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Transplantation of bone marrow-derived mesenchymal stem cells ameliorated dopamine system impairment in a D-galactose-induced brain ageing in rats

Gehan El-Akabawy et al., Stem cell transplantation in ageing rats

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ABSTRACT

Ageing is the primary risk factor for Parkinson's disease. Progressive motor and coordination decline that occurs with ageing has been linked to nigrostriatal dysfunction. Few studies have investigated the efficacy of mesenchymal stem cells in ameliorating the structural and functional alterations in the ageing nigrostriatal system. This study is the first to evaluate the effects of intravenous injection of bone marrow-derived mesenchymal stem cells (BMMSCs) in a D-galactose-induced rat model of nigrostriatal ageing. BMMSCs were intravenously injected once every 2 weeks for 8 weeks. The transplanted cells survived, migrated to the brain, and differentiated into dopaminergic neurones and astrocytes. BMMSC transplantation improved locomotor activity, restored dopaminergic system function, preserved atrophic dopaminergic neurones in the substantia nigra, exerted antioxidative effects, and restored neurotrophic factors. Our findings demonstrate the efficacy of BMMSC injection in a nigrostriatal ageing rat model, and suggest that these cells may provide an effective therapeutic approach for the ageing nigrostriatal system.
Key words: bone marrow-mesenchymal stem cells, D-galactose, rat, nigrostriatal dysfunction

INTRODUCTION

Ageing is associated with several biochemical and molecular changes that eventually lead to cognitive and somatosensory impairments. This can be considered a primary risk factor for the development of neurodegenerative diseases such as Parkinson’s disease (PD) [1-4]. Dopaminergic (DA) neurones are among the most vulnerable cells of the central nervous system to the deleterious consequences of ageing. The most obvious indication of DA neurone susceptibility to ageing is the degradation of nigrostriatal DA neurones [5,6].

Ageing has also been associated with structural alterations of the nigrostriatal system. Several reports have shown that pathogenic alterations associated with PD are identical to age-related changes in DA neurones. The loss of DA neurones in the substantia nigra (SN), which reduces the amount of dopamine released and the number of DA receptors in the striatum and causes bradykinesia, muscular stiffness, and shaking, is a notable neuropathological aspect of PD [5-9]. These characteristics are mostly related to ageing (also known as late-onset PD) and

are dependent on environmental and hereditary variables [10]. A substantial body of evidence suggests that brain-derived neurotrophic factor (BDNF) is essential for the survival of SN DA neurones. BDNF helps SN neurones survive in vitro and is protective against a variety of neurotoxic injuries both in vitro and in vivo [11-13]. Another neurotrophic factor, glial cell line-derived neurotrophic factor (GDNF), supports the survival of DA cells in the midbrain, increases the function of the remaining DA neurones in the SN, and inhibits degeneration and DA neuronal death [14-16]. In addition, oxidative stress appears to be a crucial risk factor for ageing-mediated neuronal and neurotransmitter changes [17,18].

The morphological and functional alterations associated with ageing are exacerbated in age-related diseases; therefore, therapies that attenuate primary and/or secondary ageing are the main focus of ageing research [19-21]. Both preclinical and clinical trials of stem cell treatment have demonstrated its effectiveness in the treatment of Alzheimer’s disease (AD) and PD. Mesenchymal stem cells (MSCs) are the most promising type of stem cells owing to their ability to differentiate into the neuronal phenotype, secrete neurotrophic cytokines, and promote endogenous brain repair. In addition, they have immunomodulatory, neuroprotective, and angiogenic capabilities [22-24]. In rodent PD models, bone marrow-derived MSC (BMMSC) transplantation has been demonstrated to improve behavioural performance, ameliorate DA system degeneration in the SN and striatum, attenuate histopathological alterations, reduce the inflammatory response, and induce the release of neurotrophic factors [25-31]. Based on a study in 2021, a single-centre, open-label phase 1 clinical study was conducted to assess the safety and feasibility of intravenous injection of allogeneic BMMSCs delivered in escalating doses to patients with idiopathic PD [32].

Most of the research on the effectiveness of stem cells has been conducted on preclinical animal models or on patients with AD and PD, whose structural and functional brain capabilities have significantly deteriorated. It is possible that early intervention to address neuropathological changes during primary ageing will stop or at least delay the pathological processes leading to secondary ageing, thus lowering the prevalence of age-related disorders [19-21,33-35]. Few studies have evaluated the efficacy of MSC transplantation in animal models of ageing [36-42]. Therefore, we aimed to evaluate, for the first time, the potential beneficial effects of systemic transplantation of BMMSCs on the

nigrostriatal system in a D-galactose (D-gal)-induced rat model of brain ageing to evaluate their potential as a protective approach for age-related neurodegeneration.

MATERIALS AND METHODS

Animal

Thirty male Sprague Dawley rats (8 weeks old, 180–200 g) were obtained from the Theodor Bilharz Research Institute, Imbaba, Egypt, and housed in the animal facility of the Faculty of Medicine, Menoufia University, Egypt. The rats were housed in standard polycarbonate cages with two rats in each cage under standard laboratory settings (22 ± 5 °C, 60 ± 5% humidity, and a 12-h/12-h light/dark cycle). Standard laboratory chow and tap water were provided ad libitum. All experimental procedures involving animals were approved by the Institutional Review Board of Ajman University, UAE (IRB# M-F-A-14-Mar), and the Institutional Review Board of Menoufia University, Faculty of Medicine, Egypt (IRB# 191219ANAT), and were conducted in accordance with the guidelines on the ethical use of animals in the European Community Council Directive 2010/63/EU.

BMMSC isolation and culture

BMMSCs were obtained from 6–8-week-old male Sprague Dawley rats, as previously reported [43]. Briefly, bone marrow plugs were harvested from the femurs and tibias of rats using a 23-gauge needle and centrifuged for 5 min at room temperature at 1800 rpm. The cell pellets were then resuspended in Dulbecco's modified Eagle's medium (Gibco, Carlsbad, CA, USA) containing 10% foetal bovine serum (FBS) (Gibco) and 1% penicillin-streptomycin (Gibco) and seeded at a density of 1×10^6 cells/cm in 25 cm^2 cell culture flasks. Cells were incubated at 37 °C in a humidified atmosphere containing 5% CO_2. The culture medium was changed every 3–4 d to eliminate non-adherent haematopoietic cells. When the cells reached 70% confluence, they were harvested for 2–5 min using 0.25% Trypsin–EDTA (Sigma-Aldrich, St. Louis, MO, USA), neutralised with a complete medium, and centrifuged at 500 × g for 5 min. Cell pellets were resuspended in a complete medium. Cell viability was examined by adding equal volumes of the cell suspension and 0.4% trypan blue (Gibco) and loading 10 μL of the stained suspension into each chamber of a haemocytometer. Viable and dead cells

were counted within 5 min of the sample preparation. Cells with greater than 90% viability were subcultured at a 1:3 ratio (passage 1). The cells were used at passage 4.

