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MSCs ameliorates DPN induced cellular pathology via $[Ca^{2+}]_i$ homeostasis and scavenging the pro-inflammatory cytokines

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The MSCs of various origins are known to ameliorate or modulate cell survival strategies. We investigated, whether UCB MSCs could improve the survival of the human neuronal cells and/or fibroblast assaulted with DPN sera. The results showed, the co-culture of UCB MSCs with human neuronal cells and/or fibroblasts could effectively scavenge the pro-inflammatory cytokines TNF-α, IL-1β, IFN-ɤ and IL − 12 and control the pro-apoptotic expression of p53/Bax. Further co-culture of UCB MSCs have shown to induce anti-inflammatory cytokines like IL-4, IL-10 and TGF-β and anti-apoptotic Bclxl/Bcl2 expression in the DPN sera stressed cells. Amelioration of elevated $[Ca^{2+}]_i$ and cROS, the portent behind the NFκB/Caspase-3 mediated inflammation in DPN rescued the cells from apoptosis. The results of systemic administration of BM MSCs improved DPN pathology in rat as extrapolated from human cell model. The BM MSCs ameliorated prolonged distal motor latency (control: 0.70 ± 0.06, DPN: 1.29 ± 0.13 m/s DPN + BM MSCs: 0.89 ± 0.02 m/s, $p < 0.05$) and lowered high amplitude of compound muscle action potentials (CMAPs) (control: 12.36 ± 0.41, DPN: 7.52 ± 0.61 mV, DPN + MSCs: 8.79 ± 0.53 mV, $p < 0.05$), while slowly restoring the plasma glucose levels. Together, all these results showed that administration of BM or UCB MSCs improved the DPN via ameliorating pro-inflammatory cytokine signaling and $[Ca^{2+}]_i$ homeostasis.

KEYWORDS
anti-inflammatory cytokines, calcium homeostasis, diabetic peripheral neuropathy, mesenchymal stem cells, nerve conduction studies, pro-inflammatory cytokines

Abbreviations: MSCs, mesenchymal stem cells; BM MSCs, bone marrow mesenchymal stem cells; UCB MSCs, umbilical cord blood mesenchymal stem cells; CMAPs, compound muscle action potentials; DPN, diabetic peripheral neuropathy; ROS, reactive oxygen species; *i.v.*, intravenous injection; MNCV, motor verve conduction velocity; SNBF, sciatic nerve blood flow; DM, diabetes mellitus; HBA1c, glycosylated hemoglobin; EDTA, ethyl-enediaminetetraacetic acid; ADM, abductor digiti minimi; STZ, streptozotocin; NCV, nerve conduction velocity; GM, gastrocnemius muscle; rNGF, recombinant nerve growth factor; EGF, epidermal growth factor; ETC, electron transport chain; FBS/FCS, fetal bovine serum/ fetal calf serum; BDNF, brain-derived neurotropic factor; Dcx, doublecrotin.

1 | INTRODUCTION

In the diabetic world, peripheral neuropathies are foremost frequent complications, which are extremely difficult to treat pain syndromes. DPN results in irreversible cell death leading to amputation, a huge morbidity factor in the management and rehabilitation of DM

(Joseph & Levine, 2004; Obrosova, 2002). Further there is an objective evidence of more than 40% of diabetics, both type 1 and 2 DM showing peripheral neuropathy (Fatani et al., 2015). In the continuation of the therapy, clinical trials using anti-TNF drugs to reverse the pathophysiology of the DPN offers a good evidence of the cytokine mediated inflammation (Genevay, Guerne, & Gabay, 2004; Karppinen et al., 2003). The involvement of abnormal Na^+ and K^+ transport across the nerve cells has been shown to result in the impairment of the nerve function with slow nerve conduction velocity (Vague et al., 1997). The involvement of abnormal Ca^{2+} homeostasis in the generation of the ROS skewed the cells to death, has been previously shown in our other study (Mallilankaraman et al., 2012).

