PESQUISA VETERINÁRIA BRASILEIRA
Brazilian Journal of Veterinary Research
ISSN 0100-736X (Print)
ISSN 1678-5150 (Online)

Pesq. Vet. Bras. 44:e07286, 2024
DOI: 10.1590/1678-5150-PVB-7286

Original Article
Small Animal Diseases
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Safety and efficacy of allogeneic bone marrow mesenchymal stem cells for treatment of canine leukopenia induced by canine parvovirus infection[1]

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ABSTRACT.- Tian Y., Zhang X., Wang Y., Guo J., Zeng M. & Yan Y. 2023. **Safety and efficacy of allogeneic bone marrow mesenchymal stem cells for treatment of canine leukopenia induced by canine parvovirus infection.** *Pesquisa Veterinária Brasileira 44:e07286, 2024.* College of Veterinary Medicine, Yunnan Agricultural University, Kunming 650000, People's Republic of China. E-mail: yanyulin333@163.com

This study aims to establish a therapy strategy for canine leukopenia induced by canine parvovirus (CPV) infection through intravenous infusion of allogeneic bone marrow mesenchymal stem cells (BMMSCs) and to evaluate the therapeutic effect of BMMSCs on canine parvovirus. Forty healthy 2-month-old dogs were randomly divided into four groups including the BMMSC treatment group (A), conventional treatment group (B), CPV infection group (C), and a normal control group (D). Then the A, B, and C groups were orally infected with CPV ($10^{3.25}$ $TCID_{50}$/mL) at 1mL/kg, and the D group received the same dose of saline. After the onset of infection, Group A received mesenchymal stem cells (MSCs) and rehydration as the treatment; Group B was treated with anti-inflammatory therapeutics and rehydration; and Group C and D were injected with the same dose of physiological saline. The level of leukocytes rebounded significantly after the treatment with BMMSCs and returned to reference numbers on Day 3 after treatment, which was significantly higher than that in the conventional treatment group. The concentrations of IL-2 and IFN-α were gradually increased during the treatment, and the BMMSC treatment group exhibited significantly higher IL-2 and IFN-α concentrations than the conventional treatment group on Days 3 and 4. The expression of the virus in the blood gradually decreased during the treatment, and the BMMSC treatment group displayed a faster decrease than the conventional treatment group. These results showed the advantages of BMMSC treatment over conventional treatment. This study provides a new BMMSC treatment strategy for canine leukopenia induced by CPV infection and reveals the mechanism by which BMMSC increases leukocytes after CPV infection.

INDEX TERMS: Allogeneic, bone marrow mesenchymal stem cells, dog, leukopenia, parvovirus infection.

RESUMO.- [**Segurança e eficácia das células-tronco mesenquimais alogênicas da medula óssea para o tratamento da leucopenia canina induzida pela infecção por parvovírus canino.**] Este estudo tem como objetivo estabelecer uma estratégia terapêutica de leucopenia canina induzida pela infecção por parvovírus canino (CPV) através de infusão intravenosa de células tronco mesenquimais da medula óssea alogênica (BMMSCs) e avaliar o efeito terapêutico de BMMSCs no parvovírus canino. Quarenta cães saudáveis de dois meses de idade foram divididos aleatoriamente em quatro grupos: o grupo de tratamento de BMMSCs (A), o grupo de terapia convencional (B), o grupo de infecção por CPV (C) e um grupo controle (D). Os grupos A, B e C foram infectados oralmente com CPV ($10^{3.25}$ $TCID_{50}$/mL) a 1mL/kg e o D recebeu a mesma dose de soro fisiológico. Após o início da infecção, o grupo A recebeu uma dose de derivadas da medula óssea (CTMMO) e hidratação como tratamento, o grupo B foi tratado com terapia anti-inflamatória e hidratação, e grupos C e D foram injetados com a mesma dose de soro fisiológico. As concentrações de IL-2 e IFN-α aumentaram gradualmente durante o tratamento, e o grupo de tratamento BMMSC mostrou concentrações significativamente maiores de IL-2 e IFN-α do que o grupo de tratamento convencional

[1] Received on June 25, 2023.
Accepted for publication on September 9, 2023.
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nos Dias 3 e 4. A expressão de vírus no sangue diminuiu gradualmente durante o tratamento, e o grupo de tratamento BMMSC mostrou uma diminuição mais rápida do que o grupo de tratamento convencional. Esses resultados mostraram as vantagens do tratamento BMMSC em relação ao tratamento convencional. Este estudo fornece uma nova estratégia de tratamento da BMMSC para leucopenia canina induzida pela infecção por VCP e revela o mecanismo pelo qual a BMMSC aumenta os leucócitos após infecção por VCP.

TERMOS DE INDEXAÇÃO: Alogênico, células-tronco mesenquimais da medula óssea, cão, caninos, leucopenia, infecção por parvovirus.

