Heliyon 6 (2020) e05833

Contents lists available at ScienceDirect

Heliyon

journal homepage: www.cell.com/heliyon

Research article

Prolonged lifespan in a spontaneously hypertensive rat (stroke prone) model following intravenous infusion of mesenchymal stem cells

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ARTICLE INFO

Keywords:
Mesenchymal stem cell
Cellular therapy
Lifespan

ABSTRACT

Intravenous infusion of mesenchymal stem cells (MSCs) has been reported to provide therapeutic efficacy via microvascular remodeling in a spontaneously hypertensive rat. In this study, we demonstrate that intravenous infusion of MSCs increased the survival rate in a spontaneously hypertensive (stroke prone) rat model in which organs including kidney, brain, heart and liver are damaged during aging due to spontaneous hypertension. Gene expression analysis indicated that infused MSCs activates transforming growth factor-β1-smad3/forkhead box O1 signaling pathway. Renal dysfunction was recovered after MSC infusion. Collectively, intravenous infusion of MSC may extend lifespan in this model system.

1. Introduction

vAging is a well-known risk factor for vascular-related pathologies [1, 2, 3] and the vascular-related diseases are also associated with increased risk of death [4, 5]. For example, structural and functional changes in small arteries are observed during normal and accelerated aging [6]. Such changes are associated with a higher risk for adverse health outcomes [7]. Small vessel disease affects highly vascular-rich organs including the kidney [8], brain [9], heart [9] and liver [10], and have a negative effect on the quality of life while also shortening life expectancy. However, the mechanisms that underlie the association between vascular-related diseases and high mortality are not fully understood and there are little effective evidence-based therapies to improve life expectancy in the older patients with vascular disease.

Intravenous delivery of bone marrow-derived mesenchymal stem cells (MSC) has been reported to have therapeutic effects on small vessel diseases via microvascular remodeling in several animal models [11, 12]. MSCs have been proposed as a treatment for diseases of the kidney [13], brain [11], cardiac system [14], and liver [15]. Therefore, intravenous infusion of MSCs could be a promising candidate for repairing systemic vascular dysfunction to improve life expectancy in patients with vascular disease.

In this study, we investigated whether intravenous infusion of MSCs has a capability to prolong the life span of rats in a model of small vessel disease. This rat model presents with severe hypertension and multi-system organ failure as a consequence of microangiopathy, with progressive involvement of the kidney [16, 17], brain [18], heart [17] and liver [19] throughout the aging process [16, 17]. Gene expression analysis was performed to elucidate the underlying mechanisms of MSC treatment in extending the lifespan of rats with small vessel disease.

2. Methods

2.1. Animals

The use of animals in this study was approved by the Animal Care and Use Committee of Sapporo Medical University, and all procedures were carried out in accordance with institutional guidelines. Male spontaneously hypertensive rats (stroke prone) (n = 66) and Wistar Kyoto (n = 17) rats (control), used in this study at 21 weeks of age. SHR/Izm (SHRSP/Izm, WKY/Izm, etc.) are provided (supplied) from the Disease Model Cooperative.

Research Association, Kyoto, Japan. Model rats were developed by the selective crossbreeding [20] to produce rats with systemic hypertension [18, 21, 22], causing multi-organ failure. The animals used in this

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https://doi.org/10.1016/j.heliyon.2020.e05833
Received 4 July 2020; Received in revised form 21 October 2020; Accepted 21 December 2020
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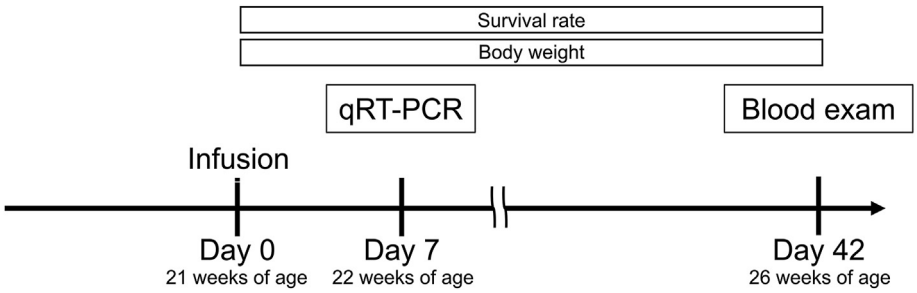

