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Research article

The efficacy of bone marrow mesenchymal stem cells on rat intestinal immune-function injured by ischemia/reperfusion

He Wang [a], Kun Wang [a], Bo Liu [b], Xiaoqian Bian [a], Xiaojie Tan [a], Haitao Jiang [a,*]

[a] Department of Gastrointestinal Surgery, Affiliated Hospital of Qingdao University, China
[b] The Second Clinical Medical College, Lanzhou University, Lanzhou, China

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ABSTRACT

Background: Transplantation of bone marrow mesenchymal stem cells (BMSCs) has a promising therapeutic efficiency for varieties of disorders caused by ischemia or reperfusion impairment. It has been shown that BMSCs can mitigate intestinal ischemia/reperfusion (I/R) injuries, but the underlying mechanism is still unclear. This study aimed at investigating the efficacy of BMSCs on the immune function of intestinal mucosal microenvironment after I/R injuries.

Methods: Twenty adult Sprague-Dawley rats were randomly assigned to a treatment or a control group. All the rats underwent superior mesenteric artery clamping and unclamping. In the treatment group, BMSCs were implanted into the intestine of ten rats by direct submucosal injection whereas the other ten rats in the control group were injected with the same volume of saline. On the fourth and seventh day after BMSCs transplantation, intestinal samples were examined for the CD4 (CD4-positive T-lymphocytes)/CD8 (CD8-positive T-lymphocytes) ratio of the bowel mucosa via flow cytometry, and for the level of Interleukin-2 (IL-2), Interleukin-4 (IL-4) and Interleukin-6 (IL-6) via ELISA. Paneth cell counts and Secretory Immunoglobulin A (SIgA) level were examined via immunohistochemical (IHC) analysis. Real time PCR (RT-PCR) was used to detect the expression levels of tumor necrosis factor-α (TNF-α) and trypsinogen (Serine 2) (PRSS2) genes. White blood cell (WBC) count was measured by manual counting under the microscope.

Results: The CD4/CD8 ratio in the treatment group was significantly lower compared with that in the control group. The concentration of IL-2 and IL-6 was lower in the treatment group compared with the control group, while the level of IL-4 is the reverse between the two groups. The number of Paneth cells in intestinal mucosa increased significantly, while the level of SIgA in intestinal mucosa decreased significantly, after BMSCs transplantation. The gene expression levels of TNF-α and PRSS2 in intestinal mucosa of treatment group were significantly lower than those of control group. The WBC count in the treatment group was significantly lower than that in the control group.

Conclusion: We identified immune-relevant molecular changes that may explain the mechanism of BMSCs transplantation efficacy in alleviating rat intestinal immune-barrier after I/R.

* Corresponding author. No. 16 Jiangsu Road, Qingdao, Shandong Province, 266003, China.
E-mail address: jianght67122@sina.com (H. Jiang).

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1. Introduction

Abstract Guidance – remove this box before submitting!

- No more than approx. 500 words (or 3000 characters).
- Self-contained and concisely describe the reason for the work, methodology, results, and conclusions. Uncommon abbreviations should be spelled out at first use. Do not include footnotes or references.
- Headings in structured abstracts should be bold and followed by a period. Each heading should begin a new paragraph. For example:

Background. The background section text goes here. Next line for new section.
Methods. Methods section here, then new line.
Results. Results section here, then new line.
Discussion. Discussion section here.

Intestinal I/R is a common phenomenon in abdominal surgery and is associated with a high occurrence of morbidity. I/R can occur in a variety of clinical events, such as small intestine transplantation [1], extracorporeal circulation [2], or a strangulated hernia [3]. The intestinal mucosal immune barrier is mainly composed of intestinal related lymphoid tissues and their secreted substances such as SIgA [4] and cytokines [5]. This immune barrier can prevent large molecules, especially bacteria and toxins from being absorbed into the blood. Intestinal I/R involves a complex mechanism. In the event of I/R, intestinal immune barrier dysfunction will lead to cytokine release, intestinal inflammatory injury and bacterial invasion and migration [6], resulting in serious body damage. Many studies have demonstrated successful application of BMSCs in reducing intestinal mucosal I/R damage in animal models [7,8], which proposing a new strategy for the management of intestinal I/R impairments. Therefore, the application of BMSCs in IR may be fruitful and have great clinical significance.

MSCs cultured from bone marrow are called BMSCs. BMSCs can differentiate into endoderm (hepatocyte), mesoderm (bone cells and chondrocytes), or ectoderm (neural precursor) and have a paraclinical function to accelerate tissue regeneration [9,10]. It has been reported that MSCs preferentially migrate to damaged tissue and therefore have therapeutic potential [10]. The possible therapeutic mechanisms are: (1) differentiating into mature tissue and organ cells to replace the injured cells; (2) saving injured cells by fusion with cells; (3) secreting cytokines to promote the proliferation of stem progenitor cells in tissues and organs; (4) delivering mitochondria and other substances to injured cells through special tunnel tubes (TNTs); (5) secreting exosomes or tiny vesicles that fuse through the membrane and enter the injured cell [11–13].

In our previous studies, we have demonstrated that BMSCs can reduce rat I/R impairment and the potential mechanism of which might attribute to a decreased inflammatory response of mucosa that accelerates tissue regeneration to ameliorate intestinal mechanical barrier function injuries [7]. In this study, we are trying to investigate the efficacy of BMSCs on intestinal immune-barrier function following I/R injury.