INTRODUCTION

Canine parvovirosis is caused by canine parvovirus (CPV) (Meunier et al. 1985, Fan et al. 2016). At present, canine parvovirosis exhibits a high mortality rate, and it is most common in puppies from six weeks to four months of age (Goddard & Leisewitz 2010, Freisl et al. 2017). Canine parvovirus usually appears in rapidly dividing cells and tissues, such as intestinal crypt epithelial cells, bone marrow precursor cells cardiomyocytes, and lymphoid tissues, leading to obvious clinical symptoms (Proksch & Hartmann 2015). The rate of lymphoid and intestinal cell turnover appears to be the main factor that determines the severity of the disease and high turnover rates are directly correlated with virus replication and cell destruction (O'Sullivan et al. 1984, Decaro & Buonavoglia 2012). The intestinal crypt epithelial cells are more susceptible to parvovirus invasion because of their fast-dividing microbial community. After the virus invasion, the virus will destroy the reproductive epithelium at the tip of the villus of the small intestine, so that the small intestine loses the ability to absorb nutrients, and multiple symptoms appear such as vomiting, diarrhea, intestinal mucosal bleeding, temporary elevation of white blood cells. Vomiting, diarrhea, and hematochezia tend to cause electrolyte imbalance massive protein loss, dehydration, and anemia, especially in the late stage of infection, and they also result in a significant reduction in white blood cells and red blood cells, thus leading to hypovolemic shock, potentially eventually causing death. At present, there is no specific antiviral drug to treat CPV infection in dogs. The cure rate of canine parvovirosis is low and the treatment cycle is long. Therefore, the development of new supportive treatment methods is urgently needed to reduce the mortality caused by this disease.

Bone marrow mesenchymal stem cells (BMMSCs) are multi-potent stromal cells that can differentiate into multiple types of cells, such as osteoblasts, chondrocytes, endothelial cells, and stromal fibroblasts (Djouad et al. 2014, Prodinger et al. 2017). These cells can produce various hematopoietic cytokines and growth factors through cell-cell contact to maintain hematopoietic homeostasis in the marrow, thus promoting hematopoietic stem cell proliferation and homing (Bernardo et al. 2012, Kato et al. 2014). BMMSCs can secrete adhesion molecules to identify hematopoietic cells and extracellular matrix components, which further promote hematopoietic cell proliferation and homing. BMMSCs can also secrete a variety of cytokines to maintain hematopoietic stem cells' (HSCs) self-renewal and promote HSC maturation (Majumdar et al. 2000, Broglie et al. 2017, Hira et al. 2018). The niche regulates HSC self-renewal and fate decisions, whereas HSCs modulate dynamic interactions between HSCs and their specific bone marrow microenvironments to coordinately maintain hematopoiesis and hematopoietic reconstitution (Pontikoglou et al. 2008, Lu et al. 2017). A growing number of studies have shown that BMMSC transplantation has a certain positive effect on the treatment of aplastic anemia, that co-transplantation of BMMSCs and HSCs can treat leukemia, and that BMMSCs exhibit a repair effect on the damaged gut (Psaila et al. 2012). This study is designed to establish a safe and effective therapeutic strategy for canine parvovirosis through intravenous infusion of allogeneic BMMSCs based on several clinical and laboratory tests.

MATERIALS AND METHODS

Animal Ethics. All animal experiments were approved by the Animal Care and Use Committee of Yunnan Agricultural University in China (IACUC: YNAU2019llmyh010).

Animals and chemicals. Forty healthy beagle dogs (male, two months old, body weight 2±1kg) used in this experiment were purchased from Qingdao Bolong Beagle Breeding Co., Ltd. (Shangdong province, China), with the license number SCXK (LU) 20170006. BMMSCs were obtained from three Chinese parental dogs in the same litter (three months old, male). All of the chemicals were supplied by Sigma Chemical Co. (St. Louis/MO, USA) unless otherwise stated.