Figure 1. Experimental protocol is shown. MSC: mesenchymal stem cell; qRT-PCR: quantitative reverse transcription-polymerase chain reaction.

study took the AIN-76A rodent diet (Na: about 2.86g/100g, Oriental Yeast Co., Ltd., Tokyo Japan) until one day before infusion. Then, we changed to the MF (Na: 0.19g/100g, Oriental Yeast Co., Ltd.) during the study period. All methods and data in this study were reported in accordance with guidelines provided by Animals in Research: Reporting in Vivo Experiments (Kilkenny et al., 2010). All evaluations were performed by multiple independent observers who quantified the results to avoid experimental bias.

2.2. Experimental protocols

The overall experimental outline is shown in Figure 1. The inclusion criteria of animals were consistent with our previous study [11]. Rats were randomized into two experimental groups: vehicle- and MSC-infused groups. On day 0 (21 weeks of age), rats in the MSC-infused group were infused intravenously with MSCs (1.0×10^6 cells each) in 1 ml of fresh Dulbecco's modified Eagle's medium (DMEM) (Sigma, St. Louis, MO, USA). Rats in the vehicle-infused group were infused with DMEM only at day 0. All intravenous infusions were administered through the left femoral vein. Beginning one day before infusion, rats were administered cyclosporine A (10 mg/kg, i.p.) and amlodipine (10 mg/kg, p.o.) daily [11]. After infusion, we evaluated the survival rate and physiological changes of the rats. The physiological data, including body weight and blood pressure, were measured 2 days prior to the infusion of vehicle or MSC and at weekly intervals thereafter until 42 days after the infusion. Blood pressure was recorded using a tail-cuff microsensor device (model MK-2000A; Muromachi KIKAI, Tokyo, Japan) [23, 24]. Quantitative reverse transcription-polymerase chain reaction (qRT-PCR) was conducted on day 7 (22 weeks of age), and a blood exam was performed to evaluate renal function on day 42 (26 weeks of age).

2.3. Preparation of mesenchymal stem cells from rat bone marrow

The preparation and culture of MSCs was conducted as previously published [25]. Briefly, rat bone marrow, obtained from the femoral bone of adult, 6–8 week old Wistar rats, was diluted in 15 ml of DMEM supplemented with 10% heat-inactivated fetal bovine serum (Thermo Fisher Scientific Inc., Waltham, MA, USA), 2 mM l-glutamine (Sigma), 100 U/ml penicillin, and 0.1 mg/ml streptomycin (Thermo Fisher Scientific Inc.). Samples were then incubated for 3 days at 37 °C in a humidified atmosphere containing 5% CO_2. When the cultures had almost reached confluence, adherent cells were detached with a trypsin-ethylenediaminetetraacetic acid solution (Sigma) and sub-cultured at 1×10^4 cells/ml of medium. After three passages, MSCs were used for the present study. A previous phenotypic analysis of surface antigens revealed MSCs were cluster of differentiation (CD) 45-, CD73+, CD90+, and CD106- [26].

