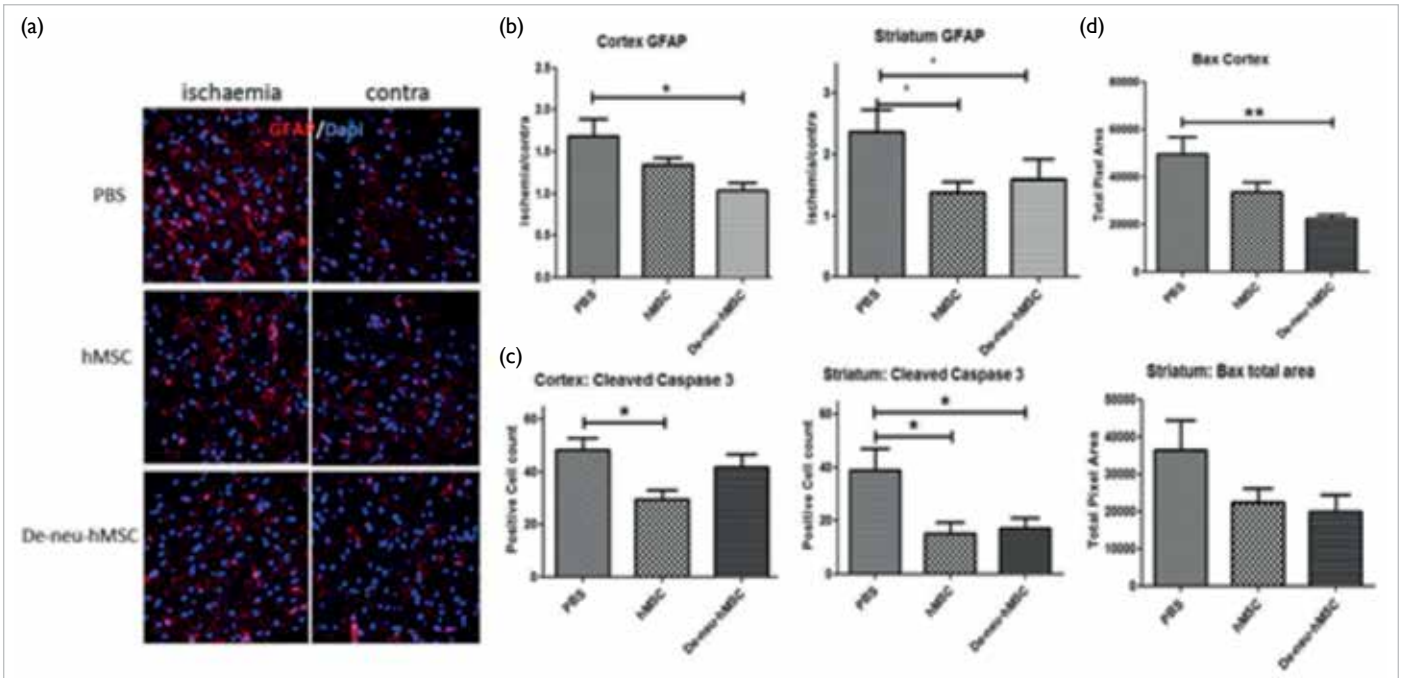

FIG 3. Dedifferentiation-reprogrammed human bone marrow-derived mesenchymal stem cells (De-neu-hMSCs) alleviates brain damage and decreases Bax expression in rats with middle cerebral artery occlusion. (a) hMSC and De-neu-hMSC transplantation reduce GFAP$^+$ cells in the cortex and striatum. (b) GFAP$^+$ cells are calculated in both ischaemia and its contra areas and the ratio of ischaemia/contra are used to evaluate the increased GFAP positive cells in both cortex and striatum areas. (c) Cleaved Caspase 3 positive is significantly decreased in hMSC or De-neu-hMSC groups. (d) Bax expression intensity is significantly decreased in the De-neu-hMSC group.

hMSC and De-neu-hMSC groups were mildly lower than that in the PBS group. By contrast, at day 10, the foot fault rate in the hMSC group and De-neu-hMSC group was significantly lower than that in the PBS group, while there was no significant difference between hMSCs and De-neu-hMSCs groups (Fig. 2d).

To further determine the molecular mechanism underlying the therapeutic effects of MSCs/De-neu-hMSCs, rats were deeply anaesthetised and the brains were fixed by trans-cardiac perfusion of 4% PFA for immunofluorescent staining. GFAP-staining was used to identify reactive astrocytes in the brain after MCAo. When neurons are damaged by ischaemia, astrocytes will generate to fill the space of dead neurons. Thus, the expression level of GFAP indicates the degree of brain damage. MCAo alone significantly increased the percentage of GFAP$^+$ cells in the lesion boundary zone of the injured hemisphere compared to the contra-side. hMSC/De-neu-hMSC treatment significantly reduced the GFAP$^+$ astrocyte in the injured striatum compared to the PBS treatment. De-neu-hMSCs (but not hMSCs) significantly reduced the GFAP$^+$ astrocyte in the injured cortex (Fig. 3). Our results suggest that stem cell treatment alleviates brain damage, more prominent in the De-neu-hMSC group. We then stained the brain tissue with cleaved caspase-3 to evaluate the apoptotic response in untreated or stem cell–treated MCAo models. Both hMSC and De-neu-hMSC treatment significantly decreased cleaved caspase-3 positive cells compared to PBS treatment, indicating stem cell treatment alleviates apoptosis-induced damage in MCAo model (Fig. 3c). We then determined the expression intensity and positivity of Bax and Bcl-2, which are apoptosis regulators. Stem cell treatment significantly decreased expression intensity and positivity of Bax (but not Bcl-2). The suppressive effect on Bax expression was more prominent in De-neu-hMSC group (Fig. 3d).

Conclusion

Human MSCs can be reprogrammed via in vitro neuronal differentiation and dedifferentiation, with enhanced cell survival, neural differentiation capacity, and migration. De-neu-MSCs exhibit enhanced therapeutic effects in treating stroke, probably through suppression of Bax-induced apoptosis pathway. Further studies are warranted to identify the molecular mechanism underlying the enhanced therapeutic effects of De-neu-hMSCs. With easy culture manipulation and low tendency of tumour formation, dedifferentiation strategy provides a feasible approach to enhance therapeutic efficacy for

stroke and stem cell based-regenerative medicine. The proposed research work has strong clinical relevance and great potential for clinical applications.

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Disclosure

The results of this research have been previously published in:

1. Yang FY, Zhang XH, Tsang LL, Chan HC, Jiang XH. Dedifferentiation-reprogrammed mesenchymal stem cells for neonates with hypoxic-ischaemic brain injury. Hong Kong Med J 2019;25(Suppl 5):12-6.

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