Cells and viruses. F81 cells were obtained from Guangzhou Jennio Biotech Co., Ltd (Guangzhou city, China). F81 cells were cultured in an incubator (37°C, 5% CO_2) containing dulbecco-modified eagle medium supplemented with 10% (v/v) fetal bovine serum (DMEM and FBS, Gibco/Life, USA). The CPV-2a strain was isolated at the College of Veterinary Medicine, Yunnan Agricultural University. BMMSCs were isolated and cultured according to the patent "Method for Simultaneously Separating and Cultivating Dog Bone Marrow Mesenchymal Stem Cells and Multifunctional Hematopoietic Stem Cells" (patent number ZL 201410405353. X). Conventional anesthetized experimental dogs were subjected to bone marrow aspiration using a bone marrow puncture needle containing sodium heparin. Approximately 10mL of bone marrow was extracted and transferred to a 50mL sterile centrifuge tube. The 10mL bone marrow was mixed with an equal amount of DMEM/F12. The 5mL of lymphocyte separation solution was added into 10 centrifuge tubes, respectively, and then an equal amount (5mL) of bone marrow dilution solution was added. The mixture was centrifuged at 4°C and 500 × g for 20 minutes to remove adipocytes in the upper layer. Afterward, white membrane cells at the junction between adipocytes and lymphocyte separation solution were extracted, washed with DMEM/F12, and centrifuged again for 20 minutes at 250 × g. Subsequently, the supernatant was removed, and the cells were resuspended in a DMEM/F12 medium (Gibco, USA) containing 15% FBS, 100U/mL penicillin, and 100U/mL streptomycin. The obtained cell suspension was inoculated to a 25cm^2 disposable cell culture bottle at 1×10^6/mL cell density, and cultured in a cell incubator at 37°C and 5% CO_2. Three days after the culture, the medium was changed to remove bone marrow stem cells that were not attached to the wall. Afterward, the medium was changed. When the cells grew to 70% to 80% confluence, they were digested for 2 min with 0.25% trypsin (Gibco, USA), and inoculated into a new culture bottle at a ratio of 1:3. After three culture passages, cryopreservation solution was prepared at the ratio of 10% DMSO, 40% FBS, 50% DMEM/F12 and the cell density was adjusted to 1×10^6/mL. The cells were added to sterile cell cryopreservation tubes with 1mL cells per tube. The cell cryopreservation tubes were frozen in a program cooling

box (-80°C) and stored in liquid nitrogen (-196°C). BMMSCs were identified using antibodies including anti-canine CD 44 APC (RD, USA), anti-canine CD 34 PE (eBioscience, USA), anti-canine CD 45-FITC (eBioscience, USA), anti-canine CD 11a-FITC (Abcam, USA), anti-canine CD 90-APC (Bios, USA). The differentiation of BMMSCs was induced using the Bone Marrow Mesenchymal Stem Cells Osteogenic Differentiation Kit (Cyogen, USA) and Bone Marrow Mesenchymal Stem Cells Adipogenic Differentiation Kit (Cyogen, USA).

Immunofluorescence assay (IFA). After infection, the presence of the virus in cells was visualized by immunofluorescence. The fifth and tenth generation F81 cells (F81 F5 and F10) were infected with CPV-2a collected, supplemented with 4% paraformaldehyde, then fixed for 15 min at room temperature, and washed three times with phosphate-buffered saline (PBS). Afterward, F81 cells were added with 0.5% Triton X-100 permeabilized at room temperature for 30 min and washed three times with PBS. Subsequently, F81 cells were blocked with 10% FBS blocking solution for 60 min at room temperature. F81 cells were added with diluted primary antibody (CPV-2a mouse monoclonal antibody at a dilution ratio of 1:200) and incubated overnight at 4°C. F81 without primary antibody was used as a negative control. After three washes with PBS, F81 cells were added with secondary antibody (FITC labeled sheep anti-rat IgG, at the dilution ratio of 1:100), incubated at room temperature for 60 min, and washed three times with PBS. Cell nuclei were stained with DAPI, and cells were incubated at room temperature for 10 min, washed three times with PBS, and observed under a fluorescence microscope (Olympus, Tokyo, Japan)

Construction of an animal CPV infection model. The basic physical status of 40 healthy beagle dogs including skin state, body temperature, mental state, and others was examined. After passing the physical examinations, they were randomly divided into two groups, of which 30 dogs orally received CPV with a virus content of $10^{3.25}$ $TCID_{50}$/mL at 1mL/kg (experimental group), and the remaining 10 dogs received the same dose of saline (control group). Blood samples were collected from 9:00 a.m. to 12:00 a.m. (Beijing time), and the blood samples were automatically analyzed using a fully automated animal blood cell analyzer (Mindray, China). After selecting the breed of dog on the blood cell analyzer, the blood samples were automatically analyzed to obtain a blood cell count. General statuses such as behavior, appetite, and mental state of the dog were closely observed, and the typical clinical symptoms of canine parvovirosis such as diarrhea, vomiting, and bloody stools, as well as the occurrence of other adverse reactions or complications were monitored and recorded.