2.4. Quantitative reverse transcription-polymerase chain reaction

Animals (n = 5/group) were sacrificed at day 7 (22 weeks of age) under deep anesthesia with ketamine (75 mg/kg, i.p.) and xylazine (10 mg/kg, i.p.), and the kidney, brain, heart, and liver were removed. The tissues were stored at −80 °C until further use. After homogenization, total RNA was purified using the RNeasy Plus mini kit (QIAGEN, Venlo, The Netherlands). RNA quality was assessed using the Bioanalyzer RNA 6000 Nano kit (Agilent Technologies, Santa Clara, CA, USA). Samples with an RNA integrity number >8.0 were used in this study. The Super Script® $VILO_{TM}$ cDNA Synthesis Kit (Invitrogen, Carlsbad, CA, USA) was used for reverse transcription. Approximately 100 ng of mRNA was used for qRT-PCR analysis. TaqMan® Universal Master Mix II with Uracil-N glycosylase (UNG) and specific sets of primers and TaqMan probes were purchased from Thermo Fisher Scientific Inc (Table 1). qRT-PCR analysis was performed in triplicate using PRISM7500 with 7500 software v2.3 (Thermo Fisher Scientific Inc.). Thermal cycling was carried out at 50 °C for 2 min and 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. The delta cycle threshold (Ct) (ΔCT) was calculated against the endogenous control (*Gapdh*), and the delta-delta Ct (ΔΔCT) was calculated against the ΔCT of the vehicle-treated samples. Fold change (FC) was calculated using the comparative Ct method [27].

2.5. Measurement of BUN and creatinine

Serum blood urea nitrogen (BUN) and creatinine (Cre) concentrations were measured using the urease glutamate dehydrogenase method and the enzymatic method, respectively, with commercially available kits on a Hitachi 7180 Autoanalyzer (Hitachi Ltd., Tokyo, Japan).

2.6. Statistical analysis

All statistical analyses were performed using JMP 12.2 for Windows (SAS Institute Inc., Cary, NC). Survival was analyzed using a standard

Table 1. PCR primer sequences used in this study.

Primer	TaqMan gene expression assay number	GenBank accession number
Forehead Box O1 (FOXO1)	Rn01494848_m1	NM_001191846.2
Transforming growth factor (TGF)-β1	Rn00572010_m1	NM_021578.2
TGF- β Receptor 1	Rn00688966_m1	NM_012775.2
Smad3	Rn00565331_m1	NM_013095.3
Glyceraldehyde-3-phospate dehydrogenase (GAPDH)	Rn01775763-g1	NM_017008.4

