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Original Article

Efficacy of human bone marrow mesenchymal stem cell transplantation in repair of radiation-induced damage to the immune system

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Abstract: Background and objectives: Human bone marrow-derived mesenchymal stem cell (hBMSC) transplantation is a potential therapy for treating radiation damage. We evaluated the ability of hBMSC transplantation to repair radiation damage affecting the immune system of mice. Methods: hBMSCs were isolated from uremic patients and injected into mice after short-term or long-term irradiation. Cellular proliferation and apoptosis were analyzed by flow cytometry. Thymic and splenic expression levels of P53 were characterized by immunohistochemistry (IHC). In addition, murine thymi were histologically analyzed via hematoxylin-eosin (HE) staining. Results: hBMSC transplantation inhibited irradiation-induced cellular apoptosis, promoted thymic and splenic cellular proliferation, and suppressed P53 expression in mice. Histological analysis also showed that hBMSC transplantation repaired extensive necrosis and precancerous thymic lesions. Conclusion: hBMSC transplantation promoted immune cell proliferation and inhibited cellular apoptosis after irradiation. Mice that received hBMSC transplantation recovered rapidly after irradiation without showing signs of precancerous lesions. hBMSC transplantation may have clinical applications for the treatment and repair of tissue damage induced by radiation.

Keywords: Human bone marrow mesenchymal stem cells (hBMSCs), transplantation, radiation damage, P53, immunohistochemistry (IHC), hematoxylin-eosin (HE)

Introduction

Radiation exposure induces mutagenic effects and can cause organ-specific alterations. Radiation sickness is characterized by acute radiation syndrome, which is caused by exposure to a short-term high dose of radiation, or chronic radiation syndrome, which is caused by exposure to long-term low doses of radiation. Generally, acute radiation syndrome appears after exposure to a dose > 1 Gy. There are currently no effective therapeutic treatment options for radiation damage. The recent nuclear crisis that occurred in Japan underscored the need for an effective therapy to treat radiation damage.

The thymic and splenic compartments of the immune system are highly sensitive to radiation. Reports have shown that exposure to radiation remarkably decreased splenic weight and cell numbers [1, 2]. The immune system plays an essential role in the clearance of senescent/damaged cells [3]. Suppression of the immune system may contribute to tumorigenesis after irradiation. Some scholars have proposed that human bone marrow-derived mesenchymal stem cells (hBMSCs) could be used in therapeutic interventions for radiation syndrome [4]. However, the effects of hBMSC transplantation on the repair of radiation damage to the immune system have not been fully evaluated.

Due to their potential clinical applications, many recently published studies have focused on mesenchymal stem cells (MSCs). Bone marrow is the main source of MSCs in clinical studies. MSCs, which maintain stem cell characteristics, are used to improve the healing of injured tissues. The use of MSCs for bone repair has

recently been extensively studied [5-7]. Considering their regenerative properties and migration ability, MSCs are also used to treat dysfunction of other tissues, such as tissues of the nervous system, the cardiovascular system and lunate bone [8-10]. Tissue engineering has rapidly developed in recent years. MSCs constitute a multipotent population that can differentiate into several connective tissues and, thus, can be used in developing suitable materials for tissue engineering [11]. A recent pioneering study suggested that MSCs can serve as an effective alternative to autografts for the regeneration of bone defects [12]. Clinically, MSC transplantations have already been used to treat amyotrophic lateral sclerosis and steroid-refractory graft-versus-host disease (GVHD) [13, 14]. Additionally, injected hBMSCs have the ability to specifically migrate to radiation-damaged tissues [15]. Furthermore, transplantation of MSCs also reduces cellular apoptosis, reduces anti-inflammatory responses and enhances angiogenesis after radiation exposure [16-19]. Thus, MSCs are viewed as a potential therapeutic candidate for treatment of radiation damage.

In the present study, we investigated the effects of hBMSC transplantation on the repair of radiation damage to the immune system. Cellular proliferation, cellular apoptosis, immunohistochemistry and histological analyses revealed that transplantation of hBMSCs not only reduced apoptosis but also induced cellular proliferation in the immune system. Moreover, we provide evidence that the decreased cellular apoptosis due to hBMSC treatment may result from the suppression of P53 expression.