BMMSC administration and safety evaluation. At three days after CPV infection, the typical symptoms were observed in the dogs, the experiment group (30 dogs) was randomly divided into the BMMSC treatment group, CPV infection group, and conventional treatment group, with 10 dogs in each group. When white blood cell count was lower than reference numbers, especially when neutrophils were decreased below reference range, treatment began. The BMMSC treatment group received a mixture of BMMSC and physiological saline at the dose of 2×10^6 cells/kg through intravenous infusion (Weiden et al. 1976), followed by administration of sodium lactate ringer (dose of 50mg/kg), once a day for three consecutive days. The conventional treatment group received an intravenous infusion of ampicillin (dose 50mg/kg), metronidazole (dose 50mg/kg), and sodium lactate ringer (dose 50mg/kg). The CPV infection group and control group (without CPV) received the same dose of physiological saline for seven consecutive days. After BMMSC transplantation, changes in general physical signs such as behavior, appetite, and mental state of the dog were closely observed, especially related symptoms of canine parvovirosis. The intravenous infusion was performed by a veterinary team, who stayed with the dogs throughout the 7-day procedure. The BMMSC treatment group underwent long-term follow-up observation after recovery including monitoring and recording the occurrence of adverse reactions, side effects, or complications and evaluating the long-term safety of BMMSC transplantation.

Quantitative real-time polymerase chain reaction (qPCR). To investigate the expression changes of target genes during treatment, total DNA was extracted from the CPV vaccine (Vanguard Plus 5. Zoetis Inc., Lincoln, USA. Production lot number: 432114A) using a DNA extraction kit according to the manufacturer's protocol. The target gene primers were designed by Beijing Genomics Institute (BGI, China), and the primer sequences were as follows: Forward: 5'-CCTTGGTCATTGGTTGATG-3' and Reverse: 5'-TTAGTTGGTGGCTGAGTAG-3'. The extracted total DNA was amplified through PCR by using a Bio-Rad thermocycler. The amplified fragments were subjected to gel recovery and ligation with the pMD18-T vector. The vector with the target gene was diluted at a gradient of 10^9-10^5, after which qPCR was performed using BIO-RAD-CFX fluorescence quantitative PCR thermal cycler (BIO-RAD, USA) following the manufacturer's instructions, and then a standard curve was drawn. The qPCR was performed on a 25μL reaction system: 1μL DNA samples, 1μL forward primer, 1μL reverse primer, 12.5μL premix Taq (Takara, Japan), and 9.5μL sterilized distilled water. The qPCR amplification conditions were as follows: 95°C for 5 min, followed by 30 cycles of 95°C for 30 s, 56.9°C for 30 s, and 72°C for 30 s. Cycle threshold (CT) values were calculated from the standard curve.

Enzyme-linked immunosorbent assay (ELISA). A canine sandwich ELISA kit (Lifespan, USA) was used to determine the concentrations of canine interleukin (IL)-2 and interferon (IFN)-α. The samples in serum separator tubes were allowed to clot for 2 h at room temperature or overnight at 4°C, followed by centrifugation for 20 min at approximately 1000 × g. The supernatants were collected for subsequent assay. A standard was diluted according to the canine sandwich ELISA kit instructions, and the standard curve was generated. The standard curve was then used to determine the concentration of the target antigen in the unknown samples. All reagents, samples, and standards were prepared following the user's manual. First, 100μL of sample, standard, or blank was added to each well and incubated for 1 h at 37°C, and then the added sample and standard were removed by aspiration. Subsequently, 100μL of Solution A was added to the well plate, incubated for 1 h, and washed three times. Then 100μL of Solution B was added to the well plate, and incubated for 30 min, and washed five times. The 90μL of TMB chromogen Solution was incubated for 20 min. Finally, 50μL of stop solution was added to each well, and optical density (OD) values were immediately determined at 450nm.

Statistical analysis. All data were expressed as mean ± standard deviation (SD), using SPSS 20.0 software, and one-way analysis of variance (ANOVA) was performed to determine the significant difference between groups, $P<0.05$ was considered as statistically significant, and graphs were plotted using Prism 6.0.

RESULTS

BMMSC culture

Under an inverted microscope, BMMSCs adhered to the wall presented the shape of a long spindle, with uniform morphology and good refractive index. The cells clearly clustered and grew in a vortex-like manner. An overwhelming majority of cultured BMMSCs (>98%) consistently expressed their classical surface markers, whereas hematopoietic

cell markers were negative (Fig.1). At three weeks after osteogenic induction and adipogenic induction, BMMSC matrix mineralization was evaluated by alizarin red S staining and lipid droplet formation was evaluated by oil red O staining. The results showed that calcium deposition and the appearance of lipid droplets were observed in BMMSCs, suggesting that the isolated BMMSCs had the potential ability for osteogenic and adipogenic differentiation (Fig.2).

Virus vitality identification

The presence of the viral particles in the fifth and the tenth generations of F81 cells infected with CPV-2a was observed. As shown in Figure 3, no green fluorescent marker was observed in the cytoplasm and nucleus of normally cultured F81 cells (without CPV-2a). Some of the cytoplasm and nucleus of F81 cells infected with CPV exhibited green fluorescent markers. The high infection rate (>80%) of CPV-2 in F81 cells indicated that F81 was a susceptible cell line to CPV-2 with strong virus activity.