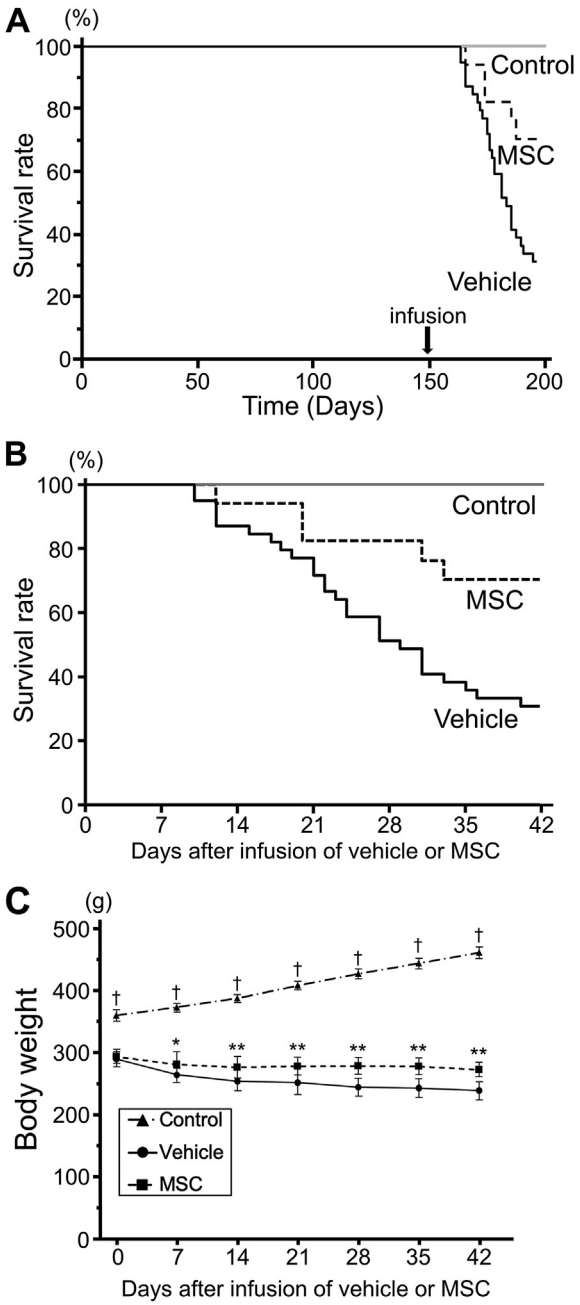

Figure 2. Survival rate and body weight. (A) Comparison of survival rates in the vehicle-infused group, the mesenchymal stem cell (MSC)-infused group, and control. (B) The lifespan after infusion of vehicle or MSC. (C) Body weight of all survived rats. MSC: mesenchymal stem cell; †: significant differed of $p < 0.01$ compared with vehicle- and MSC-infused group; *, **: significant difference of $p < 0.05$ and $p < 0.01$ compared with vehicle-infused group.

Kaplan– Meier analysis with a Mantel–Cox log-rank test. Continuous data were assessed for normality using the Shapiro-Wilk test. Normally distributed continuous data were analyzed by one-way analysis of variance with the Tukey-Kramer post-hoc test. P-values < 0.05 were considered statistically significant. All data are presented as mean ± SD.

3. Results

3.1. Intravenously delivered MSCs extend the lifespan

The survival rate of rats in this model declined rapidly after 22 weeks of age (Figure 2A). Therefore, we intravenously infused the rats with MSCs (n = 17) or vehicle (n = 39) at 21 weeks of age and observed the animals for 42 days (until 26 weeks of age). The survival rate at day 42 following infusion was 30.7 % (n = 12/39) in the vehicle infused group and 70.6% (n = 12/17) in the MSC group, respectively. Standard Kaplan-Meier analysis with a Mantel–Cox log-rank test (Figure 2B) showed that the expected average lifespan of MSC-infused group (183 ± 2.4 days) was significantly longer than the vehicle-infused group (176.1 ± 1.8 days). The estimated lifespan after infusion of MSCs or vehicle (Figure 2B) indicates that the MSC-infused group (36.5 ± 2.4 days after infusion) could live longer than the vehicle-infused group (29.1 ± 1.8 days after infusion) (P = 0.011). These results demonstrate that intravenous infusion of MSCs extends lifespan in this model system.

The body weight of all survived rats (MSC: n = 12, vehicle: n = 12) was also analyzed weekly after infusion of vehicle or MSC. The body weight of the spontaneously hypertensive rats (stroke prone) declined gradually, although the body weight of age-matched control rats was increased with age (Figure 2C). No significant difference was noted in body weight between rats in the vehicle-infused group and the MSC-infused group before infusion. However, it was observed that the progressive weight loss was significantly attenuated in MSC-infused group compared to the vehicle-infused group at days 7, 14, 21, 28, 35 and 42. Thus, the infused MSC group display attenuation in body weight loss in this model system.

Note that before controlling blood pressure, the systolic blood pressure was extremely high in both vehicle (249 ± 34/163 ± 23 mmHg) and MSCs infused groups (250 ± 24/164 ± 37 mmHg). From one day before the vehicle or MSC infusion, all rats received oral administration of amlodipine daily (10 mg/kg, p.o.). After the blood pressure treatment, the systolic blood pressure was dropped in the both vehicle (155 ± 32/93 ± 36mmHg) and MSC infused group (153 ± 30/96 ± 32 mmHg), at 7 days after the infusion of vehicle or MSCs, although there was no significant difference between the groups. At day 14, 21, 28, 35, and 42 after the infusion of vehicle or MSCs, there was also no differences in the average blood pressure (Table.2).

3.2. Activation of TGF-β1-SMAD3/FOXO1 pathway by intravenously delivered MSCs

To analyze the possible mechanism underlying lifespan extension by intravenous infusion of MSCs, gene expression analysis was performed.

Table 2. Blood pressure during study period.