Materials and methods

Animals

NOD/SCID mice aged 6 to 8 weeks and weighing 18 to 20 g were purchased from the Shanghai Laboratory Animal Center, Shanghai. All mice were housed under specific pathogen-free (SPF) conditions. All murine investigations and procedures were performed under a protocol approved by the authors' Institutional Animal Care and Use Committee (IACUC).

Isolation and culture of hBMSCs

Bone marrow was drawn from uremic patients (aged 18-30 years) prior to renal transplantation after having obtained written informed consent and ethical approval. Bone marrow was centrifuged to collect cells. Cells were resuspended in low-glucose Dulbecco's modified Eagle's medium (DMEM-LG; Gibco, USA) containing 10% fetal bovine serum (FBS; Gibco, USA). Cells were then incubated at 37°C in a humidified atmosphere containing 5% CO_2 and fed every three days until reaching confluence. The adherent cells were recovered using 0.25% trypsin (Gibco, USA) and reseeded.

Irradiation of animals

For the short-term irradiation study, mice were randomly assigned to a normal group, a control group and a treatment group (5 mice in the normal group, 20 mice in the control group and 20 mice in the treatment group). Mice in the control and treatment groups underwent sodium pentobarbital-induced anesthesia and were exposed to 5.5 Gy of 60 Coγ ray with an exposure dose rate of 3.99×10^{-2} c/(kg·min) at an irradiation distance of 4 m. Mice in the normal group were sham irradiated. Half of the exposed mice were assigned to the control group and received a caudal intravenous injection of 0.2 ml physiological saline. The remaining exposed mice were assigned to the treatment group and received a caudal intravenous injection of 1×10^8 hBMSCs resuspended in 0.2 ml physiological saline.

For the long-term irradiation study, mice were randomly assigned to a normal group, a control group or a treatment group (5 mice in the normal group, 30 mice in the control group and 30 mice in the treatment group). Mice in the control and treatment groups underwent sodium pentobarbital-induced anesthesia and were exposed to 1.75 Gy of 60 Coγ ray once per week. After four irradiation exposures, control mice received a caudal intravenous injection of 0.2 ml physiological saline immediately after irradiation, and a second injection was performed one week after the final irradiation. The treatment group was treated similarly to control animals except with 0.2 ml physiological saline containing 10^8 hBMSCs injected intravenously rather than saline alone.

Cellular proliferation and apoptosis assay

Mice were sacrificed by cervical dislocation. The thymus and spleen of each mouse was dissected and immersed in RPMI-1640 medium

Table 1. Cell cycle phase distribution of thymocytes after short-term irradiation

Groups	*Time course*	*G0-G1 (%)*	*S (%)*	*G2-M (%)*
Normal		78.50±3.20	16.75±3.90	4.75±1.10
Radiation control	6 h	88.45±5.32	6.47±1.23	5.08±1.21
	12 h	90.16±3.56	5.38±1.02	4.46±2.10
	24 h	82.82±2.64	12.93±4.11	4.30±1.42
	48 h	67.60±5.23	27.73±3.45	4.67±1.54
hBMSC treatment	6 h	86.13±4.55	8.44±2.02	5.43±1.52
	12 h	93.38±3.46#	3.89±1.44	2.73±1.22
	24 h	75.28±4.13#	19.16±2.15#	6.57±1.54
	48 h	57.59±3.66#	42.43±4.73#	4.18±2.02

#P < 0.05 compared with the radiation control group. The data were analyzed using Student's t-test.

Table 2. Cell cycle phase distribution of spleen cells after short-term irradiation

Groups	*Time course*	*G0-G1 (%)*	*S (%)*	*G2-M (%)*
Normal		74.83±5.67	18.08±4.05	6.09±0.88
Radiation control	6 h	85.86±5.76	6.46±2.12	7.69±1.46
	12 h	83.69±3.51	8.23±1.96	7.08±1.64
	24 h	78.72±3.46	12.51±2.32	8.77±1.44
	48 h	52.38±5.03	42.74±4.68	4.88±1.24
hBMSC treatment	6 h	89.24±4.26#	4.60±1.36	6.16±2.12
	12 h	84.41±3.13	7.70±1.66	7.90±1.72
	24 h	68.56±2.57#	25.76±3.55#	5.68±2.11
	48 h	40.98±3.55#	46.94±3.89#	12.08±2.46#

#P < 0.05 compared with the radiation control group. The data were analyzed using student's t-test.