MSCs are known immune modulators (Nemeth, 2014; Nemeth & Mezey, 2015) and recently they are also reported to modulate the repair process of neurons by secreting cytokines, anti-apoptotic molecules, and cell survival trophic factors. (Asami et al., 2013; Zhou, Zhang, & Qian, 2016). There exists a paucity of data on the effect of BM MSCs on the treatment of DPN at the clinical level; however, pre-clinical data has revealed beneficial effects of BM MSC administration. In rodent models of DM, intravenous injection (*i.v.*) of BM MSCs were shown to ameliorate the symptoms of DPN (Kim, Jin, & Bae, 2011). Further transplantation of BM MSCs improved hypoalgesia, delayed motor nerve conduction velocity (MNCV), reduced sciatic nerve blood flow (SNBF), and decreased axonal circularity in diabetic nerves of treated rats but without incorporation of MSCs into the tissues (Shibata et al., 2008). Subsequent studies showed the decrease of pro-inflammatory cytokines with improvements in pain due to MSCs (Waterman et al., 2012). There are studies that report on MSCs administration stimulating the mass of sympathetic and parasympathetic nerves in the ventricular myocardium of diabetic rats (Wang et al., 2013).

None of the current studies have shown direct evidence of the cellular integration of MSCs in the tissues or restoration of the β-cells in pancreas. There is no direct clinical investigations or human data to validate the claims of MSCs amelioration of the DPN. It is also very difficult to keep a track of the transplanted MSCs and indigenous resident MSCs in human studies. There are many studies suggesting various subsets of MSCs having uniform expression of surface markers (Heo, Choi, Kim, & Kim, 2016). BM and UCB MSCs are more or less same in most functions except for the fact that UCB MSCs are more naïve with rapid proliferation and differentiation ability (Jin et al., 2013). Further the mode of administration, dose, and MSC subpopulation from different sources on the functional capabilities are yet to be determined. Nonetheless, in light of the exciting preclinical evidence on the benefits of MSCs specifically on immunomodulation, angiogenesis, and neurogenesis are well documented. When these activities of the MSCs are coupled with the emerging evidence on the glucose lowering effect is more likely to improve the nerve function and alleviate the symptoms and clinical consequences of DPN. This will potentially reduce the burden of neuropathic ulceration, pain, and impaired quality of life associated with DPN.

The question here is rather management and treatment of the DPN than reversal of diabetes itself. Our study focuses is in the assessment of MSCs, an immunologically modulatory cell type in the multipotent restoration of the neuronal cells. The aim of the current investigation is to study the missing link in the complex inflammatory and oxidative stress mediated cellular damage in DPN by BM or UCB MSC treatment, which is currently sub-optimally managed with contemporary treatment strategies.

2 | MATERIALS AND METHODS

This study was undertaken at Center for Stem Cell Research and Department of Physiology, College of Medicine, King Khalid University Abha and Kasr Al Aini Faculty of Medicine, Cairo University, Egypt. Type 2 diabetic patients attending the out-patient department of Asir Central Hospital were selected for the study after obtaining their consent. Ethical clearance was obtained from the King Khalid University ethical committee, college of medicine, approval letter REC # 2015-03-07. The animal experiments were performed as collaborative work between Kasr al Aini Faculty of Medicine, Cairo University, Egypt and College of Medicine, King Khalid University, Abha, SA. The work was approved by the animal ethical committee letter REC # 2013-02-08. The electrophysiological studies were carried out in the Clinical Neurophysiology unit-Kasr Al-Aini hospital.

2.1 | Human DPN samples

Glycated haemoglobin (HbA1c) indicated the severity of diabetes and patients were selected for DPN study based on the reference criteria (Supplementary Annexure S1). Five milliliter of the blood was collected in EDTA coated vacutainer as per good phlebotomical practices. Serum was separated and stored at −86°C till further experiments. The nerve conduction data in about 50 DPN cases were retrospectively collected and only used as a reference to check the alteration of nerve conduction to confirm the DPN status.

2.2 | Measurement of HbA1c

The HbA1c levels were determined by the borate affinity assay (Nycocard, AXIS-SHIELD PoC AS, Norway) as per manufacturer instructions.