	Systolic blood pressure		Diastolic blood pressure	
	Vehicle	MSC	Vehicle	MSC
Day -2	249 ± 34	250 ± 24	163 ± 23	164 ± 37
Day 7	155 ± 32	153 ± 30	93 ± 36	96 ± 32
Day14	156 ± 18	147 ± 34	100 ± 16	81 ± 31
Day 21	156 ± 26	157 ± 30	94 ± 23	98 ± 24
Day 28	161 ± 27	166 ± 34	91 ± 27	103 ± 33
Day 35	175 ± 23	167 ± 37	104 ± 23	96 ± 23
Day 42	172 ± 31	171 ± 36	100 ± 12	96 ± 19

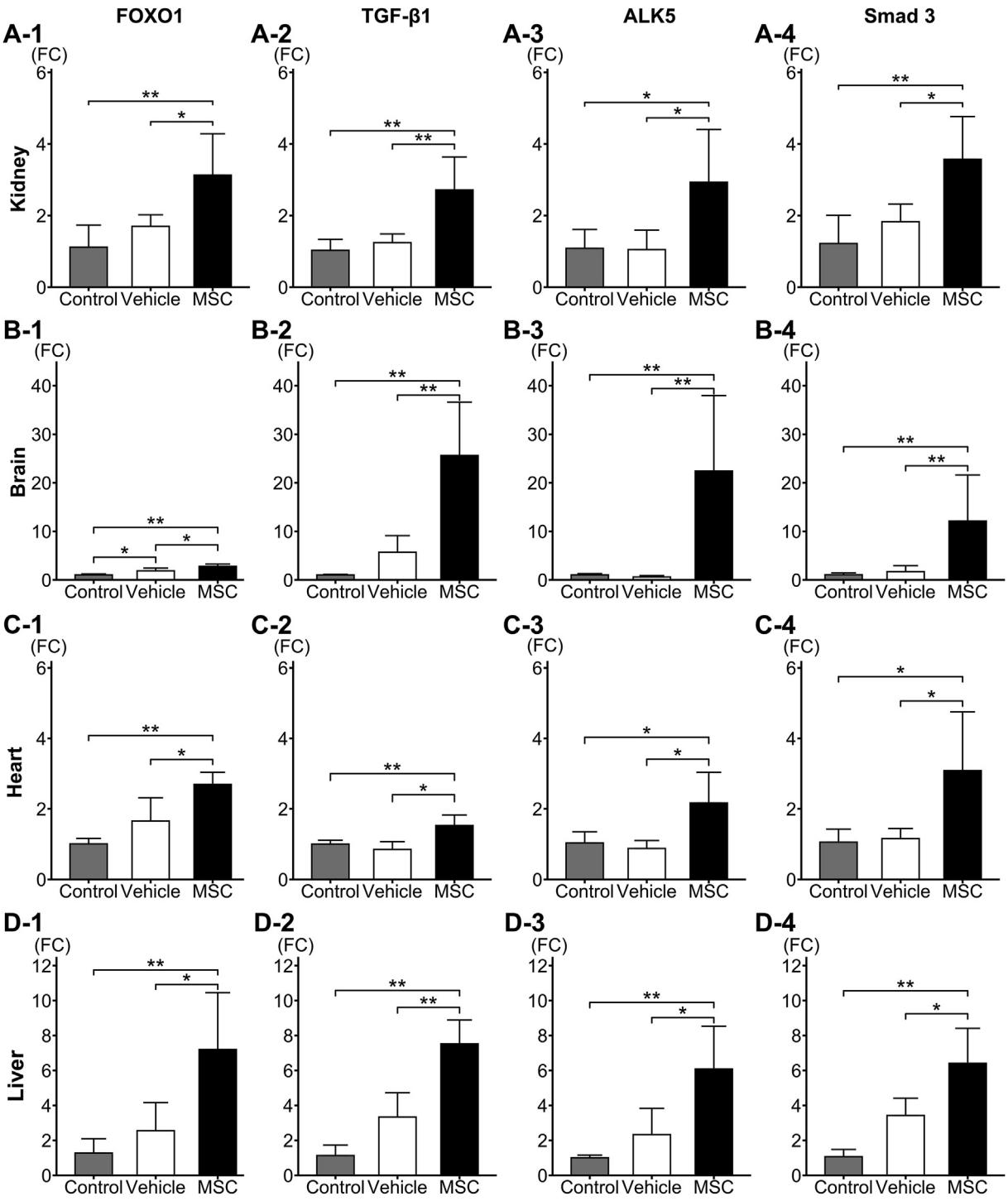

Figure 3. mRNA expression of FOXO1, TGF-β1, TGF-βR1, and Smad3 in the kidney (A), brain (B), heart (C), and liver (D). qRT-PCR: quantitative reverse transcription-polymerase chain reaction; FOXO1: forkhead box O1; TGF-β1: transforming growth factor beta 1; TGF-βR1: transforming growth factor beta receptor 1, *P < 0.05, **P < 0.01.

Previous studies have reported that MSCs exert therapeutic efficacy through activating the TGF-β pathway [11, 28]. Therefore, we evaluated the mRNA expression levels of genes in the TGF-β pathway and key players in lifespan in the kidney (Figure 3A), brain (Figure 3B), heart (Figure 3C), and liver (Figure 3D), of rats from each group (n = 