2. Materials & methods

2.1. Animals and groups

We followed our previous study: 4-week-old male Sprague Dawley (SD) rats (from Jiangsu Jicui Yaokang Biotechnology Co. LTD) were adopted as BMSCs donor animals. Female SD rats weighing about 180–220 g were treated as the recipient animals. All animals were housed in plastic-bottomed wire-lidded cages and were kept with 12 to 12 white night cycles in a controlled environment at 25 °C, with free access to water and food. The animals were acclimatized for seven days before being included in the experiment. Twenty clean adult SD rats were randomly assigned to a treatment or a control group by simple randomization, five rats in the two groups were sacrificed on day 4 and day 7, respectively. There was no protocol before the study and all the study data are available. Animal procedures were conducted in accordance with the guidelines for the review and approval of the Animal Care and Use Committee of Qingdao University (Ethical Approval No. QYFYWZLL27110), and followed the Guide for the Care and Use of Laboratory Animals issued by the USA National Institutes of Health. Effort was made to minimize pain and the number of animals used in the experiments.

2.2. BMSCs preparation and surgical procedure

The isolation, identification, and culture methods of BMSCs were consistent with our previous studies. Animal handling, surgical procedures, and cells transplantation were using the same procedure as described in our previous studies [7]. Twenty rats were divided into treatment or control groups. Under general anesthesia, the superior mesenteric artery (SMA) was occluded with an atraumatic microvascular clamp for 45 min to complete the I/R model. Submucosal injection of BMSCs was performed in the treatment groups, and the cultured cells were directly injected into the intestinal submucosa at 10 different points. Control groups were injected with the same amount of saline at ten different submucosal points of the intestine after clamping the SMA for 45 min [7].

2.3. Measuring cytokines content of the intestinal mucosa

Animals were sacrificed under general anesthesia. The complete small intestine of each animal was removed, and the intestinal

contents were rinsed and mucosal tissue was scraped. The intestinal mucosal tissues were mixed with a homogeneous solution in a ratio of 1:10 (weight/volume) and homogenized in the ice-water environment. Then the homogenate was transferred into a 1.5 ml EP tube and centrifuged at 3000 RPM for 15 min. IL-2, IL-4, IL-6 were determined by ELISA according to the detailed instruction of the kit (Thermo Fisher Thermo ScientificMK3 US).

2.4. Flow cytometry to analyze CD4CD8 lymphocyte subsets

When the cell coverage rate in the culture bottle reached 80%–90%, the original medium was removed, and trypsin (0.25%) was added for digestion for 1–2 min. After that, the same volume of serum-containing medium was added to terminate the digestion, and the cells were suspended (pipette blowing cells) and centrifuged. Then the cells were re-suspended and transferred to the culture bottle for further culture. The cells at logarithmic growth stage were digested and collected with trypsin (0.25%), washed with phosphate buffered saline (PBS) twice (centrifugation at 2000 rpm for 5 min), and 5×10^5 cells were collected. Fluorescently labeled flow cytometric lectin antibodies were added and incubated at room temperature and away from light for 15 min. After centrifugation again (1500 r/min) for 5 min, the supernatant was discarded. Cell pellet was washed twice using PBS. Then 0.5 ml PBS was added and mixed to make single-cell suspension. Cell surface antigen expression was detected by flow cytometry (Ex = 488 nm; Em = 530 nm, Becton-Dickinson FACS Calibur, USA).

2.5. Paneth cell count in a field of specific size

The intestinal mucosa sections of treatment and control mice were stained with H&E and counted under a microscope. We evaluated the functional status of Paneth cells in the intestinal mucosa by counting Paneth cells in the intestinal mucosa section at 400× magnification (Fig. 1a, b).

2.6. SIgA measurement by IHC

SIgA expression was detected by the immunohistochemical method. Intestinal mucosal tissue was sectioned by polymer method and then observed under a light microscope. The tissue was operated according to the kit instruction (SP Immunohistochemical kit: Purchased from Fuzhou Maixin Biotechnology Co., LTD. Item No.: KIT9902).

Microscopical manual counting to measure white blood cell count.

2.7. RT-PCR

Total RNA was extracted by Trizol method. OD260/OD280 were determined to be between 1.8 and 2.0, and then reverted to cDNA for amplification. Rat-GAPDH primer (111bp) – F: 5-TCAAGAAGGTGGTGAAGCAG-3, R: 5-AGGTGGAAGAATGGGAGTTG-3; Rat-GapDH Primer (111BP) Rat-tnf-α primer (198bp) – F: 5-CAAGGAGGAGAAGTTCCCAA-3, R: 5-ttgTCttTGagatCCATgC-3; Rat-PRSS2 primer (109bp) – F: 5-ATCAATGACCAGTGGGTGGT-3, R: 5-GCTTGATGATCTTGGCAGCA-3. After the preliminary test to ensure the reliability of the samples, the formal test was conducted. Rat-GAPDH was used as the internal reference gene. The reaction system and parameters were set according to the instructions of the GoTaq 3-Step RT-qPCR kit. The Ct values of the target gene and Rat-GAPDH were obtained using the sample fitting analysis results. The relative expression levels were calculated using the comparative Ct value method with the reference of Rat-GAPDH: ΔΔCT = (Ct value of target gene in test group − Ct value of reference gene in test group) − (Ct value of target gene in control group − Ct value of reference gene in control group), the relative expression level was $2^{-\Delta\Delta CT}$.