Flow cytometry

Cells were resuspended in staining buffer (2% FBS/phosphate buffer solution [PBS]) and surface-stained with fluorescein isothiocyanate-conjugated mouse anti-rat CD44 (BioLegend, UK), FITCH-conjugated mouse anti-rat CD90 (BD Pharmingen, USA), or PE-conjugated rabbit anti-rat CD34 (Abcam, UK) at 4 °C for 30 min. Isotype-matched antibodies served as controls. Cells were analysed using an EPICS XL flow cytometer (Beckman Coulter).

Experimental design

The rats were randomly divided into three groups: control, D-gal-treated, and D-gal + BMMSC-treated (N = 10 in each group). The sample size was calculated using G Power software. D-gal (300 mg/kg; Sigma-Aldrich, St. Louis, MO, USA) was administered subcutaneously to rats in the D-gal and D-gal + BMMSCs treatment groups daily for 8 weeks. Once every two weeks, 1×10^6 BMMSCs labelled with the membrane-bound fluorescent marker PKH26 (Sigma-Aldrich) were intravenously delivered to rats in the D-gal + BMMSCs group.

Behavioural tests

All animals were acclimatised for one week after arrival to behavioural testing. Tests were conducted one week after the last transplantation. The test sessions were conducted between 2 PM and 5 PM. Two observers were present throughout each session and blinded to the experimental conditions.

Open-field test. The open-field test allows the simultaneous evaluation of exploration and locomotion. A box 1 m × 1 m × 50 cm in height was made of wood. The floor of the box was divided into equal areas. Each rat was positioned in the centre of the open-field arena, and the rearing frequency and number of crossings (with both forepaws) were recorded using a

video camera installed 2.5 m above the box for 5 min. The box was then placed in a noiseless room under controlled illumination.

Beam walking test. Beam walking is a test of motor coordination [44]. The rats had to traverse a beam (100 cm long wooden beam, 4 cm wide, and 3 cm tall), which was hung between a start stage at one end and their home cage at the other end at a height of 80 cm, was suspended by two pillars. Foam padding was placed underneath the beam to protect the animals from injury during a fall. A line (20 cm) was drawn at the start of the beam. During the test, the rat was placed within this starting area facing its home cage, and a stopwatch was started upon release of the animal. The timer was halted when all four paws were fully placed on the finishing platform at the other end of the beam. The numbers of footsteps and faults were also recorded.

Measurement of body weight and the brain index

The general appearance of the rats, including behavioural activity, shininess, and hair coat colour, was observed daily. Body weights were assessed weekly. At the end of the experiment, the rats were anaesthetised by intraperitoneal injection of ketamine (90 mg/kg) and xylazine (15 mg/kg), and decapitated. Brains were immediately collected from all rats and weighed. Brain indices were calculated using the following formula: brain tissue weight (mg)/final body weight (g).

Assessment of oxidative stress and antioxidants indices

A spectrophotometre was used to assess the levels of malondialdehyde (MDA) and glutathione (GSH) in brain tissues. To assess the extent of lipid peroxidation, rat striata (100 mg) were homogenised in 1 mL of PBS (pH 7.0), and the MDA concentration was measured [45]. The homogenates were centrifuged after mixing with trichloroacetic acid (20%) at 5000 rpm for 15 min. The supernatants were treated with a 5% thiobarbituric acid solution before boiling in a water bath for 10 min. The absorbance at 532 nm was measured, and the MDA concentration was estimated using a standard curve. The results are expressed in nmol per mg of protein. Ellman's method [46] was used to assess the GSH levels. A solution of dithiobis nitrobenzoate was added to the striatal tissue homogenate and incubated for 1 h. The

absorbance was measured at 412 nm. A standard curve was used to measure the GSH concentration. The findings are expressed in mmol per mg of protein.

Quantitative reverse-transcription polymerase chain reaction

Total RNA was extracted from homogenised striata of rats in each group using RNeasy Purification Reagent (Qiagen, Valencia, CA, USA), according to the manufacturer's protocol. RNA purity was assessed with a spectrophotometre; the wavelength absorption ratio (260/280 nm) was between 1.8 and 2.0 for all preparations. RNA was reverse-transcribed into cDNA using Superscript II (Gibco Life Technologies, Grand Island, NY, USA). Quantitative polymerase chain reactions were run and analysed using a StepOneTM instrument with software version 3.1 (Applied Biosystems, Foster City, CA, USA). The reaction mixtures contained SYBR Green Master Mix (Applied Biosystems), gene-specific primer pair (Table I), cDNA, and nuclease-free water. The cycling conditions were as follows:10 min at 95 °C, followed by 40 cycles of 15 s at 95 °C, and 60 s at 60 °C.

The ABI Prism sequence detection system software was used to analyse the data, and quantification was performed using Sequence Detection Software v1.7 (PE Biosystems, Foster City, CA). Relative target gene expression was calculated using the comparative cycle threshold method [47]. All values were normalised to β-actin mRNA levels.

Immunofluorescence analysis

For immunofluorescence staining, brains were dissected and fixed at 4 °C for 24 h, then cryoprotected in 30% sucrose at 4 °C. Serial sections (40 μm) were cut by a cryostat and stored at –20 °C until use. The sections were incubated in 10 % blocking solution (10% normal goat serum in 0.3% Triton X-100 in PBS) at RT for 1h, then incubated at 4 °C overnight in the primary antibodies rabbit anti-GFAP (1:1000, Abcam, Cat. #ab7260), or rabbit anti-tyrosine hydroxylase (TH) (1:500, Abcam, Cat. #ab112). The sections were then rinsed in PBS and a secondary antibody was applied (1:500, Alexa-488, Cat. #A-11034, Molecular Probes) at RT for 1h. Finally, the sections were rinsed in PBS and mounted in Fluoroshield mounting medium with DAPI (Abcam, Cat. #ab104139).