5/group). qRT-PCR analysis showed that the relative mRNA expression of forkhead box O1 (*FOXO1*), which is associated with lifespan, was significantly higher in the brain (Figure 3B1), and trended upward in the kidney (Figure 3A1), heart (Figure 3C1), and liver (Figure 3D1) in the vehicle-infused group compared to the control group. However, the expression of *FOXO1* in the MSC-infused group was significantly elevated in the kidney (Figure 3A1), brain (Figure 3B1), heart (Figure 3C1), and liver (Figure 3D1), compared with the vehicle-infused group. Moreover, while the mRNA expression of *TGFB1*, action receptor-like kinase 5 (*ALK5*, receptor 1 of TGF-β), and *SMAD3* in the vehicle group did not differ from the control group, their expression was significantly elevated in the MSC-infused group in the kidney (Figure 3A2-4), brain (Figure 3B2-4), heart (Figure 3C2-4), and liver (Figure 3D2-4).

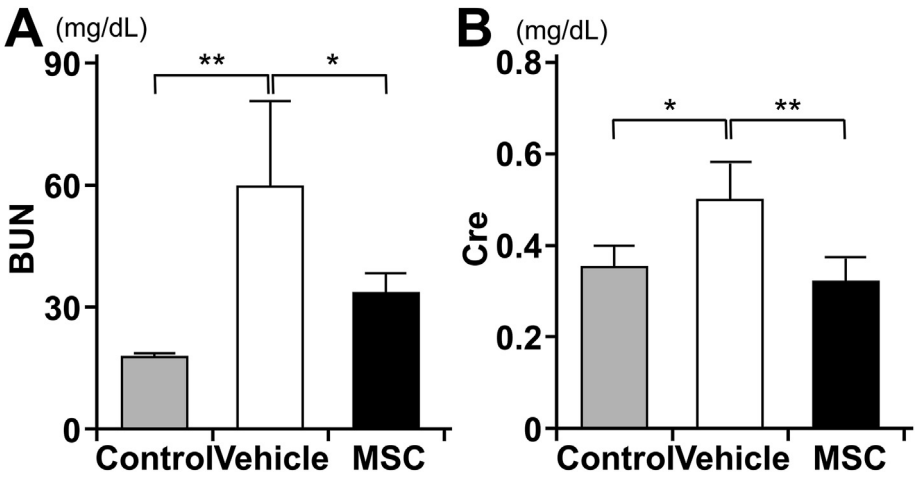

Figure 4. Renal function assessed by blood urea nitrogen (BUN: A) and creatinine (Cre: B) at day 42 after infusion. *P < 0.05, **P < 0.01.