(Gibco, USA). After cutting into pieces, the tissues were crushed through 100-μm cell strainers (BD Pharmingen) to obtain single-cell suspensions. The cells were then washed with PBS and treated with red-blood cell (RBC) lysis buffer (Applygen, China) to lyse RBCs. Cells were collected by centrifugation and resuspended, followed by cell counting. In total, 1×10^6 cells were resuspended in PBS containing 1.5% new-born bovine serum (Gibco, USA) and 70% ethanol. The aqueous solution was fixed at -20°C for 24 h, followed by treatment with RNase solution (200 μl, 1 mg/ml) at 37°C for 30 min. The aqueous solution was incubated with propidium iodide (PI) dye (2 μg/mL) (BD Biosciences, USA) at 37°C for 20 min and then analyzed using a LSRII flow cytometer.

Determination of P53 protein expression

Thymic and splenic tissues were dissected and fixed in a 4% paraformaldehyde solution. The tissues were embedded in paraffin and cut into 5-μm-thick sections. Slides with deparaffinized sections were incubated for 5 min in a 3% hydrogen peroxide solution to quench endogenous peroxidase activity. Antigen retrieval was performed by microwaving for 20 min. Anti-P53 mouse antibody was used as the primary antibody (Santa Cruz, USA). HRP-conjugated goat anti-mouse secondary antibody was supplied by Santa Cruz Co. Ltd., USA. Immunoreaction was visualized by using 3, 3'-diaminobenzidine (DBA) (DAKO, Denmark).

Histological analyses

Mice were sacrificed via cervical dislocation, and thymic tissues were removed and fixed in Bouin's solution. After dehydration using a graded series of ethanol solutions, samples were embedded in paraffin, sectioned serially at 5 μm thickness and mounted on slides. The slides were stained with hematoxylin-eosin (HE).

Statistical analysis

Statistical analysis was performed using SPSS 13 (SPSS Inc., USA). All data were expressed as the mean ± S.D. and analyzed using Student's *t* test with significance considered for $P < 0.05$.

Results

Increased cellular proliferation in the thymus and spleen after hBMSC treatment

To investigate the effect of hBMSCs on the repair of radiation damage to the immune system, we first examined cellular proliferation in the thymus and spleen. The cell cycle stage of thymocytes and spleen cells was determined by flow cytometry at 6 h, 12 h, 24 h and 48 h after short-term irradiation (**Tables 1** and **2**). At

Table 3. Cell-cycle phase distribution of thymocytes 1 month after long-term irradiation

Groups	G0/G1 (%)	S (%)	G2+M (%)
Normal	66.5±6.64	30.3±6.61	3.82±1.05
Control	65.8±8.54	33.6±8.03	0.72±0.68*
hBMSC treatment	51.3±6.95*,#	37.6±8.71	0.07±0.03*,#

*P < 0.05 compared with the normal group; #P < 0.05 compared with the radiation control group. The data were analyzed using Student's t-test.

Table 4. Cell cycle phase distribution of thymocytes 2 months after long-term irradiation

Groups	G0/G1 (%)	S (%)	G2+M (%)
Normal	65.7±6.56	30.1±6.47	3.77±1.22
Radiation control	67.3±1.74	30.2±4.18	4.41±2.56*
hBMSC treatment	55.1±0.37*	44.8±0.19*,#	0.18±0.13*,#

*P < 0.05 compared with the normal group; #P < 0.05 compared with the radiation control group. The data were analyzed using Student's t-test.

Table 5. Cell cycle phase distribution of thymocytes 3 months after long-term irradiation

Groups	G0/G1 (%)	S (%)	G2+M (%)
Normal	67.6±6.46	29.6±6.56	3.72±1.35
Radiation control	63.9±2.07	36.3±2.06	1.15±0.64*
hBMSC treatment	56.7±2.11*,#	42.1±2.12*	1.98±1.12*

*P < 0.05 compared with the normal group; #P < 0.05 compared with the radiation control group. The data were analyzed using Student's t-test.