Quantitative histological assessments

Four non-overlapping images per section were randomly captured from the striatum and SN and analysed for each brain section for each marker. Immunofluorescence images were captured using a Leica DM5500 B/11888817/12 microscope equipped with a Leica DFC450C camera, using a Leica HI PLAN 10/0.25 objective. For each image, the region of interest was the field of view at a magnification of 10x. From at least three sections/rat, immunopositive cells were counted using ImageJ software (National Institutes of Health, Bethesda, Maryland, US) by a manual approach using the plugin/cell counter tool [48] and then averaged per field for each rat. The calculated numbers for the 10 animals/experimental group were used for comparison and statistical analyses. Concerning TH immunoreactive striatal fibres, the immunoreactivity of TH fibres in the striatum was measured by densitometry as described by Febbraro et al. [49]. Photos were converted to grayscale using Image J program (1.51 version; National Institute of Health, Bethesda, MD, USA) and analysed for gray intensity after calibrating the Image J program by assessing the optical density (OD). OD values for the treatment groups are presented as a percentage of the control groups.

Statistical analysis

Data are expressed as the mean ± standard error of the mean. Normal distributions were evaluated using the D'Argostino and Pearson normality tests, and data were analysed using one-way or two-way analysis of variance followed by a post hoc Bonferroni test. Statistical significance was set at $p<0.05$. Statistical analyses were performed using the GraphPad software.

RESULTS

Characterization of BMMSCs

MSCs derived from the bone marrow of Sprague-Dawley rats were spindle-shaped, fibroblast-like cells after 10 days of culture. At passage 4, cells were evaluated by flow cytometry for the expression of CD90, CD44 (mesenchymal cell marker), and CD34 (haematopoietic lineage marker). More than 90% of the cells were CD90+ and CD44+, and

less than 10% were CD34+ (Fig. 1). These results indicated that the cells were mostly non-haematopoietic MSCs.

BMMSC transplantation improved the physical characteristics and brain indices

Rats in the D-gal group showed physical signs of general ageing, such as reduced activity and rough, dull, yellow hair coat with hair loss, whereas rats in the transplanted group exhibited signs of normal activity and smooth, glossy, brightly coloured hair coat, indicating that BMMSC treatment had beneficial effects on D-gal-induced ageing. In the current study, the body weights of rats in the control, D-gal, and transplanted groups were not significantly different (Fig. 2a). However, the brain index was significantly reduced in D-gal-treated rats compared with that in the control rats, whereas the brain index of the BMMSC-treated group was significantly improved compared with that in the aged rats (Fig. 2b), demonstrating that transplanted cells reversed D-gal-induced brain atrophy.

BMMSCs recovered locomotion and motor coordination in D-gal ageing rats

Aged rats showed significantly decreased locomotor activity, as indicated by a significant decline in the number of line crossings compared with those in the control group. This was significantly improved by BMMSC injection, as indicated by the significant increase in the number of line crossings compared to the values in the ageing group (Fig. 3a). In addition, aged rats showed increased rearing, which was significantly reversed in the BMMSC-injected group (Fig. 3b). In aged rats, beam walking tests exhibited a significant increase in crossing time (Fig. 4a), foot fault number (Fig. 4b), and footstep number (Fig. 4c), with a marked reduction in velocity (Fig. 4d), when compared to control rats. These observations demonstrate marked changes in motor coordination during ageing. Interestingly, D-gal + BMMSC-treated rats showed significant improvement in these parameters.

BMMSCs restored DA system function

The impact of age on several DA targets, including receptors, transporters, and relevant enzymes in the striatum, has been reported [50-54]. Gene expression of the main functional components of DA neurones was assessed in the different groups. Gene expression

of TH for dopamine synthesis, vesicular monoamine transporter-2 (VMAT2) for dopamine transport into the vesicle, presynaptic dopamine transporter (DAT), and main postsynaptic receptors, D1 and D2, were downregulated in the striatum of aged rats compared to control rats (Fig. 5). These declines in DA markers' expression were significantly prevented in the striatum of the D-gal + BMMSCs group.

BMMSCs differentiated into TH-positive cells and astrocytes, and protected DA neurones in the D-gal ageing brain

To examine the underlying mechanisms by which BMMSCs improve motor deficits and restore functional DA system alterations, we first examined whether systemically administered BMMSCs homed to and survived in the brains of transplanted rats. PKH-labelled BMMSCs were extensively found in all brain regions in the transplanted group. Studies have shown that age-related changes in DA neurones are comparable to the pathogenic changes observed in PD. A decline in the number of TH-positive cells has been reported in the SN of healthy aged subjects. TH staining demonstrated a significant decrease in TH-positive cells in the SN and TH fibre density in the striatum of aged rats compared to that in the controls (Fig. 6). Transplantation of BMMSCs significantly increased the number of TH neurones in the SN and TH fibre density in the striatum compared to that in aged rats. Interestingly, some of the PKH-labelled BMMSCs co-expressed TH in SN, indicating their differentiation into DA neurones. Furthermore, in the striatum, approximately 20% of the PKH-labelled BMMSCs co-expressed GFAP, indicating their differentiation into astrocytes (Fig. 7).

BMMSCs induced antioxidative effects and restored neurotrophic factors

The modification of neuronal and neurotransmitter functions that accompanies ageing seems to be linked to oxidative stress. The specific susceptibility of SN neurones to ageing accumulated reactive oxygen species that may be the cause of the age-associated reduction in DA and motor function in elderly rats [17,18]. Aged rats had higher levels of MDA, an index of lipid peroxidation, in the striatum than control rats. Furthermore, GSH levels were significantly downregulated in aged rats compared with those in control rats. In D-gal +

BMMSC-treated rats, MDA levels significantly declined, whereas GSH levels were upregulated when compared with the levels in aged rats (Fig. 8a and b). Neurotrophic factors, such as BDNF and GDNF, are crucial for the synaptic activity, survival, and function of DA neurones, and their decline has been linked to motor deficits associated with aged animals [68-75]. In the striatum of aged rats, the expression of BDNF, GDNF, and VEGF was remarkably reduced when compared with the expression in control rats, and these declines were reversed by BMMSC transplantation (Fig. 8c).