2.3 | Electrophysiological studies in humans

Male, right handed patients with known history of type 2 diabetes of 5–10 years duration and those who were on treatment were tested for glycated haemoglobin (HbA1c) levels. Based on the HbA1c levels, 50 diabetic patients with HbA1c levels of <7.0 and 50 patients with HbA1c levels of >7.0 were selected and grouped into group 1 and group 2 categories respectively. Fifty age matched male, right handed volunteers, who were non diabetics and healthy were selected as controls and were grouped as group 3. The NCV was studied for the ulnar nerve of the right hand in all the 150 individuals. The neuro physiological measurements were performed in a warm room with the participants in a sitting position, with their forearms partially flexed.

NCV measurements were made by using Medtronic Keypoint® 2 EMG EP software. The surface metal plated stimulating electrodes were placed at 5 cm below the medial epicondyle and at 5 cm above the medial epicondyle and the recording electrodes were placed over the abductor digiti minimi (ADM) muscle on the ulnar side of the hand that is between the fifth meta carpophalangeal joint and the pisiform bone. The compound muscle action potentials (CMAPs) were evoked by the electrical stimulation (0.1 ms duration, constant current pulse) of the ulnar nerve, starting with a minimum and progressing to the maximum intensity of the stimuli. Latency, amplitude, and nerve conduction velocity were assessed.

2.4 | Animals

The experiments were performed on healthy male wistar rats of 10 weeks old and weighting 200–250 g. The rats were fed with standard laboratory diets, given water ad libitum and maintained under laboratory conditions of temperature 22°C (±3°C), with 12 hr light and 12 hr dark cycle. All experimental procedures involving the handling and treatment of animals were approved by the Ethical Committee and were conducted in accordance with the National Institute of Health's Guide for the Care and Use of Laboratory Animals. The animals were divided into control and DPN groups ($n = 7$). Induction of type 2 diabetes in rats was by a single i.v. low dose of streptozotocin (STZ) (Sigma, St Louis, Mo) 40 mg kg − 1 body weight into the tail vein. The rats were fed with high fat diet for 15 days pre STZ injection. Blood glucose above 300 mg/dl indicated diabetes in STZ treated animals (Kambiz et al., 2015). After the confirmation of the DPN through nerve conduction studies, the rats with DPN were injected with 5×10^6 MSCs by the tail vein (i.v.) as two doses 1 week apart. The animals were rested for 6 weeks prior to repeated blood sugar and nerve conduction studies. No mortality was observed during the study period.

2.5 | Electrophysiological studies in animals

Anesthesia, equipment, approach of the sciatic nerve and placement of electrodes were carried out in the same way as described elsewhere (Rupp et al., 2007). Care was taken to ensure that the rats' core temperature was maintained at between 36° and 38°C. The rats were prepared for nerve conduction by shaving the hind limbs, and cleaning with alcohol. All stimulations and recordings were done using Nihon Kohden, 2 channels unit (Japan).

2.6 | Calculation of nerve conduction velocity (NCVs)

NCVs were measured in the sciatic-tibial nerve. Stimulation was carried out two monopolar needle electrodes positioned directly on the nerve at the gluteal fold (length 12 mm; diameter 0.3 mm; Viasys Healthcare Supplies 2003 Catalogue No.: 019-404700; Nicolet, Germany). Electrical square wave pulses of 0.2 msec. duration were delivered at a rate of 10 Hz and the intensity was increased to be just supra-maximal (1.9 mA) until a potential could be recorded in the gastrocnemius muscle (GM).

The provoked motor response was recorded from the GM of the hind limb using bipolar (concentric) needle electrodes for intramuscular recordings of amplitude of CMAPs and latency of NCV.

Concentric needles are preferable to monopolar electrodes for intramuscular recordings (Horning, Kraft, & Guy, 1972) as it narrows down the source of error (Gassel, 1964) due to the fact that smaller CMAPs are recorded and the background noise is suppressed (Cuddon, 2002; Scholle et al., 2005).

The CMAP latencies, defined as the lag between stimulus and onset of the first deflection of the action potential from the baseline, were measured at supramaximal stimulation intensities. Then the rats' hind limbs were straightened and the distances between the stimulating active electrodes were ascertained with a tape measure to the nearest millimeter. These values were subsequently used to calculate the individual NCVs. (Dallak et al., 2008; Rupp et al., 2007).