6 h and 12 h after irradiation, the percentage of thymocytes and spleen cells in the S phase was markedly decreased in both the control and hBMSC treatment groups compared to the normal group. The percentage of cells in the S phase was increased in both the control and hBMSC treatment groups at 24 h after irradiations, and the increase in the hBMSC treatment group was higher than that in the control group. Thereafter, the percentage of cells in the S phase in both the control and hBMSC treatment groups continued to increase and exceeded that in the normal group at 48 h after irradiation. The percentage of cells in the S phase was dramatically increased in the hBMSC treatment group relative to the control group. Moreover, the percentage of arresting cells in the G0-G1 phase in the hBMSC treatment group markedly decreased at 24 h and 48 h. These results indicate that hBMSC treatment promoted cellular proliferation in the thymus and spleen after irradiation.

To evaluate the long-term effect of hBMSCs on radiation damage to the immune system, the cell cycle stage of thymocytes was determined 1 month, 2 months and 3 months after exposure (**Tables 3**-**5**). The percentage of cells in the G2+M phase markedly decreased one month after irradiation. Among hBMSC-treated animals, the percentage of cells in the S phase was modestly increased, and there were fewer cells in the G0-G1 phase one month after irradiation, indicating that hBMSC treatment promoted cell proliferation. The percentage of cells in the S phase in the hBMSC treatment group continued to increase for up to 2 months after irradiation. At 3 months after irradiation, the percentage of cells in the S phase in the hBMSC treatment group had slightly decreased, but the percentage of cells in the S phase remained significantly higher compared to the normal group. There was no significant difference between the normal group and the control group after long-term exposure.

Decreased cellular apoptosis in the thymus and spleen after hBMSC treatment

The rate of cellular apoptosis was examined in splenic cells after short-term irradiation and in thymocytes after long-term irradiation. Following short-term irradiation, the cellular apoptotic rate increased in splenocytes and peaked 12 h after irradiation in both the control and hBMSC treatment groups (**Table 6**). At 24 h and 48 h after irradiation, cellular apoptosis in the control and hBMSC treatment groups gradually decreased but remained higher than that in the normal group.

Interestingly, in the long-term, thymocyte apoptosis in the control group remained high at 1 month, 2 months and 3 months after irradiation. In contrast, cellular apoptosis in hBMSC-treated animals only showed a significant increase at 1 month and had rapidly returned to normal levels at 2 months and 3 months (**Table 7**).

As previous studies have shown that P53 is required for radiation-induced apoptosis [20, 21], we investigated P53 expression in the thymus and spleen via immunohistochemistry. As

Table 6. Apoptosis of thymocytes and spleen cells in mice at different time points after irradiation

Groups	*Time course*	*Thymocytes (%)*	*Spleen cells (%)*
Normal		1.21±0.42	0.41±0.12
Radiation control	6 h	7.78±1.25	6.25±0.23
	12 h	10.48±2.13	24.95±2.73
	24 h	3.12±0.33	5.32±0.87
	48 h	2.49±1.06	4.89±1.15
hBMSC treatment	6 h	6.12±2.12	7.03±1.56
	12 h	8.26±1.56*	13.01±2.65*
	24 h	2.43±4.20	2.58±0.57*
	48 h	2.19±0.97	2.75±0.81

*P < 0.05 compared with the normal control group. The data were analyzed using Student's t-test.

Table 7. Apoptosis of thymocytes in mice at different time points after irradiation

Groups	1 month	2 months	3 months
Normal	0.73±0.48	0.68±0.51	0.71±0.56
Radiation control	9.66±5.51*	9.58±6.21*	5.58±4.65*
hBMSC treatment	4.28±3.91*,#	0.31±0.22#	0.96±0.74#

*P < 0.05 compared with the normal control group, #P < 0.05 compared with the radiation control group. The data were analyzed using Student's t-test.