DISCUSSION

The most important risk factor for PD is ageing, and progressive motor and coordination deterioration associated with normal ageing has been linked to nigrostriatal degeneration. Several studies have shown that the DA system, both structurally and functionally, is affected during normal ageing [1-8]. The capability of stem cells to replace lost or malfunctioning cells has been the focus of recent research [36-42]. This study is the first to examine the potential beneficial effects of systemic transplantation of BMMSCs on the DA system in a D-gal-induced rat model of brain ageing. In this study, we demonstrated that intravenous transplantation of BMMSCs into D-gal-aged rats ameliorated behavioural deficits, restored DA dysfunction, differentiated into TH-positive cells in the SN, protected TH immunoreactivity in the aged striatum, and induced antioxidative and neurotrophic effects. These data demonstrate the therapeutic effectiveness of BMMSCS in the aged brain.

Ageing is characterised by a gradual decline in locomotion and motor coordination. In our study, BMMSCs improved motor alterations in D-gal + BMMSCs. Our results are consistent with those of previous studies. Implantation of BMMCs into the striatum of naturally aged rats increased the functional recovery of swimming performance as measured by the Marshall scale for vigour and success, as well as motor coordination as measured by the transverse bridge test [36]. In various animal models of PD, BMMSCs implantation improved motor impairments [25, 29-31, 55,56]. The beam walk test, which examines limb movements, such as accurate stepping, coordination, and precise positioning of the paw, is particularly susceptible to DA depletion [57-59]. In our study, the detected decrease in striatal DA levels resulted in significant motor incoordination in D-gal rats, as indicated by an

increased number of footsteps and foot slips and a significant decline in velocity. Intravenous injection of BMMSCs decreased the number of footsteps and foot faults with an increase in velocity. The observed motor improvements were associated with increased DA and TH levels in the striatum, suggesting that BMMC transplantation improved motor dysfunction in aged rats, possibly through DA upregulation in the striatum.

Several studies have demonstrated the significance of DA signalling in the maintenance of motor function, and that declines in dopamine functional component availability might be responsible for age-related behavioural deficits [50-54]. In the treated group, the gene expression of DA markers demonstrated an overall increase compared to that in aged rats. A study evaluating the striatal implantation of rat adult bone marrow MSCs in a 6-hydroxydopamine rat model of PD reported a partial albeit significant recovery of DA presynaptic markers such as D1, D2, DAT, and VMAT2 in treated animals compared to non-treated ones [60]. Therefore, it can be concluded that the detected improvements in motor and coordination activities might be due to the recovery of the DA system.

The survival, migration, and differentiation capacity of the injected BMMSCs in D-gal-aged rats were investigated to explain the mechanism behind the behavioural and DA functional improvements observed following BMMSC transplantation. Repeated intravenous BMMSC injection resulted in significant cell migration across all brain areas studied. These findings corroborate prior findings. Ageing has been associated with increased blood-brain barrier permeability in both animals and humans, which might be caused by numerous ageing-related processes, including increased oxidative stress and greater microglial activation [61,62]. In our study, a few transplanted cells differentiated into TH+ cells in SN. Substantial evidence suggests that BMMSCs can differentiate into neurones, particularly DA neurones, both in vitro and in vivo [63-67]. Moreover, the nigrostriatal pathway is known to be involved in ageing and PD progression, and the striatum is well established to be the target brain structure for DA projections from the SN. We further assessed whether the DA fibres of the striatum were re-innervated in the transplanted group. Our results revealed that transplantation of BMMSCs significantly increased the number of endogenous TH neurones in the SN and TH fibre density in the striatum compared to that in aged rats. These results suggest endogenous restoration of the host DA system in the SN of transplanted rats. Therefore, we

next sought to investigate the potential paracrine mechanisms mediated by transplanted BMMSCs that could contribute to the endogenous revival of the host DA system.

MSCS can be considered as mini-bioreactors capable of secreting a wide range of cytokines and neurotrophic factors that are crucial in the treatment of neurodegenerative disorders. There is a substantial body of evidence that MSCs express a variety of neurotrophic factors, including nerve growth factor, GDNF, BDNF, insulin-like growth factor-1, and basic fibroblast growth factor at both the mRNA and protein levels [68-70]. Synaptic plasticity, as well as the survival and function of midbrain dopamine neurones, are dependent on BDNF. By comparing BDNF (+/-) with wild-type mice at various ages, the effects of a partial genetic deletion of BDNF on motor activities and DA level measurements were studied. With age, a decrease in BDNF expression becomes more important for DA circuits and associated behavioural performance [71]. Depletion of BDNF leads to declined TH expression in the SN [72]. GDNF is a secretory protein that protects DA neurones both in vitro and in vivo. GDNF treatment increases striatal dopamine levels and potentiates striatal DA fibre regeneration in preclinical animals [73]. As a result, intracranial ectopic administration of GDNF has been attempted in multiple PD clinical studies with promising but equivocal outcomes [74]. We observed decreased BDNF and GDNF expression in D-gal aged rats, and these decreases were remarkably restored in BMMSC-transplanted rats. GDNF is primarily produced by astrocytes in the brain. GDNF expression is increased in astrocytes in the striatal region of PD animal models with DA innervation, reflecting a process of endogenous regeneration [75]. Interestingly, in the present study, 20% of transplanted cells differentiated into astrocytes in the striatum. The biological features of BMMSC production of neurotrophic factors such as GDNF, as well as the ability of these cells to differentiate into astrocytes, indicate their potential for treating age-related neurodegenerative diseases.

CONCLUSIONS

This study demonstrated that intravenous transplantation of BMMSCs prevented locomotion and coordination deficits in a D-gal ageing rat model by restoring DA system function, protecting atrophic DA neurones in the SN, inducing antioxidative effects, and secreting neurotrophic factors. Our study provides proof of principle that the systemic

transplantation of BMMSCs is a potential therapeutic approach for the protection of nigrostriatal changes associated with ageing.

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Table 1. List of primers used in RT-qPCR

Gene name	Gene accession	Primer sequence forward/Reverse 5'→3'
TH	NM_012740.3	TCGGAAGCTGATTGCAGAGA TTCCGCTGTGTATTCCACATG
DAT	NM_012694.2	CCAGCAATTCAGTGATGACATCA CAGCATAGCCGCCAGTACAG
VMAT2	NM_013031	CGC AAA CTG ATC CTG TTC AT 5-AGA AGA TGC TTT CGC AGG TG
D1R	NM_012546.2	GGAGGACACCGAGGATGA ATGAGGGACGATGAAATGG
D2R	NM_012547.1	TGGGTCAGAAGGGAAGG GATGATAAAGATGAGGAGGGT
BDNF	NM_012842	TGTCCGAGGTGGTAGTACTTCATC CATGCAACCGAAGTATGAAATAACC
VEGF	AF062644	GAGGAAAGGGAAAGGGTCAAAA CACAGTGAACGCTCCAGGATT
GDNF	NM_019139.1	CCAGAGAATTCCAGAGGGAA CTTCACAGGAACCGCTACAA
BETA ACTIN	NM_031144	ATTTGGCACCACACTTTCTACA TCACGCACGATTTCCCTCTCAG

Figure 1. Characterization of the BMMSC population. The cell-surface phenotype of the BMMSCs was assessed by flow cytometry using antibodies against CD90, CD44, and CD34. In total, 92.3%, and 92.1% of the cells expressed CD90 and CD44, respectively, whereas only 9.5% expressed CD34.