3.3. Improvement of kidney function by intravenously delivered MSC

To investigate whether intravenous infusion of MSCs could prevent the progressive kidney dysfunction, biochemical analysis of the serum was performed at day 42 after infusion of vehicle or MSC. The concentration of both serum BUN (Figure 4A) and Cre (Figure 4B) were elevated in the vehicle-infused group compared to those in the control group. However, MSC-infused rats showed a significantly lower level of both serum BUN and Cre compared to the vehicle-infused group (BUN, Control, 17.6 ± 1.0 mg/dL, vehicle-infused group, 59.5 ± 21.1 mg/dL, MSC-infused group, 33.2 ± 4.7 mg/dL, P = 0.002; Cre, Control, 0.35 ± 0.05 mg/dL, vehicle-infused group, 0.50 ± 0.08 mg/dL, MSC-infused group, 0.32 ± 0.05 mg/dL, P = 0.003). Thus, intravenous delivered MSCs could prevent progressive renal dysfunction in this model system.

4. Discussion

The present study indicates that intravenous infusion of MSCs extends the lifespan of rats with spontaneous hypertension manifesting multi-system end-organ damage. The body weight of rats in the MSC group was higher than that in the vehicle group. Our data suggest that infused MSCs activated the TGF-β-SMAD3/FOXO1 pathway across different tissues (kidney, brain, heart and liver), which could play a role in the MSC-induced prolonged lifespan and contribute to the preservation of renal function. Collectively, the systematic delivery of MSCs could have promising anti-aging effects in this model system.

Intravenous injection of MSCs prolonged the lifespan of rats in this study; however, the underlying molecular mechanisms for this finding have not been elucidated. Here, the expression of genes in the TGF-β1-SMAD3/FOXO1 pathway was activated in multiple organs including the kidney, heart, liver, and brain. TGF-β1 activates TGF-receptor I and II kinases and SMAD transcription factors including SMAD3. The TGF-β1-SMAD3 pathway facilitates the activation of FOXO1 to modulate cell metabolism [29, 30, 31]; this pathway also regulates several biological processes including cell proliferation and death [32].

Although hypertension provokes systemic microvasculature dysfunction in this model system, increased expression of ALK5, Smad 3 following intravenous infusion of MSCs was observed following infused MSCs. The ALK5/Smad 3 pathway might facilitate remodeling of endothelial cells leading to restoration of damaged microvasculature through TGF-β pathway in the vascular-rich organs and may be associated with the promotion of lifespan prolongation (Figure 5A).

The FOXO subfamily has been reported inhibit the life-shortening effects of insulin/insulin-like growth factor-I receptor signaling pathways, which accelerate aging through the suppression of FOXO. Although this pathway is strongly conserved from nematodes through mammals, FOXO has been well investigated in *Caenorhabditis elegans* (*C. Elegans*) and Drosophila which have only one FOXO homologous gene, named DAF-16 and dFOXO, respectively [33, 34]. Stress resistance has been observed in both *C. elegans* and Drosophila with mutated insulin receptor-like transmembrane tyrosine kinases, and is associated with an increase in DAF-16 or dFOXO, respectively. These genes activate several