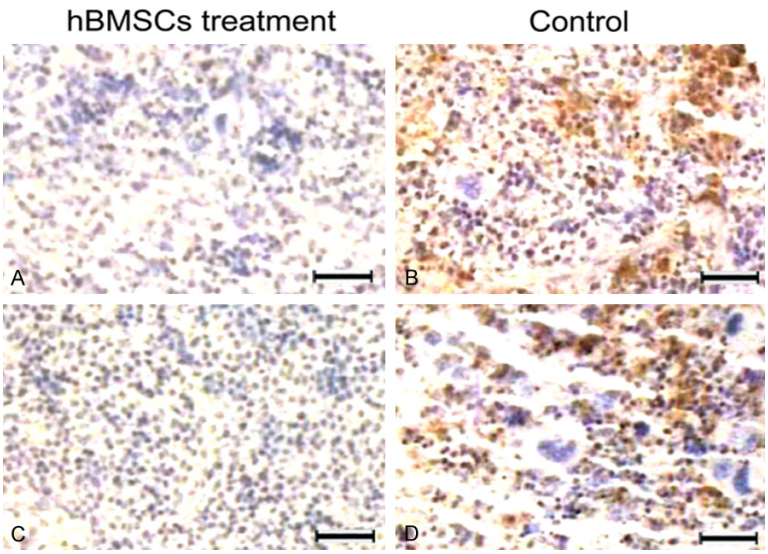

Figure 1. Immunohistochemical staining of P53 in the thymi and spleens of mice at 12 h after short-term irradiation. Representative P53 immunostaining in the thymus of a mouse from the hBMSC treatment group (A) and a mouse from the control group (B); P53 immunostaining in the spleen of a mouse from the hBMSC treatment (C) and a mouse from the control group (D). Bar = 50 μm.

shown in **Figure 1**, significantly stronger P53 immunostaining was detected in thymic (**Figure 1A** and **1B**) and splenic (**Figure 1C** and **1D**) tissues in the radiation control groups than in the hBMSC treatment groups.

Histological staining of the thymus

Histological analysis showed extensively increased necrosis of thymocytes in the radiation control group compared to the normal group (**Figure 2A**) at 1 month after irradiation. At 2 months after irradiation, regenerative repair of thymocytes in the radiation control group was observed, and at 3 months, precancerous lesions of the thymus were found (**Figure 2B**). However, cortical enlargement and medulla shrinkage were observed in the hBMSC treatment group, and there were no significant differences between the hBMSC treatment group and the normal group at 2 months and 3 months (**Figure 2C**).

Discussion

The present study provides the first evidence that transplantation of hBMSCs reduces cellular apoptosis and promotes cellular proliferation in the immune system after irradiation. Previous studies have shown that exposure to radiation remarkably reduces splenic cell numbers [1, 2]. However, cellular proliferation was not previously assessed. Here, using cellular proliferation and apoptotic assays, we showed that suppression of cellular proliferation and induction of apoptosis occurred after irradiation. The rate of cellular apoptosis in the spleen was elevated to 24.95±2.73% in the control group at 12 h after irradiation. Nevertheless, the rate of cellular apoptosis decreased to relatively low levels at 24 h