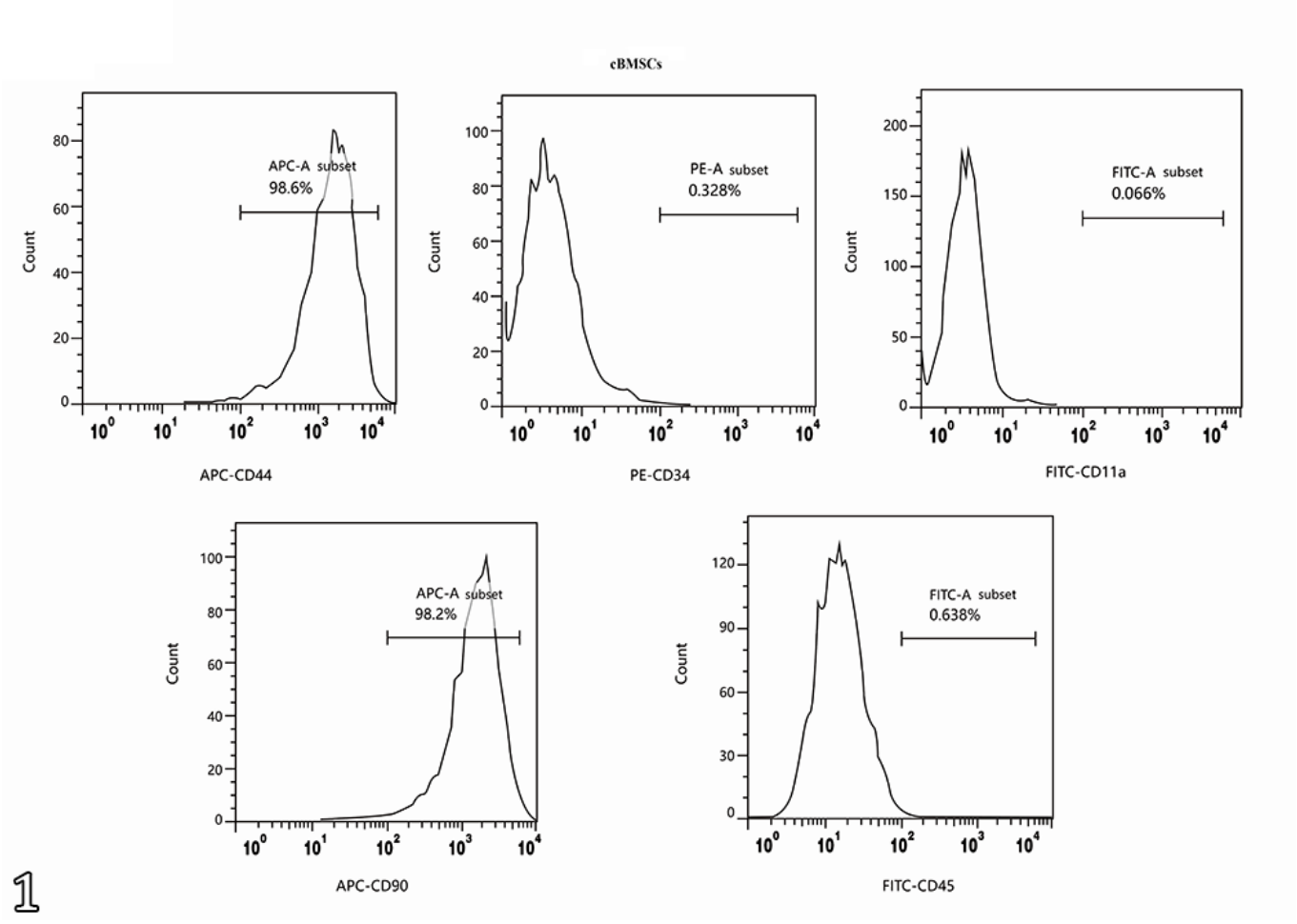

Fig.1. Characterization of dog bone marrow-derived mesenchymal stem cells (MSCs) by flow cytometry.