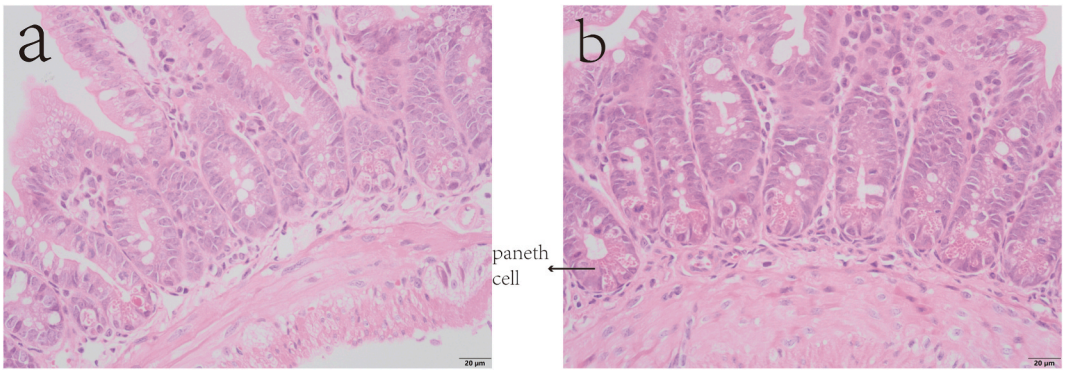

Fig. 1. The counts and morphology of Paneth cells which is stained by H&E. The number of Paneth cells in the control group (a) was significantly lower than that in the treatment group (b). The repair effect of BMSCs was also observed in the morphology (Villus length and crypt depth) of intestinal mucosa.

2.8. Statistical analysis

SPSS 26.0 software was used for statistical analysis. Measurement data with normal distribution and homogeneity of variance were expressed as mean ± SD, and independent sample *t*-test was used for comparison between the two groups. Median was used for comparison between groups that did not meet the above requirements, and rank sum test was used for comparison between two groups. Statistical data were expressed as frequency (percentage) and calculated by chi-square test or Fisher exact probability method ($p < 0.05$ was considered statistically significant).

3. Results

3.1. Characterization of BMSCs

Mature BMSCs are either fusiform or spindle-shaped and have a swirling or fish-like growth (Fig. 2a–h). We determined the purity of cultured BMSCs by assaying the ratio of CD90 (+) CD45 (−) cells by Flow cytometry. In the third generation of cultured BMSCs, most of the hematopoietic stem cells have been removed and relatively purified BMSCs are obtained (Fig. 3a–d).

3.2. The CD4/CD8 lymphocyte subsets were altered

T test was used to compare CD4 (F value = 0.322, $p > 0.05$) (Fig. 4 a) and CD8 lymphocyte subtypes (F value = 1.538, $p > 0.05$) (Fig. 4 b). *U* test was used to compare CD4/CD8 ratio (F value = 6.646, $p < 0.05$) (Fig. 4 f).

The proportion of $CD4^+$ lymphocytes in the treatment group (4.840 ± 3.647) was significantly decreased compared with control group (20.700 ± 3.511). For $CD8^+$ lymphocytes, similar results were found in the treatment (3.255 ± 1.200) and control groups (12.806 ± 1.345). The CD4/CD8 ratio was higher in the control group (1.606) than that in the treatment group (1.189) (*U* test = 0.02, $p < 0.05$). BMSC can affect the proportion of CD4/CD8 lymphocyte subsets in the intestinal mucosa. In general, we can infer that the BMSC is immunosuppressive (Table 1) (Fig. 4).

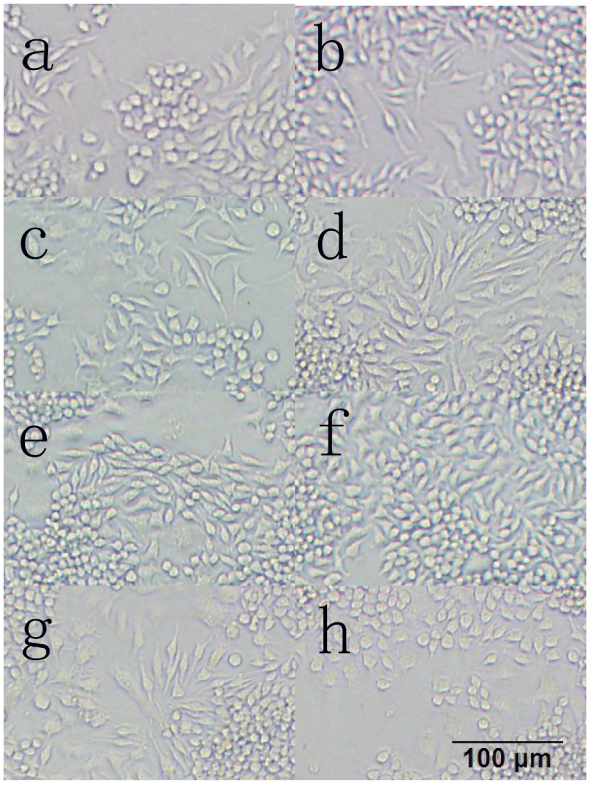

Fig. 2. The morphology of BMSCs in passage 3 generation (a–f). The cell states are different at different growth stages. Most BMSCs are typical spindle-shaped, fibroblast-like cells, and a small number of BMSCS may be approximately circular.