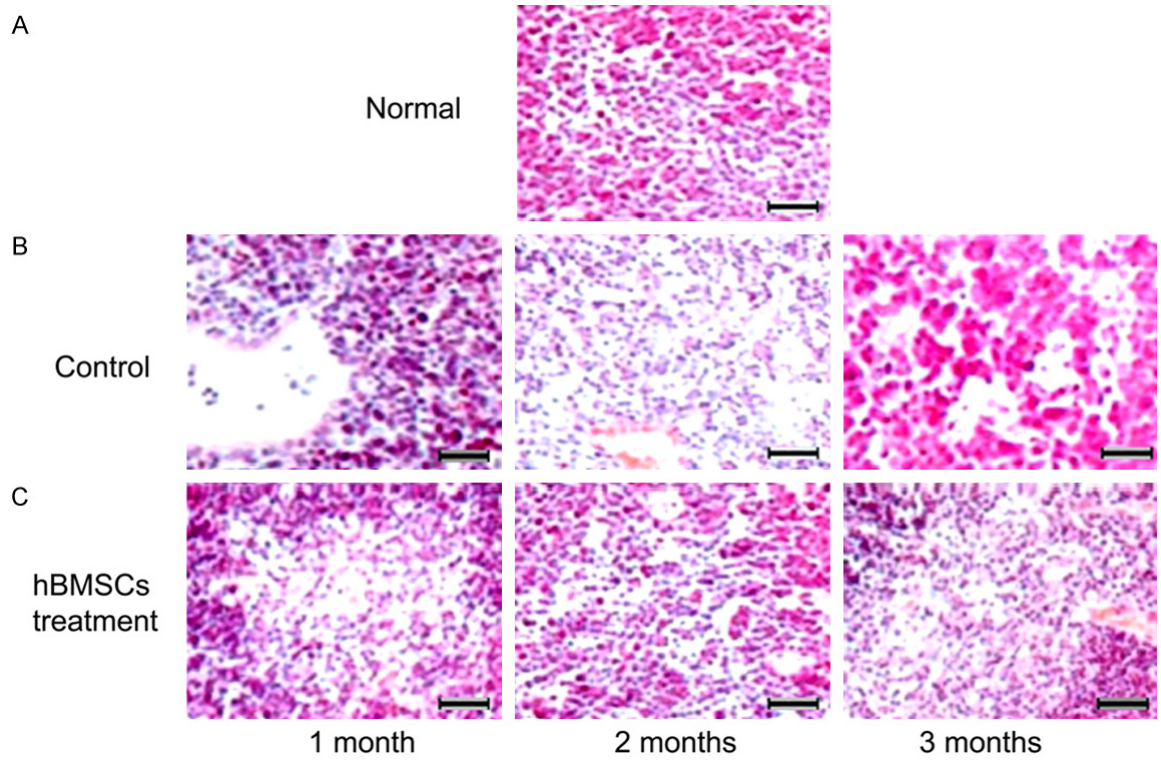

Figure 2. Histological analysis of the thymus. Representative histological sections of the thymus of mice from the normal group (A), the radiation control group (B) and the hBMSC treatment group (C) at 1 month (left panel), 2 months (middle panel) and 3 months (right panel) after long-term irradiation. Bar = 50 μm.

and 48 h after injury, indicating that self-recovery occurred 12 h after irradiation. The transplantation of hBMSCs seemed to promote recovery. Additionally, cellular proliferation was suppressed at 6 h and 12 h and recovery was initiated at 24 h and 48 h. A larger percentage of cells in the S phase was observed in the hBMSC treatment group than in both control groups at 48 h, indicating that hBMSC treatment promoted recovery. In the long-term irradiation study, control mice were administered a low dose of irradiation at each exposure but a higher total dose. These mice exhibited no marked suppression of cellular proliferation, indicating that the effects of irradiation depended on the mechanism of administration. Nonetheless, hBSMC treatment after long-term exposure promoted cell proliferation and suppressed cell apoptosis. These results indicate that the effect of irradiation on cellular proliferation was dependent both on the number of exposures and the total dose. However, transplantation of hBMSCs promoted cellular proliferation and reduced cell apoptosis after any amount of radioactive exposure.

Our data suggest that the promotion of cellular apoptosis contributed more to radiation damage of the immune system than the suppression of cell proliferation. Because P53 is involved in cellular apoptosis after irradiation, we investigated whether hBMSC treatment suppressed P53 expression. Using immunohistochemistry, we provide the first evidence that P53 expression is inhibited in the thymi of mice treated with hBMSCs. Further studies should be carried out to elucidate the mechanism of increased P53 expression after hBMSC transplantation.

Radiation exposure increases long-term cancer risk [22, 23]. In this study, histological analyses showed that precancerous lesions of the thymus were observed at 3 months after irradiation. However, hBMSC treatment decreased the thymic tissue damage detected at 1 month. At 2 months and 3 months, no abnormal lesions in the thymus were observed in the hBMSC treatment group. The feasibility of human MSC transplantation has already been extensively evaluated. Human MSCs allow unrelated donor stem cell transplantation and suppress T-cell

activation, permitting their use in clinical applications [24, 25]. Therefore, based on these characteristics, hBMSCs are an ideal candidate for transplantation as a therapeutic treatment.

In conclusion, this study evaluated the effects of hBMSC transplantation on the repair of radiation damage to the immune system. Transplantation of hBMSCs promoted cellular proliferation and reduced apoptosis in the immune system after irradiation. Mice treated with hBMSC transplantation recovered rapidly after irradiation and showed no signs of precancerous lesions. Moreover, P53 expression was suppressed in the hBMSC treatment group. Thus, hBMSC transplantation is a promising future strategy for the treatment of radiation damage.

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Disclosure of conflict of interest

None.

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