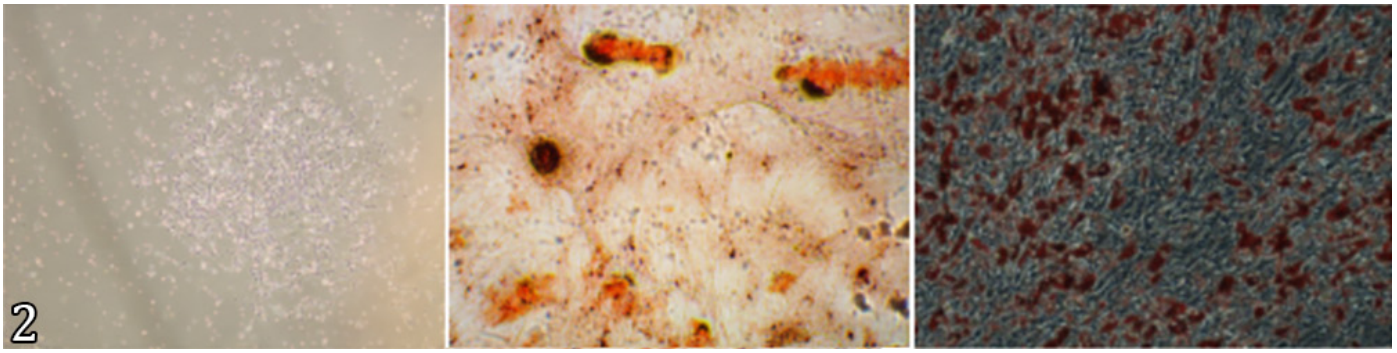

Fig.2. Induced differentiation of dog bone marrow-derived mesenchymal stem cells (MSC). The left picture shows the 7th day of primary culture of MSCs, the middle picture shows induced-osteoblastation by alizarin red staining, and the right picture shows induced-adipogenic by oil red O staining. Magnification 40x.

Clinical symptoms

The dogs in experiment groups (30 dogs) began to display vomiting, bloody stools, depression, and other symptoms on the third day after CPV infection, while the control group (10 dogs) showed no obvious symptoms. After treatment, the 10 dogs in the BMMSC treatment group were cured. On the third day after BMMSC treatment, with the white blood cell count recovered to reference numbers, symptoms of diarrhea were relieved, no vomiting and no convulsive symptoms were observed, and mental state was gradually improved. In the conventional treatment group (10 dogs), symptoms such as bloody stools, vomiting, and mental depression appeared, six dogs were cured and the remaining four died, and the white blood cell count of the cured dogs returned to reference numbers on the fourth day. In the CPV infection group (10 dogs), severe symptoms were observed, including bloody stools, vomiting, and mental depression; eight dogs died, while the remaining two cases survived. Long-term follow-up observation including regular physical examination and the health status monitoring of the BMMSC treatment group was conducted to evaluate the long-term safety of BMMSC transplantation. The results showed that there were no adverse clinical symptoms (including no convulsions or mental depression) during follow-up observation. Our results of the safety and efficacy of BMMSC treatment were consistent with the previous reports on the application of allogeneic BMMSCs to the treatment of human aplastic anemia (Pang et al. 2017, Xu et al. 2018).

Complete blood count (CBC)

During the CPV infection process, the white blood cell count of the dogs began to rise after the onset of infection and reached the maximum of 18.95×10^9/L on the second day. Then the level decreased from the third day and reached the minimum of 2.32×10^9/L, which was below the reference number, with significantly lower blood cell count. The leukocytes rebounded significantly after treatment with BMMSCs and recovered to reference ranges on the third day after treatment (Fig.4). In the conventional treatment group, the white blood cells gradually began to rise after treatment, and the white blood cell count of six dogs returned to reference numbers, and then increased more slowly, compared to the BMMSC treatment group, and the other four dogs died on the fourth day in the conventional treatment group (Fig.4). The rise of leukocytes