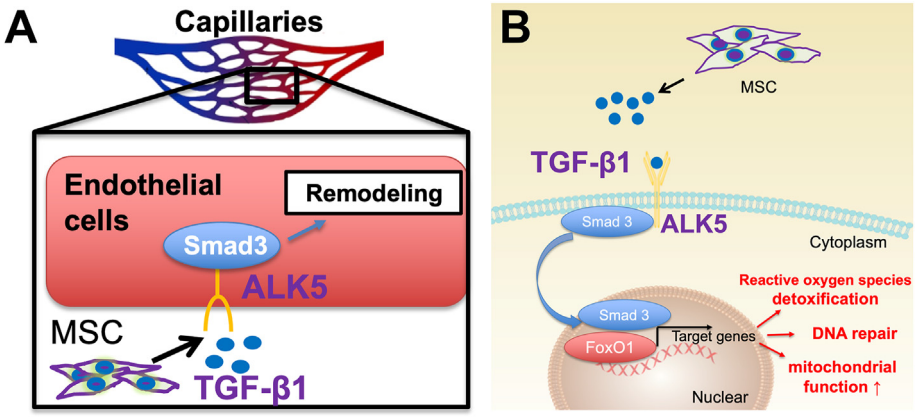

Figure 5. Potential mechanism of intravenous infusion of MSC for prolonged lifespan (A, B). MSC: mesenchymal stem cell; TGF-β: transforming growth factor beta; ALK5: activin receptor-like kinase 5; FOXO1: forkhead Box O1.

enzymes for the detoxification and removal of reactive oxygens species, resulting in a deceleration of aging [34]. Moreover, it was reported that overexpression of dFOXO in the Drosophila fat body represented the functional equivalent of upregulation in the mammalian liver and adipose tissue, and significantly extended the animal's lifespan [35].

Consistent with these findings, the upregulation of *FOXO1* in rats after intravenous infusion of MSCs in this study might be associated with prolonged lifespan. MSC infusion could upregulate the TGF-β1/SMAD3 pathway and FOXO1 in major organs, which could promote lifespan extension in this model. These potential mechanisms are summarized in Figure 5B.

Here, we also observed preserved renal function in MSC-infused animals. Due to low vascular resistance in the kidney, the arterioles are highly susceptible to the endothelial injury of small vessels [36, 37], which is often associated with a high prevalence of chronic kidney disease and poor prognosis among the elderly population [36, 38, 39, 40]. In this study, the concentrations of serum BUN and Cre were elevated in the vehicle-infused group compared to those in the control group, which was also observed in other previous studies [41, 42]. However, MSC-infused rats showed significantly lower levels of both BUN and Cre compared to the vehicle-infused group. Previous studies reported that the systemic infusion of MSCs improved renal function and inhibited the progression to end-stage renal failure in both renovascular hypertension [43] and diabetic nephropathy models [13]. Recent work has also demonstrated the therapeutic effects through diffusible factors including nanoparticle exosomes which contains proteins and micro RNAs in several disease models [44]. Indeed, we recently demonstrated that intravenously delivered exosomes derived from MSCs may mediate at least some of the effects of IV MSC administration in the injured spinal cord [45]. Taken together, intravenous infusion of MSCs could have therapeutic effects on progressive renal dysfunction induced by systemic hypertension that could lead to prolonged lifespan in this model system.

5. Conclusions

Intravenous infusion of MSCs extends lifespan in a spontaneously hypertensive rat (stroke prone) model. Prolonged lifespan could be associated with the activation of the TGF-β-SMAD3/FOXO1 signaling pathway by MSCs.

Declarations

Author contribution statement

Masahito Nakazaki: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Shinichi Oka: Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data.

Masanori Sasaki: Conceived and designed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Yuko Kataoka-Sasaki: Performed the experiments; Analyzed and interpreted the data.

Hiroshi Nagahama: Analyzed and interpreted the data.

Kazuo Hashi: Conceived and designed the experiments.

Jeffery D. Kocsis: Conceived and designed the experiments; Wrote the paper.

Osamu Honmou: Conceived and designed the experiments; Analyzed and interpreted the data; Wrote the paper.

Funding statement

This work was supported by the JSPS KAKENHI (19K11794), the Grants-in-Aid for Regional R&D Proposal-Based Program (H30 S-2–4) from the Northern Advancement Center for Science & Technology of Hokkaido Japan, and the Merit Review Award 1 I01 BX003190 from the US Department of Veterans Affairs (BLRD and the RRD Services).

Data availability statement

Data will be made available on request.

Declaration of interests statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

Acknowledgements

We are thankful to the Disease Model Cooperative Research Association (Kyoto, Japan) for providing the rats (SHRSP/Izm, Wistar Kyoto rats/Izm) for this study.

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