2.7 | Rat mesenchymal stem cell

MSCs were isolated from bone marrow of the rats. The protocols for the isolation/phenotyping BM MSCs were as per protocol standardized in our laboratory and previously published (Karaoz, Genc, Demircan, Aksoy, & Duruksu, 2010; Zhang & Chan, 2010). Briefly, femur and tibia were aseptically removed from the age, gender matched donor rats. The flushed bone marrow was strained through 70 μm sterile cell strainer, the cells were washed with ice-cold phosphate buffered saline (PBS) once at 1200 RPM, 4°C without brakes. The adherent cells were incubated in 100 mm tissue culture dish at 37°C in 5% humidified CO_2 for 12–14 days. The cells of the large colonies were trypsiniszed, washed and enriched for CD54 and CD90 using positive selection magnetic cell sorting. The enriched cells were checked for purity and positive expression of CD54 and CD90 and negative for CD34 and CD45 phenotypic markers using flowcytometery before transplantation purposes (Haidara et al., 2015).

2.8 | Human umbilical cord blood mesenchymal stem cells

The UCB MSCs derived from donor umbilical cord blood was used in ex vivo assays and for neuronal cell differentiation protocols. Briefly mononuclear cells were isolated from umbilical cord blood by using Ficoll-Paque (GE Healthcare Life Sciences, Milan, Italy) density gradient centrifugation. The adherent cells were cultured for 2 weeks in 100 mm tissue culture treated dish at 37°C in a humidified environment with 5% CO_2. Every alternate day the media was replenished with fresh mesenchymal growth media. Once the culture reached 80% confluence, UCB MSCs were trypinized and enriched for CD 73, CD29, and CD105 using positive selection magnetic cell sorting. The enriched UCB MSCs were checked for purity and phenotyped positive for CD 73, CD29, CD105 and negative for CD34 and CD45 before used in subsequent ex vivo assay and neuronal differentiation (Bieback, Kern, Kluter, & Eichler, 2004; Hussain, Magd, Eremin, & El-Sheemy, 2012).