Figure 2. Body weight (a) and brain index (b) were evaluated in the control, aged (D-gal), and transplanted (D-gal + BMMSCs) rats. $^{**}p<0.01$ vs. control rats; $^{\#}p<0.01$ vs. aged rats. Data are expressed as mean ± SEM. N = 10/group.

Figure 3. Locomotion was evaluated in control, aged (D-gal), and transplanted (D-gal + BMMSCs) rats. Locomotion (horizontal locomotion and vertical rearing) were assessed for 5 min in an open-field test $^{***}p<0.001$ vs. control rats; $^{###}p<0.001$ vs. aged rats. Data are expressed as means ± SEMs. N = 10/group.

Figure 4. Motor coordination was evaluated using a beam walking test in control, aged (D-gal), and transplanted (D-gal + BMMSCs) rats. $^{***}p<0.001$ vs. control rats; $^{#}p<0.05$ and ##p<0.01 vs. aged rats. Data are expressed as means ± SEMs. N = 10/group.

Figure 5. Gene expression of dopaminergic system markers in the striatum in control, aged (D-gal), and transplanted (D-gal + BMMSCs) rats as measured by RT-qPCR. $^{***}p<0.001$ vs. control rats; $^{#}p<0.05$, $^{##}p<0.01$, and $^{###}p<0.001$ vs. aged rats. Data are expressed as means ± SEMs. N = 10/group.

Figure 6. Number of TH positive neurones in the substania nigra and the density of the TH fibres in the striatum in control, aged (D-gal), and transplanted (D-gal + BMMSCs) rats. Scale bar = 500 µm. $^{***}p< 0.001$ vs. control rats; $^{##}p<0.01$ vs. aged rats. Data are expressed as means ± SEMs. N = 10/group

Figure 7. Survival and differentiation of transplanted BMMSCs into TH positive neurones in the substania nigra (a-d) and astrocytes in the striatum (e-h) in the transplanted (D-gal + BMMSCs) group. A number of PKH-labelled BMMSCs (red) (b, f) co-expressed TH (green) (c) and GFAP (green) (g). The insets display the boxed area at a higher magnification. PKH-labelled cells (red) (b, f), TH-positive cells (green) (c), GFAP-positive cells (green) (g), DAPI-stained nuclei (blue), (a, e) and merged images (d, h). Scale bar = 500 µm.

Figure 8. Status of MDA and GSH and gene expression of BDNF, NGF, and VEGF in the striatum of control, aged (D-gal), and transplanted (D-gal + BMMSCs) rats. $^{***}p<0.001$ vs.

control rats; $^{\#\#}p<0.01$ and $^{\#\#\#}p<0.001$ vs. aged rats. Data are expressed as means ± SEMs. N = 10/group.

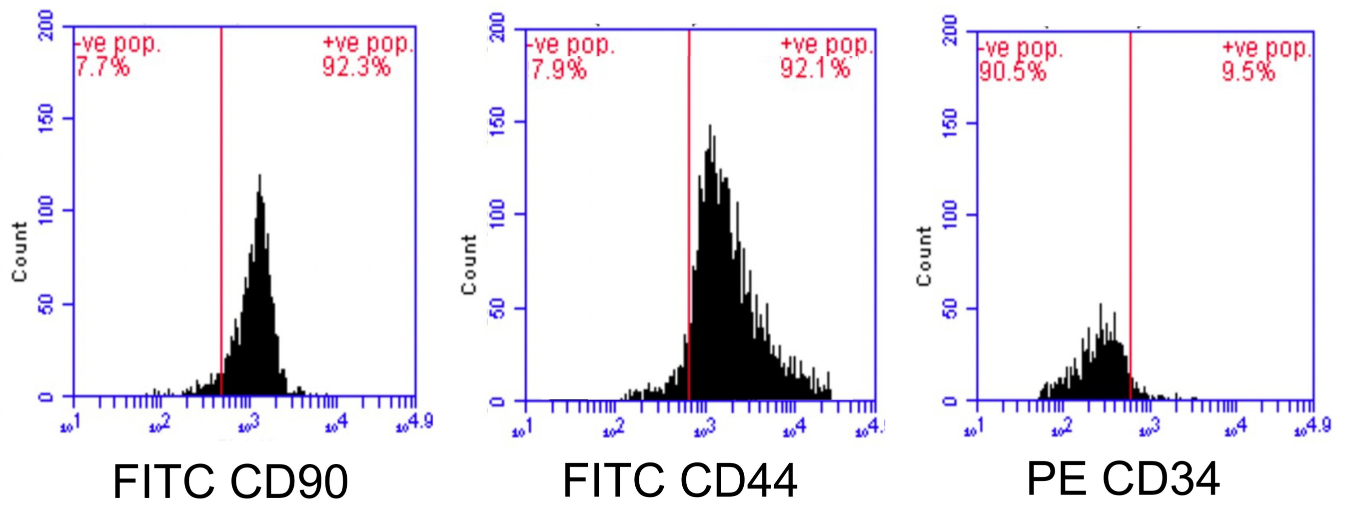
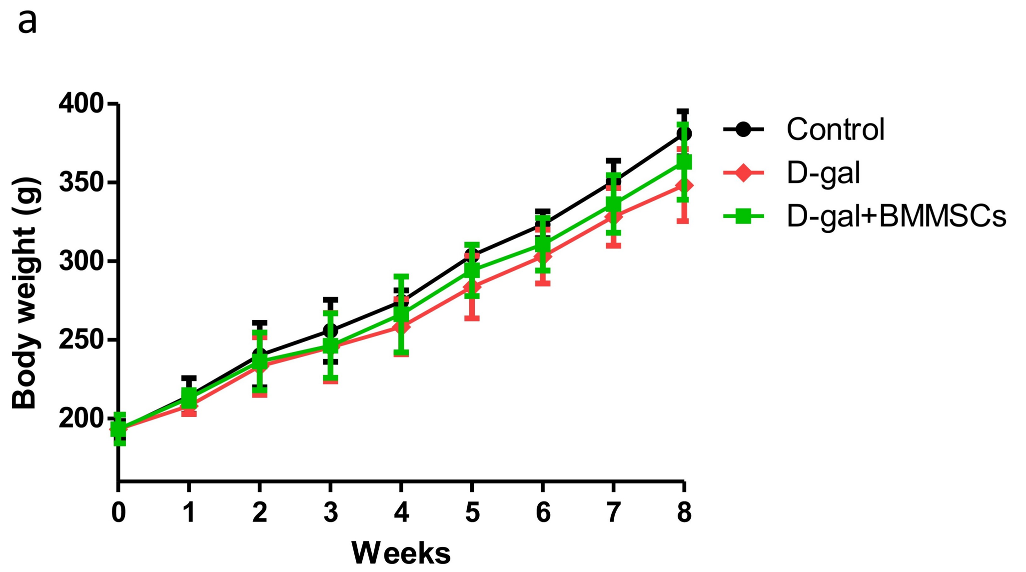