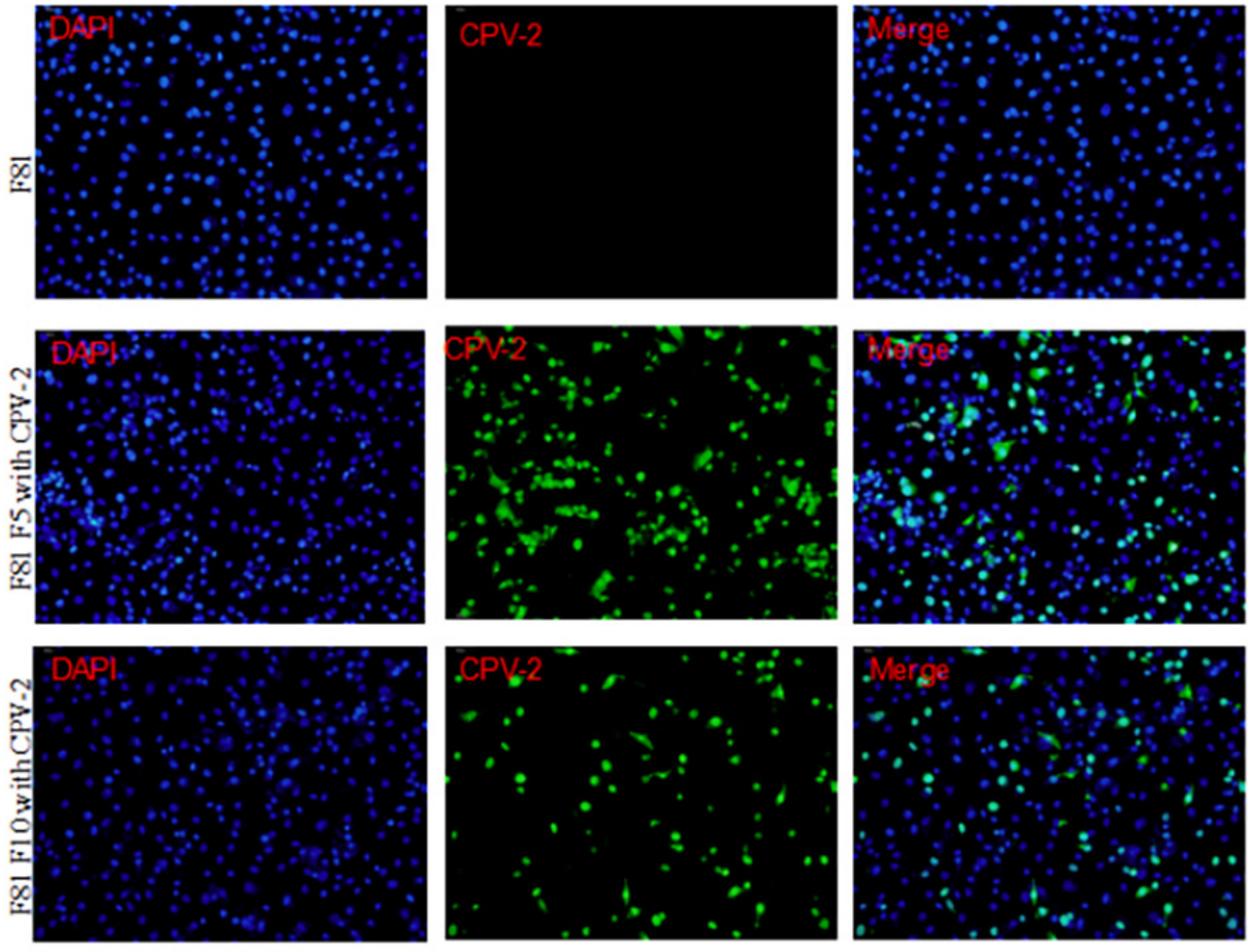

Fig.3. The distribution of canine parvovirus type 2 (CPV-2) after infection with F81 cells was determined by Immunofluorescence assay (IFA). Magnification 200x.

in the CPV infected group (without treatment) was slow, and the level of leukocytes was returned to reference numbers on the sixth day and declined continuously. The eight dogs died on the fourth and fifth days of infection, and two dogs gradually recovered in this group. The data of dead dogs were no longer recorded and the Kaplan Meier survival curve was used to record the time of death of sick dogs (Fig.5). The comparison between the BMMSC treatment group and the conventional treatment group revealed that the BMMSC treatment could quickly return leukocytes to reference numbers, thus improving immunity and achieving a rapid recovery. Both CPV infection group and conventional treatment group showed significantly lower levels of red blood cells than reference range (5.1-8.5×10^{12}/L), and red blood cell count in the CPV infection group returned to reference numbers on Day 5, while those in the conventional treatment group returned to reference numbers on Day 6 (Fig.6). The BMMSC treatment group, CPV infection group, and conventional treatments group all reached their lowest platelet count on Day 3, but they were all within the reference range (Fig.7). For the dog blood routine statistics, all the blood indicators of the BMMSC treatment group were within the reference range, indicating the safety of this BMMSC treatment. Our result was consistent with one previous report (Lan et al. 2021).

Interleukin-2 (IL-2) and interferon-α (IFN-α)

A reduction in white blood cell numbers in dogs indicates that the functions of the immune system are weakened, thus decreasing IL-2 and IFN-α secretion. Standard curves show that the correlation between OD value and IL-2 concentration or IFN-α concentration was R^2=0.999 and R^2=0.9975 respectively, with the corresponding regression equations being $y = 417.52x^2 + 267.96x - 3.4734$ and $y = 431.22x^2 + 233.14x - 9.1399$, respectively. After the onset of treatment, the number of white blood cells was increased, and the concentrations of IL-2 and IFN-α were also increased, with significantly higher concentrations in the BMMSC treatment group than in the conventional treatment group. The BMMSC treatment group demonstrated a significantly faster increase than the conventional treatment group (Fig.8 and 9). The BMMSC treatment group serum IFN-α reached a maximum level (215.23pg/mL) on Day 4, whereas the conventional treatment group serum IFN-α level reached a maximum level (186.48pg/mL) on Day 5, and then returned to the reference range. BMMSC treatment group serum IL-2 concentration reached the maximum level (25.93pg/mL) on Day 3, while the conventional treatment group achieved the maximum level (23.29pg/mL) on Day 4, and then returned to the reference range. Taken together, the serum IL-2 and IFN-α concentrations showed a faster recovery in the BMMSC treatment and conventional treatment groups than in the CPV infection group.