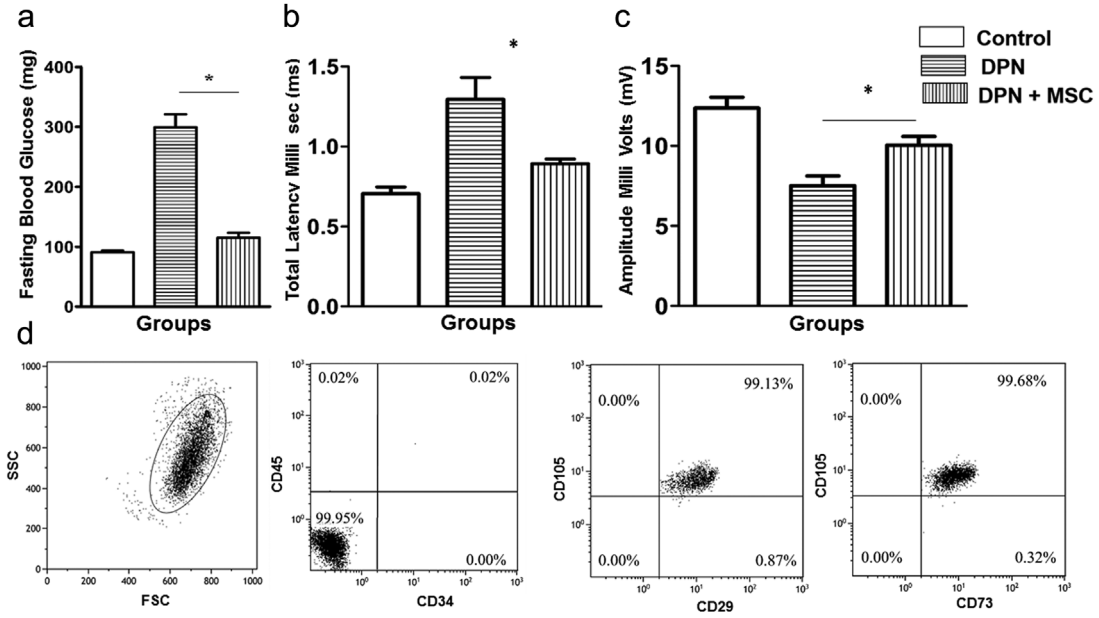

FIGURE 7 Nerve conduction studies in DPN rats. The graphs are combined to compare between DPN and DPN + BM MSCs and the values are expressed as mean ± SE and $^*p < 0.05$ was considered as significant. A. Fasting blood glucose levels of control, DPN after induction with STZ and restoration of blood glucose in DPN rats received i.v. BM MSCs. (b,c) Comparison of standardized latencies and amplitude of CMAPs. (b) Total latency of nerve conduction which is high in DPN and ameliorated in + BM MSCs treatment. (c) The amplitude which was recorded low in DPN improvised with + BM MSCs treatment. (d) The purity of the enriched rat BM MSCs used for *i.v* injections

Though the mechanism of the neuronal cell death has been well established, the work of BM MSCs in the niche or at the site of inflammation are supportive and not inclined to the traditional understanding of homing of BM MSCs at the site repopulating and differentiating into neuronal cells (Hidmark et al., 2014; Rouq, Hammad, & Meo, 2014). We have studied skin biopsy to investigate the labeled BM MSCs to be located among the damaged tissues without success (Zhou et al., 2016). Further to be noted that in DPN, predominantly small myelinated and unmyelinated fibers are affected resulting in pain and sensation to environmental factors. The involvement of the small fibers rather than the major nerves is a complicating factor in the chasing of the MSCs to check its colonization at the site of inflammation or nerve damage in DPN (Didangelos, Doupis & Veves, 2014; Javed, Alam, & Malik, 2015). It is evident from our results that the transplantation of MSCs rescues the neuronal damage which is well evidenced with the nerve conduction studies. Previous studies have shown localized sciatic nerve injection of the hematopoietic stem cells (HSCs) or endothelial progenitor cells (EPCs) to reverse the diabetic complications especially DPN with improved nerve conduction and vascularization (Hasegawa et al., 2006; Naruse et al., 2005).

4.3 | UCB MSCs triggers off anti-inflammatory cytokines improving DPN in-vitro.

The results of the in-vitro studies with the DPN sera treatment to the fibroblast and neuronal differentiated cells initially proved the pro-inflammatory mediated cell death. Co-culture of UCB MSCs with these cells for more than 48 hr showed significant reduction of the cell death. It has been well known that metabolic disease like diabetes causes neuronal damage via insulin resistance and dyslipidemia through the process of inflammation (Kalupahana & Moustaid-Moussa, 2012) wherein increased expression of the pro-inflammatory cytokines like TNF-α, IL-1β, MCP-1 and IL-6 (Medzhitov, 2008) mediates cell damage. Our in-vitro experiments too showed increased expression of pro-inflammatory cytokines (TNF-α, IL-1β, and IL-6) both in DPN sera and in the cell culture supernatant of DPN sera treated neuronal cells. The expression of the anti-inflammatory cytokines (IL-4, IL-10, and IFN- α) were observed subsequently due to the co-culture of UCB MSCs. Anti-inflammatory cytokines at the site of peripheral nerve damage during simultaneous episodes of the stress in DPN is an excellent pro-survival signal and therapeutic option. These results were well reproducible in our studies.

4.4 | Increase in basal $[Ca^{2+}]_i$ mediated ROS is key determinant of cell death.

Our results also showed increased pro-inflammatory cytokines and cell death which raised the question on nature of cell death. Next, we investigated on the mechanism of cell death. From the earlier studies and in a condition like hyperglycemia, it is well known that excessive influx of glucose can lead to over loading of the mitochondrial electron transport chain (ETC) resulting in the excessive ROS (Fernyhough & Calcutt, 2010; Fernyhough, Roy Chowdhury, & Schmidt, 2010; Gonzalez et al., 2016; Naziroglu, Dikici, & Dursun 2012; Premkumar & Pabbidi, 2013). However it has be noted that excessive inflammation which is also observed in prolonged hyperglycemia results in oxidative stress (Haidara, Yassin, Rateb, Ammar, & Zorkani, 2006). Our