Fig. 1

a



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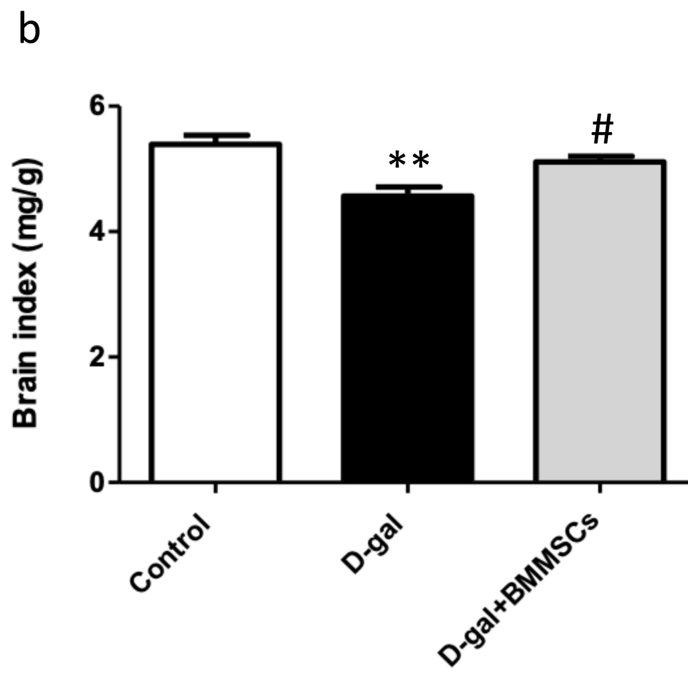
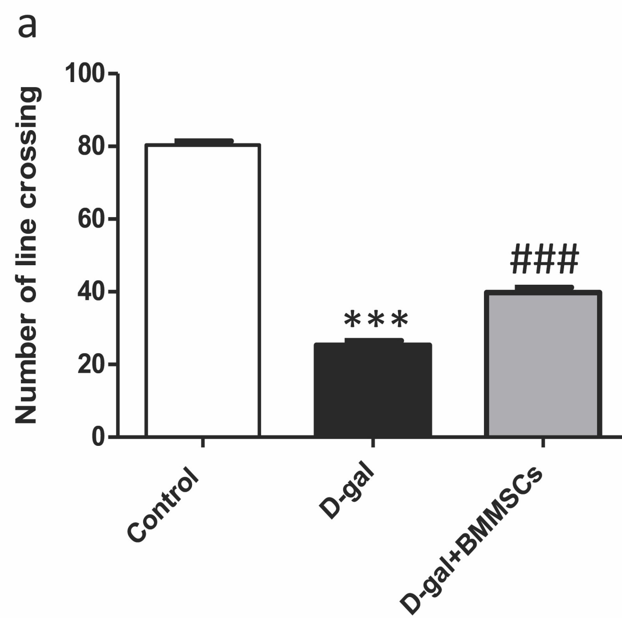