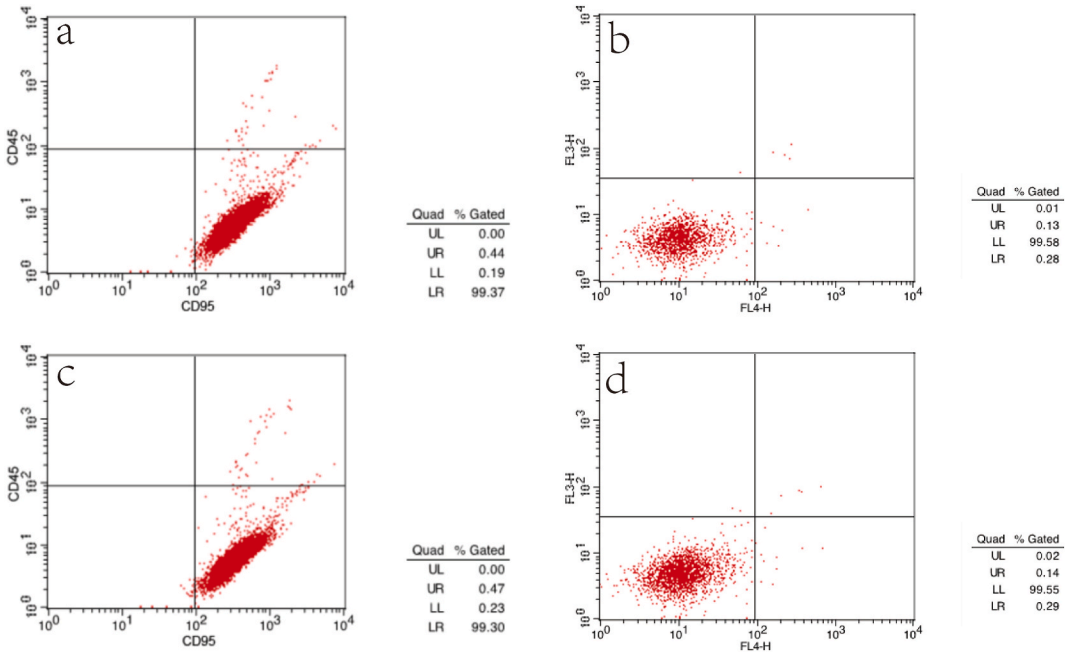

Fig. 3. Purity identification of BMSCs. After completing isotype control for CD45 and CD95 (b, d), we obtained the ratio of CD90 (+)CD45(−) cells in passage 3 generation BMSC was more than 98% (a, c), indicating that most of the cells obtained were bone mesenchymal stem cells.

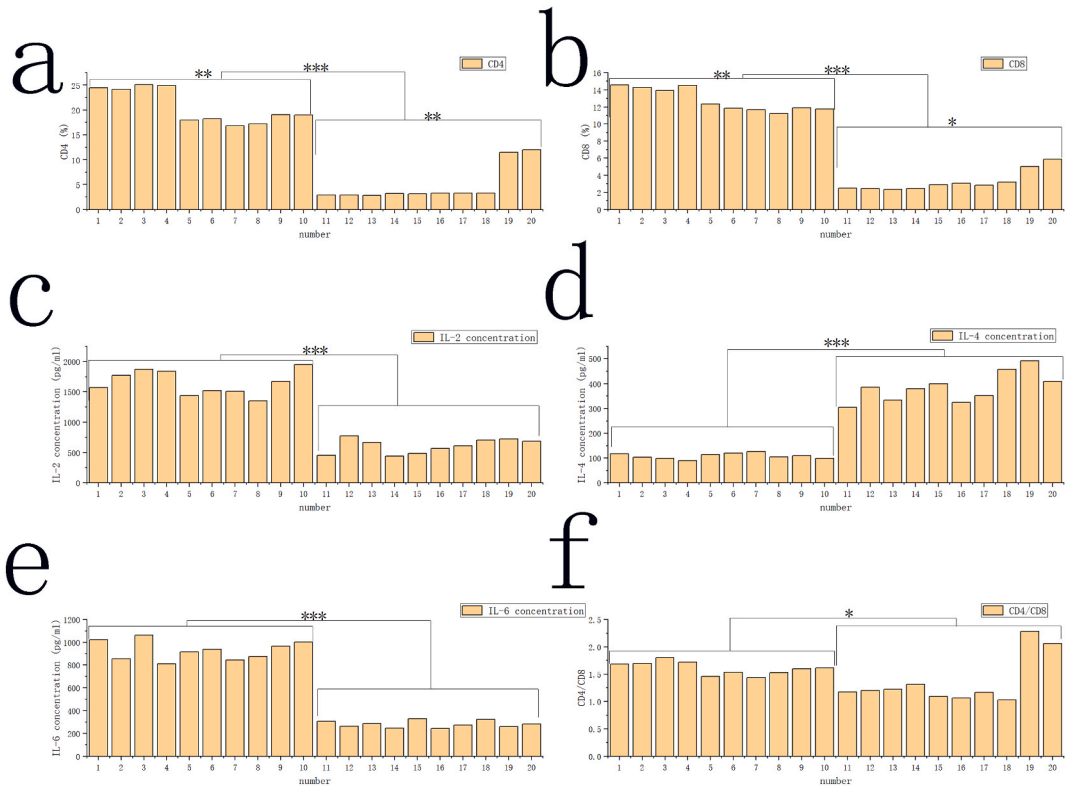

Fig. 4. Effects of BMSCs on $CD4^+$ lymphocytes (a), $CD8^+$ lymphocytes (b), IL-2 (c), IL-4 (d), IL-6 (e) and CD4/CD8 ratio (f) in rats intestinal mucosa. Number 1–20 refers to 20 rats from the control group and the treatment group in sequence. Number 1–5, 6–10, 11–15, 16–20 is the control group rats sampled on day 4 and day 7, treatment group rats sampled on day 4, day 7. The data were reported as mean ± SD or median, values were compared by T test or *U* test. *Significant difference, $P < 0.05$; **significant difference, $P < 0.01$; ***significant difference, $P < 0.001$.

Table 1
Intestinal mucosal immune index.

Variables	Control (n = 10)	Treatment (n = 10)	P-value (*U* test/*t*-test)
CD4 (%)	20.700 ± 3.511	4.840 ± 3.647	0.000
CD8 (%)	12.806 ± 1.345	3.255 ± 1.200	0.000
CD4/CD8 ratio	1.606	1.189	0.023
IL-2 (pg/ml)	1622.505	638.925	0.000
IL-4 (pg/ml)	107.881	382.891	0.000
IL-6 (pg/ml)	927.005	278.897	0.000
SIgA			
Control	treatment	P-value	
Variables	(n = 21)	(n = 21)	(*U* test)
positive area	395,096	143,603	0.000
average gray scale	68151.961	16460.09	0.000
density volume	53,823,144	18,769,544	0.000