Fig. 2



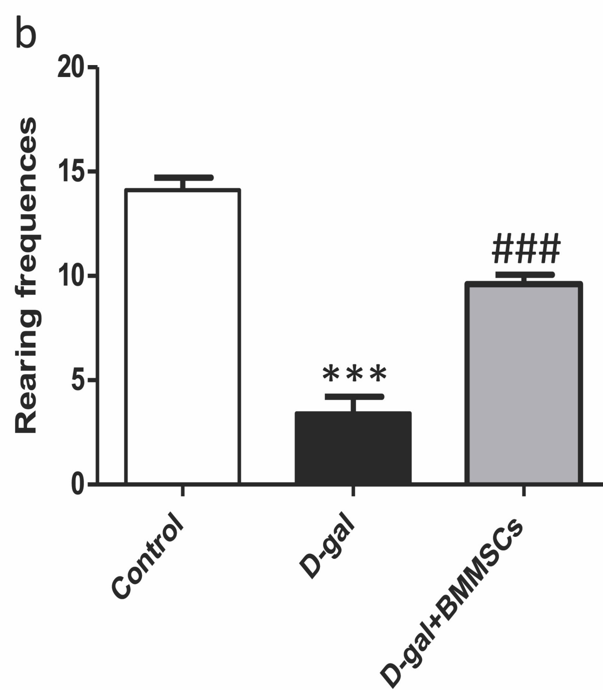


Fig. 3

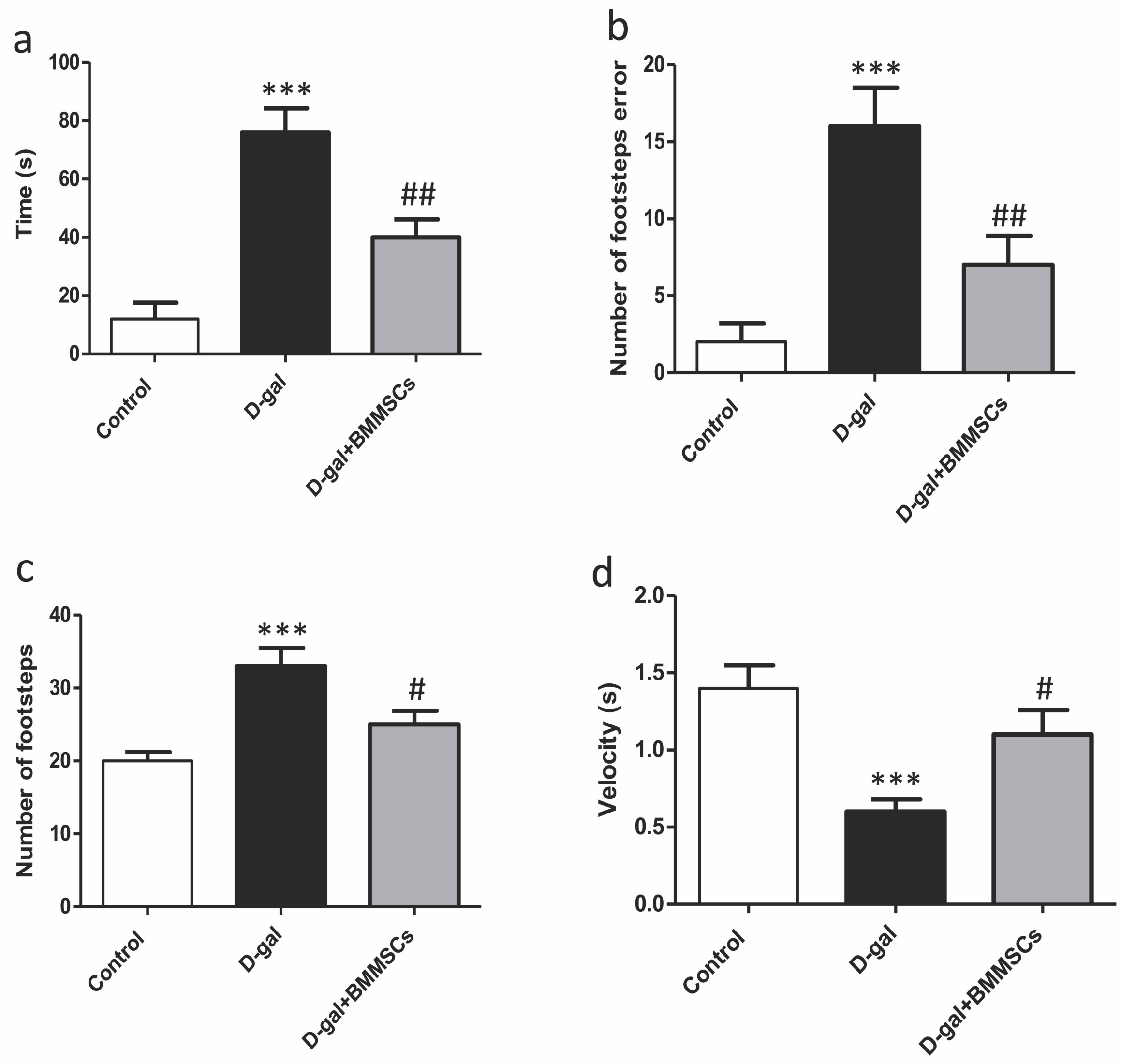


Fig. 4

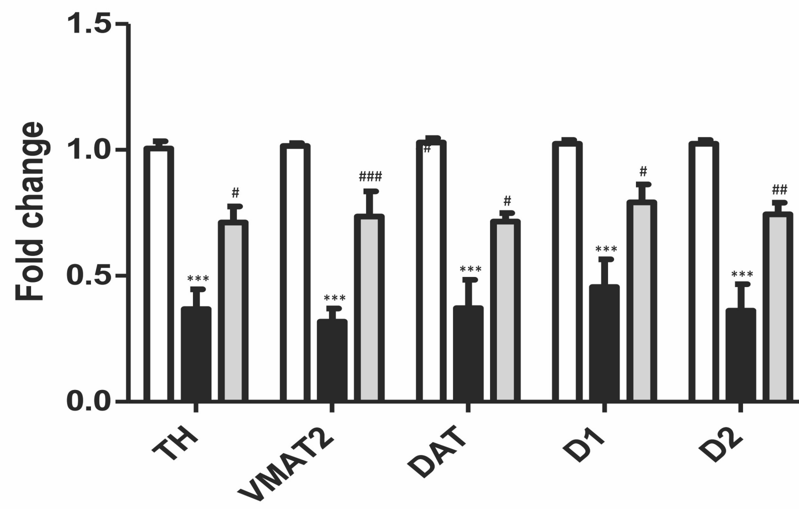