3.3. BMSCs alter the content of immune-related cytokines in the intestinal mucosa

1) As shown in Table 1, the IL-2 (Fig. 4 c) and IL-6 (Fig. 4 e) expressions in the control group (1622.505, 927.005) were higher than those in the treatment group (638.925, 278.897). (F value = 4.564 $p < 0.05$; *U* test = 0.000, $p < 0.05$. F value = 10.197 $p < 0.05$; *U* test = 0.000, $P < 0.05$)
2) IL-4 levels were lower in the control group (107.881) than in the treatment group (382.891). (F value = 10.122 $p < 0.05$; *U* test = 0.000, $p < 0.05$) (Table 1) (Fig. 4 d)

3.4. BMSCs alter Paneth cells count

Paneth cells are located at the base of the gland. They have shapes filled with thick eosinophilic secretory granules toward the luminal end of the cytoplasm (Fig. 1). There was a statistically significant difference in Paneth cell counts between the two groups (control group 19.720 ± 2.886 treatment group 27.330 ± 3.181 F value = 0.013 $p > 0.05$; *t*-test = 0.000, $P < 0.05$) (Fig. 5 a). The decrease of number of Paneth cells in the treatment group compared to the control indicates that Paneth cells were involved in the effect of BMSC on the intestinal mucosal immune barrier.

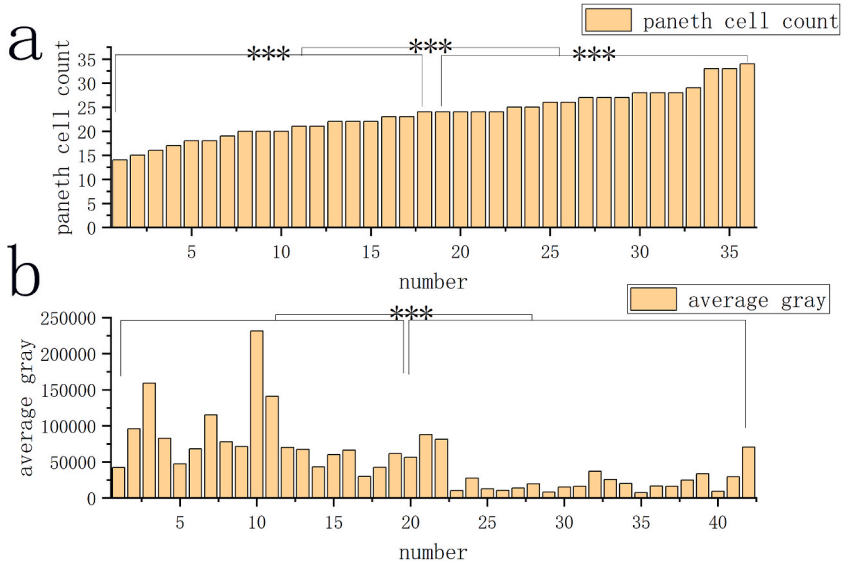

Fig. 5. Effects of BMSCs on Paneth cell (a) and SigA (b). We observed Paneth cells and SIgA with microscope, measured the amount of SIgA by mean gray scale and determined the number of Paneth cells by direct counting method. Numbers refers to different samples. For Pan's cells: Number 1–9, 10–18, 19–27, 28–36 is the control group rats sampled on day 4 and day 7, treatment group rats sampled on day 4, day 7. For SIga: Number 1–10, 11–20, 21–30, 31–40 is the control group rats sampled on day 4 and day 7, treatment group rats sampled on day 4, day 7. The data were reported as mean ± SD, values were compared by T test. *Significant difference, $P < 0.05$; **significant difference, $P < 0.01$; ***significant difference, $P < 0.001$.

3.5. BMSCs alter SIgA of humoral immunity

The level of SIgA was examined by IHC and there were statistical differences between the treatment group and the control group. SIgA levels in the control group (measured by average gray scale 68151.961) were significantly higher than those in the treatment group (average gray scale 16460.09) (F value = 6.105 $p < 0.05$; *U* test = 0.000, $P < 0.05$) (Table 1) (Fig. 5 b) (Fig. 6a, b).

3.6. BMSCs alter WBC count

WBC count in treatment group (42.400 ± 8.044) was significantly lower than that in control group (77.000 ± 14.244) (F value = 1.530 $p > 0.05$; *t*-test = −6.689, $P < 0.05$).

3.7. BMSCs alter expression levels of TNF-α and PRSS2 genes

The expression levels of TNF-α and PRSS2 gene in gut mucosa of treatment group ($2^{-\Delta\Delta CT}$: 1.350, 1.472) were significantly lower than those of control group ($2^{-\Delta\Delta CT}$: 5.294, 5.317) (TNF-α: F value = 10.846 $p < 0.05$; *U* test = 100, $P < 0.05$, PRSS2: F value = 10.846 $p < 0.05$; *U* test = 100, $P < 0.05$).

3.8. There are changes in the intestinal mucosal immune barrier on day 4 and day 7

Intestinal mucosal immunity on day 4 and day 7 (Figs. 4 and 5).

According to Sémont As research, intestinal spontaneous recovery is a very rapid process and can be completed within 7 days [1]. So we were interested in the restoration of the intestinal mucosal immune barrier within 7 days. Therefore, we analyzed the intestinal mucosal immunity on day 4 and day 7 (Fig. 4) (Fig. 5).

In the control group, compared with day 4 (CD4 + T cells 23.356 ± 3.014, CD8 + T cells 13.930 ± 0.919), there was a statistically significant decrease in the proportion of CD4 positive and CD8 positive T cells separately on day 7 (CD4 + T cells 18.044 ± 1.100, CD8 + T cells 11.682 ± 0.264) (F value = 2.462 $p > 0.05$; *t*-test = 3.736 $p < 0.05$. F value = 2.714 $p > 0.05$; *t*-test = 5.257 $p < 0.05$). There was no statistical difference in IL-2.4.6 and CD4/CD8 ratio. (day4: IL-2.4.6 and CD4/CD8 1701.090 ± 185.627, 105.023 ± 11.460, 932.724 ± 106.780, 1.671 ± 0.128 vs day7: IL-2.4.6 and CD4/CD8 1600.704 ± 225.413, 112.519 ± 10.920, 925.522 ± 64.528, 1.544 ± 0.069 F value = 0.044, 0.000, 2.220, 0.713 $p > 0.05$; *t*-test = 0.769, −1.059, 0.129, 1.953 $p > 0.05$) This result is likely reflecting the self-repair process of intestinal mucosal immunity (Table 2).

In the treatment group, there was a statistically significant increase in the proportion of CD4 positive and CD8 positive T cells separately on day 7 (day4: CD4 + and CD8 + T cells 2.930, 2.440 vs day7: CD4 + and CD8 + T cells 3.310, 3.190 F value = 85.567, 26.728 $p < 0.05$; *U* test = 25.000, 24.000, $p < 0.05$). There was no statistically significant difference in IL-2.4.6 and CD4/CD8. (IL-2.4.6, CD4/CD8 day4: 489.360, 360.450 ± 40.029, 286.734 ± 32.928, 1.206 vs day7 687.090, 406.644 ± 70.163, 276.991 ± 30.860, 1.165 F value = 7.347, 1.787, 0.079, 41.955 p = 0.027, 0.218, 0.785, 0.000; *U* test/*t*-test = 18.000, −1.279, 0.483, 11.000 $p > 0.05$). The differences between control and treatment groups suggest that BMSCs can increase the proportion of CD4 and CD8 positive T cells during the recovery of the intestinal mucosal immune barrier. However the possible bias caused by the small sample size cannot be ruled out (Table 2).

In both the control (Day 4, 17.44 ± 2.218, Day 7, 22.00 + 1.225, F value = 3.567 $p > 0.05$; *t*-test = 0.00 $P < 0.05$) and treatment group (Day 4, 25.00, Day 7, 28.00, F value = 13.288 $p < 0.05$; *U* test = 0.00, $P < 0.05$), there was a statistically significant increase in Paneth cells on day 7 compared to day 4.

The level of SIgA showed no statistical difference between the fourth day and the seventh day after surgery in two groups (control

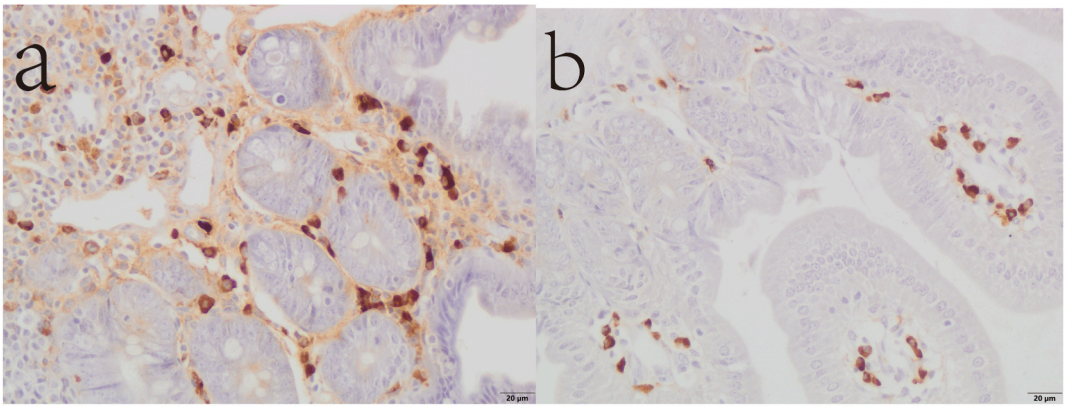

Fig. 6. SIgA was measured by IHC, compared with the control group-10 (a), the content of SIgA in treatment group-17 (b) decreased significantly. The data were reported as median, values were compared by *U* test.

Table 2
Comparison of intestinal mucosal immune indexes on day 4 and day 7.

Variables		Day 4 (n = 5)	Day 7 (n = 5)	P-value (*U* test/*t*-test)
CD4 (%)	Control	23.356 ± 3.014	18.044 ± 1.100	0.006
Treatment	2.930	3.310	0.009	
CD8 (%)	Control	13.930 ± 0.919	11.682 ± 0.264	0.001
Treatment	2.440	3.190	0.016	
CD4/CD8 ratio	Control	1.671 ± 0.128	1.544 ± 0.069	0.087
Treatment	1.206	1.165	0.754	
IL-2 (pg/ml)	Control	1701.090 ± 185.627	1600.704 ± 225.413	0.464
Treatment	489.360	687.090	0.251	
IL-4 (pg/ml)	Control	105.023 ± 11.460	112.519 ± 10.920	0.321
Treatment	360.450 ± 40.029	406.644 ± 70.163	0.237	
IL-6 (pg/ml)	Control	932.724 ± 106.780	925.522 ± 64.528	0.900
Treatment	286.734 ± 32.928	276.991 ± 30.860	0.642	

group: Day 4, 99036.00 ± 57520.92, Day 7, 65936.20 ± 29397.85, F value = 3.061 $p > 0.05$; *t*-test = 0.11, $P > 0.05$), (treatment group: Day 4, 21493.44 ± 21777.14, Day 7, 26377.65 + 17337.62, F value = 0.052 $p > 0.05$; *t*-test = 0.57, $P > 0.05$). This may indicate that SIgA has a long-term and stable immune regulation effect compared with other intestinal mucosal immune barrier components.

4. Discussion

This research aimed to investigate the expression of related growth factors in mucosal tissues after cell transplantation to reveal the potential regulatory mechanism of transplanted cells on intestinal mucosal immune cells, with the hope to provide a new theoretical basis and experimental research basis for the treatment of intestinal barrier dysfunction by cell transplantation.