Fig. 5

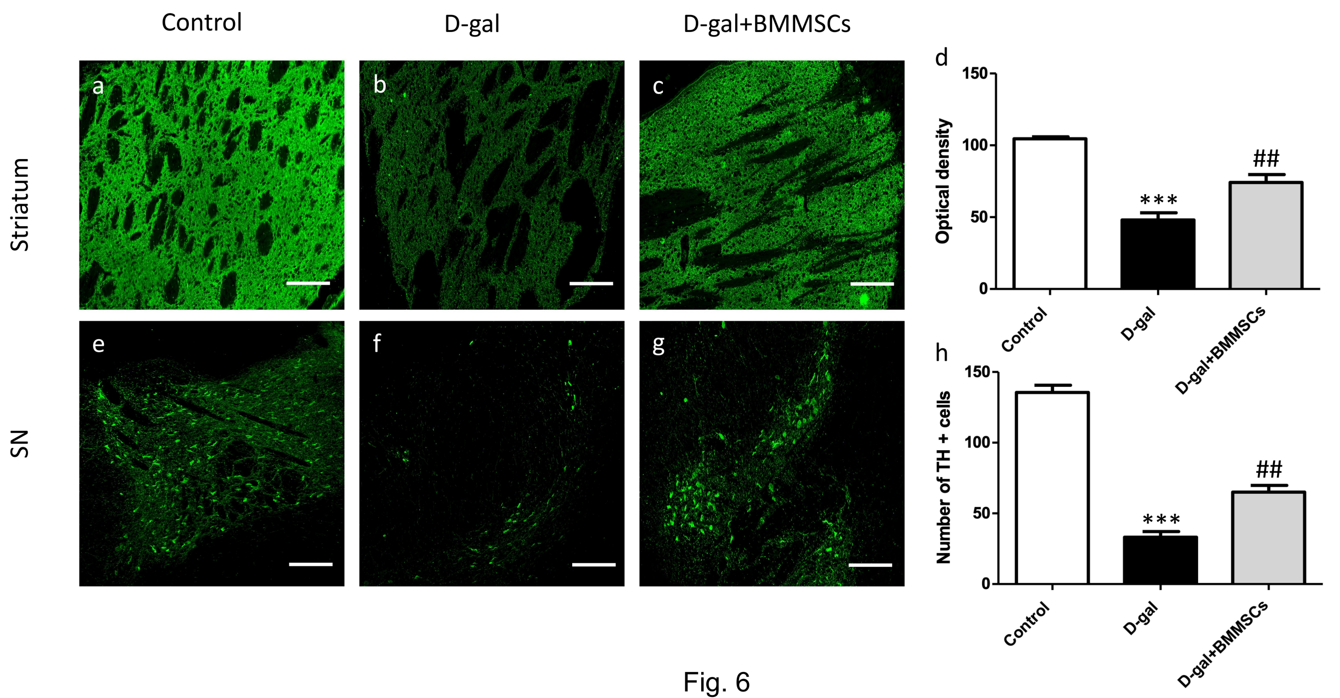


Fig. 6

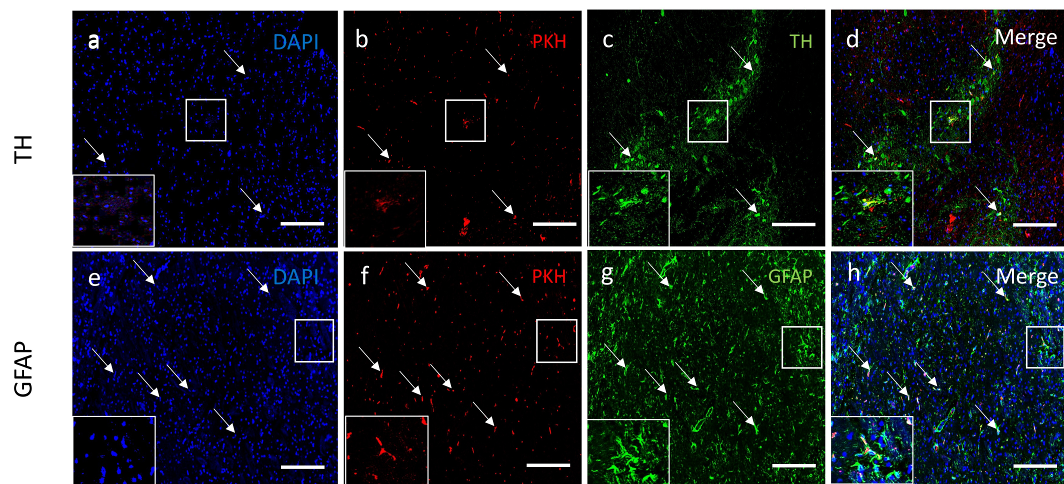


Fig. 7

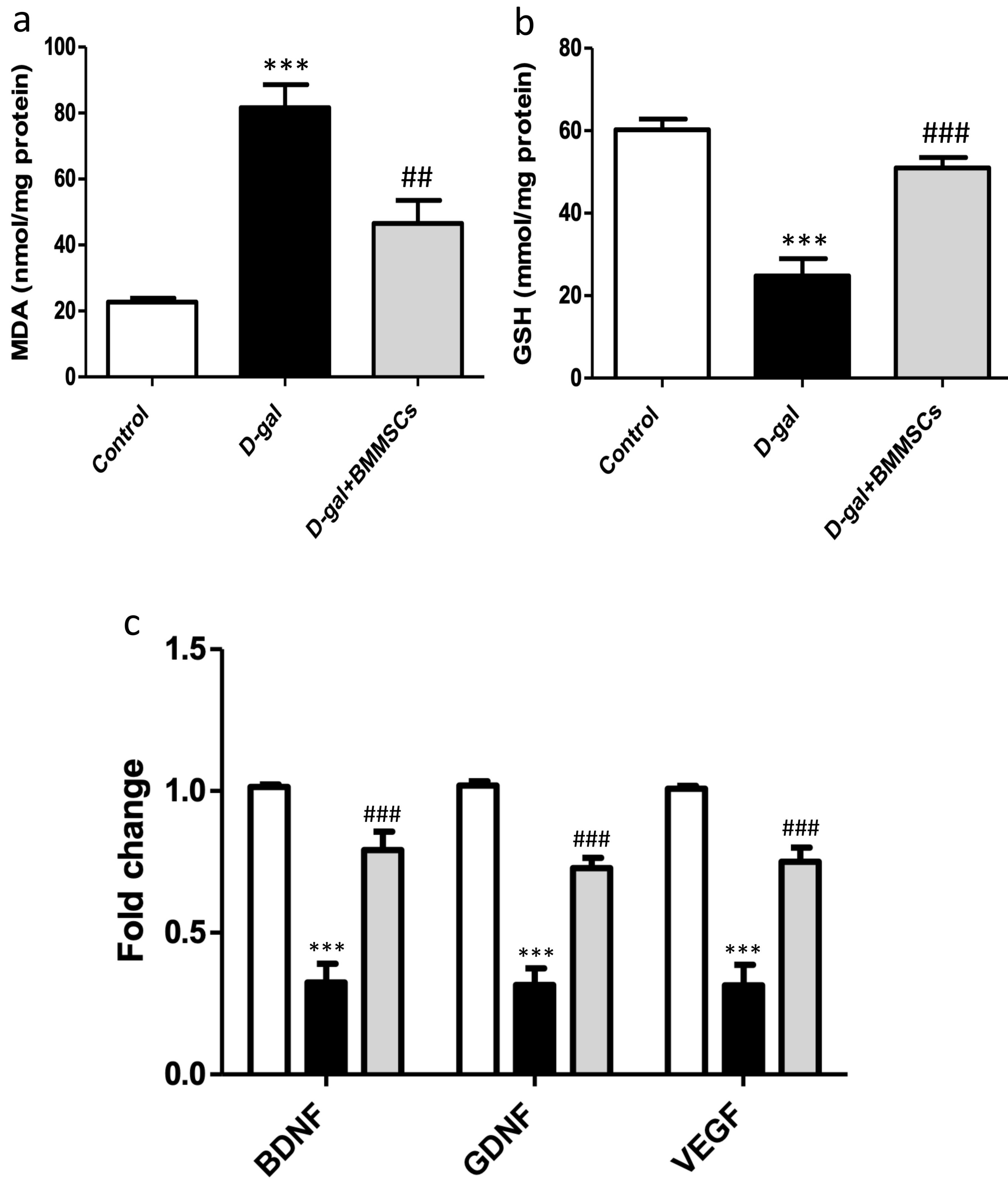


Fig. 8