Our previous studies have demonstrated that BMSCs alleviate and promote the recovery of the mechanical barrier function post intestinal I/R injury. Progenitor cells from bone marrow can travel through peripheral blood to target solid organs [14]. Our study found that submucosal injection of BMSC has a better intervention effect for intestinal mucosal repair, the mucosal tissue generally returned to normal on day 7 post I/R, and BMSCs amount decreased to normal on day 10 [7].As expected, this study showed that BMSCs have a noticeable effect on intestinal mucosal immune markers. In order to clarify the effect of BMSCs on the inflammatory response, further analysis showed that the white blood cells in the peripheral blood of the rats treated with BMSCs were significantly decreased. The decrease of TNF-α and PRSS2 gene expression in the treatment group reflected the inhibitory effect of BMSCs on inflammation in rats. Paneth are relatively abundant in the intestinal tract of rats, and can secrete immunoglobins like IgA and IgG. BMSCs implantation seems to increase the number of Paneth cells but specifically lower the level of SIgA, suggesting that the inhibition of the SIgA release by BMSCs is through other pathways. The above results indicate that the increase of Paneth cells maybe due to the restoration of the intestinal mucosal immune barrier and the decrease of SIgA level is more likely due to the immunosuppressive effect of BMSCs.

Intestinal mucosal T lymphocytes are mainly distributed in the intestinal epithelium and lamina propria. Eighty percent of intraepithelial T lymphocytes are $CD8^+$ T lymphocytes. The changes in lymphocyte subsets in the epithelium and lamina propria represent the changes in intestinal immune function. Our results showed that BMSCs affect the intestinal mucosal immune barrier by reducing the number of $CD4^+$, $CD8^+$ cells, as well as the CD4/CD8 ratio. This suggests that BMSCs suppress the immune response by inhibiting lymphocyte proliferation or trafficking, the underlying mechanism of which may be related to the programmed cell death 1 (PD-1)/programmed cell death ligand 1 (PD-L1) signal pathway [15].

IL-2 is an interleukin and a class of cell growth factors in the immune system. It can regulate the activity of white blood cells in the immune system, promote the proliferation of TH0 cells and Cytotoxic T Lymphocyte (CTL), and also participate in antibody response, hematopoiesis, and tumor monitoring. IL-2 can activate the expression of cell signaling pathways that promote mitosis, cell proliferation, migration and even inhibit inflammatory genes expression in tissue cells [16]. Through the production of PGE2, BMSCs reduce the expression of the EKONO receptor by IL-2, thereby inhibiting the clonal expansion of activated T cells. TGF-β is also an effective inhibitor of the IL-2 signaling pathway and is involved in BMSCs-mediated G1 cell cycle arrest of activated T cells. The inhibitory effect of BMSCs on T cell proliferation was partially reversed because of the decrease of IL-2 [17]. PGE2 secreted by BMSCs can also inhibit T cell proliferation by reducing IL-2 content and down-regulating IL-2 receptors, and impairing transcription factor DNA binding activity by inhibiting the Janus kinase 3 signaling pathway [18]. In our study, we suspect that the decrease of IL-2 attenuated the T cell proliferation, but eventually, the number of T cells should be reduced by a combination of other regulatory mechanisms.

IL-4 is produced mainly by activated T cells. In rats, the IL-4 receptor is present on the surface of T cells, B cells, thymocytes, bone marrow cells, macrophages, and mast cells. It has been considered an immune regulator, which plays a regulatory role in the tumor microenvironment and is an important part of the immune system. IL-4 in intestinal tissue homogenate is mainly secreted by Th2 lymphocytes and is an active factor that plays a regulatory role in the immune response [19]. It can increase the proliferation of T lymphocytes and B lymphocytes, promote the secretion and classification transformation of SIgA, and the synergistic effect of the two can improve the function of humoral immunity. IL-4 also increases the ability of macrophages to present antigens [20]. We found that

IL-4 levels in the treatment group were significantly higher than in the control group. The possible reasons are as follows: 1. IL-4 can maintain the proliferation of Th2 cells. A decrease in the number of T cells corresponds to an increase in IL-4 levels due to negative feedback regulation [21]. 2. Exosomes contain IL-4.

Paneth cell is the main producer of intestinal antimicrobial proteins (including Lysozyme, regenerating islet-derived protein 3 gamma (RegIIIγ), secretory phospholipase A2 (sPLA2)). Translocation of the flora results in changes in the number of Paneth cells. The secretion of antibacterial products is triggered by bacterial signals [22–24]. Paneth cells play an important role in controlling the mucosal penetration of symbiotic and pathogenic bacteria [24]. Clinical studies have confirmed that Low levels of Paneth cells in the intestinal mucosa are associated with a high incidence of infection and poor prognosis [23]. We hypothesize that BMSCs could promote intestinal mucosal immune barrier and functional recovery by increasing the number of Paneth cells, and consistent results were obtained. The mechanism may be the direct differentiation of BMSCs or a combination function of cytokines (IL-2, IL-4).

IL-6 is a mature pleiotropic pro-inflammatory factor, which is highly expressed in a variety of inflammatory diseases [25]. The production of IL-6 is affected by many factors, and it is crucial for BMSCs to maintain stem cell characteristics in a dose-dependent manner. Consistent with our findings in the intestinal mucosa, IL-6 levels were significantly increased in the body fluids of patients with rheumatoid arthritis and osteoarthritis [26,27]. There are many target cells of IL-6, including macrophages, hepatocytes, quiescent T cells, activated B cells and plasma cells. Its effect is very complex, and it has been called B cell stimulator 2, 26KD protein, B cell differentiation factor (bCDf), hepatocyte stimulator (hsf), etc. Studies have shown that BMSCs directly produce IL-6 protein to activate the IL-6/gp130/STAT3 signaling pathway [28], thereby accelerating cell migration and cell number. We found that the level of IL-6 in the treatment group was lower than that in the control group, consistent with the observed less inflamed, and faster restoration of the intestinal mucosal microenvironment by BMSC implantation. TNF-α is a proinflammatory cytokine produced mainly by macrophages and monocytes and is involved in normal inflammatory and immune responses [29]. TNF-α is increased in many pathological conditions, including sepsis, malignancy, heart failure, and chronic inflammatory diseases [30,31]. Increased TNF can be found in the blood and joints of patients with severe rheumatoid arthritis [32]. The level of TNF-α can directly reflect the degree of intestinal mucosal injury during intestinal I/R injury in rats. Therefore, our results of lower TNF-α expression level in the treatment group compared to control group corroborate the repair effect of BMSCs on intestinal mucosa.

PRSS2 is a protein with a molecular weight of 25kD. Most of it is secreted into pancreatic juice in the form of pancreatic acinar zymogen, which is activated by enterokinase in the intestine and becomes an activator of other digestive enzymes with high activity. The reduction of PRSS2 can alleviate ischemia-reperfusion injury. PRSS2 is an inflammatory marker that is closely associated with pancreatitis and induces episodes of acute viral myocarditis [33,34]. The lower expression level of PRSS2 demonstrated the protective effect of BMSCs on intestinal mucosa and the repair of immune barrier.

Eating stimulates the release of secreted IgA, which coats bacteria in the lumen and prevents them from sticking to epithelial cells [35]. Intestinal mucosa SIgA is the most important immunoglobulin in the intestine, acting as the first line of defense of the intestinal immune barrier and preventing pathogens from invading into the lamina propria and mucosal layer. IL-4, IL-5, IL-6, and IL-10 can induce an increase in SIgA. The level of SIgA in the intestinal mucosa decreased after I/R injury [36]. After the application of BMSCs, intestinal mucosal tissue that had experienced I/R injury showed a decrease in mucosal injury but a further decrease in SIgA content, which was contrary to the increase of IL-4 levels. In the treatment group, the intestinal mucosal immune barrier was restored earlier, while the content of SIgA was decreased. SIgA is the most endocrine immunoglobulin in the body and the first line of defense of intestinal mucosa against pathogens. The complex formed by SIgA and the antigen stimulates the goblet cells in the intestinal mucosa to secrete a large amount of mucus, which plays the role of flushing the intestine, removing pathogens and protecting the body from allergic reactions [37].

The level of SIgA in the treatment group was relatively low for the following possible reasons: First, it may be related to the significant increase of Paneths cells, which secrete other antibacterial proteins to restore intestinal immune function earlier and reduce the burden of immune defense of SIgA. Second, autoimmune diseases are closely related to Siga, so the relatively low content of SIgA may also be related to the immunosuppressive ability of BMSCs [38]. Third, the early restoration of intestinal mucosal structure such as mechanical barrier reduces bacterial invasion and migration, which lowers the need for SIga [39]. Therefore, we infer that the increase of Paneth cells could contribute to the restoration of the intestinal mucosal immune barrier and the decrease of SIgA is more likely due to the immunosuppressive effect of BMSCs. So the application of BMSCs may increase the incidence of intestinal infections, which may be a limitation of BMSCs applications [2].

COVID-19 is still raging, so we want to do everything we can to contain it. Some reporters showed that the levels of IL-2, IL-7, IL-10, G-CSF, IP10, MCP-1, MIP-1α, and TNF-α are higher in COVID-19 patients [40,41]. The inhibitory effect of BMSCs on the secretion of IL-2 and TNF-α may slow down the progression of COVID.

In the control group, the proportion of CD4 positive and CD8 positive T cells on day 7 was significantly decreased. There were no statistically significant differences in IL-2.4.6 levels, CD4/CD8 ratio, WBC count and gene expression levels of PRSS2 and TNF-α. In the treatment group, there was a statistically significant increase in the proportion of CD4 positive, CD8 positive T cells and gene expression levels of PRSS2 and TNF-α on day 7. There were no statistically significant difference in IL-2.4.6levels, CD4/CD8 ratio and WBC count. Although we detected certain differences between BMSC treatment and control group, we cannot rule out the possibility of bias caused by the small sample size and the results reflect the self-repair process of intestinal mucosal immunity. Other limitations of our study include commonly recognized issues with tissue specific stem cells [42] and BMSCs [43].

5. Conclusions

MSCs are often referred to as “immune-privileged cells” or “universal donors” and are considered potential candidates for cell and

gene therapy, by virtue of its immune tolerance and its ability to transplant, proliferate, and differentiate in the appropriate target tissues [44]. MSCs may be an ideal source of cells for the treatment of hereditary or degenerative diseases [42]. Our study showed that BMSCs intervention resulted in a statistically significant improvement in ischemia-reperfusion intestinal mucosal immune barrier. The immune barrier of the intestinal mucosa is a complex and multifactorial system. Although we cannot completely explain the mechanism of BMSCs' influence on the immune barrier of intestinal mucosa after ischemia/reperfusion injury, the results presented here will provide a good basis for subsequent studies. Understanding the mechanism of MSCs will make it possible to be applied as a treatment in the near future.

Author contribution statement

Haitao Jiang M. D, He Wang M. D, Kun Wang M. D and Bo Liu M. D conceived and designed the experiments. All authors performed the experiments. He Wang M. D, Xiaoqian Bian M. D and Xiaojie Tan M. D analyzed and interpreted the data and all authors contributed reagents, materials, analysis tools or data. He Wang M. D wrote the paper and all authors read and approved the final manuscript.

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Data availability statement

Data associated with this study has been deposited at Figshare https://figshare.com/s/f449d3dbeea18043e454.

Additional information

No additional information is available for this paper.

Declaration of interest's statement

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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