Changes in the expression level of CPV

The expression levels of CPV were detected by qPCR (Fig.10). The correlation coefficient (R^2) was 0.999. The primer

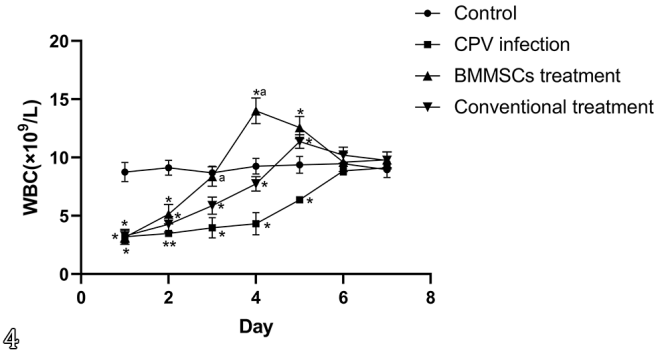

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Fig.4. Changes in white blood cell (WBC) counts during 7-day treatment. *P*<0.05 (*), *P*<0.01 (**).

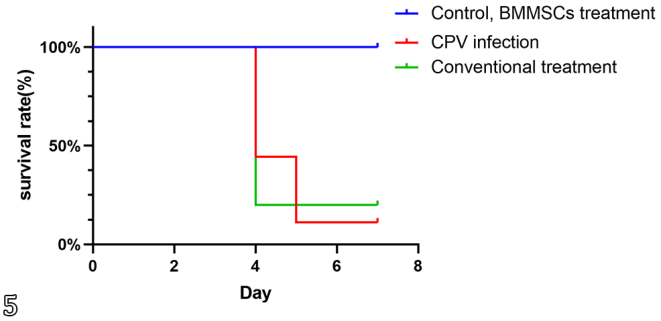

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Fig.5. Impact of canine parvovirus (CPV) infection on dog survival (with 10 dogs in each group).

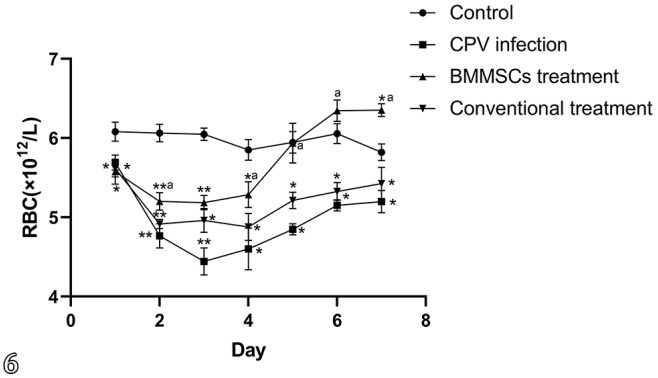

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Fig.6. Changes in red blood cell (RBC) counts during 7-day treatment. *P*<0.05 (*), *P*<0.01 (**).

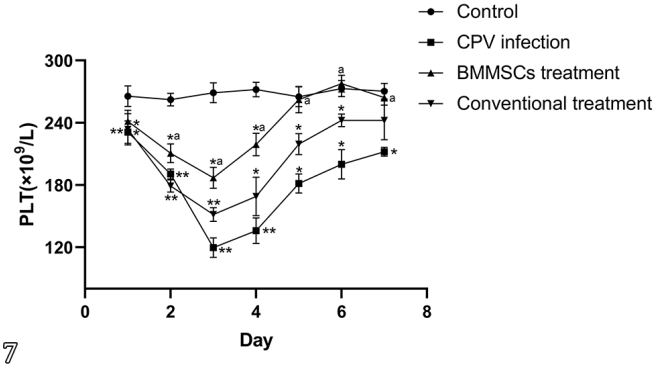

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Fig.7. Changes in platelet counts after treatment. *P*<0.05 (*), *P*<0.01 (**).

amplification efficiency (E) was 96.5%. The standard curve equation was y = −3.408x + 38.139. During the course of treatment, viral expression was gradually decreased in blood. CPV expression level was significantly lower in the BMMSC treatment group than in the conventional treatment group on Day 4 after the treatment. However, these two groups of dogs continued to produce a small amount of virus after being cured. On Day 7, the virus expression level of the BMMSC treatment group was close to that of the control group, indicating that BMMSC therapy was effective.

DISCUSSION AND CONCLUSION

With the development of science and technology, a growing number of animal models are used in the research of new drugs. However, this animal model construction is more common in human medicine and has very limited application in veterinary practice. The purpose of this study was to investigate the efficacy of BMMSCs in the treatment of CPV infection by assessing clinical sign improvements, laboratory parameters, virus expression, and recovery time.

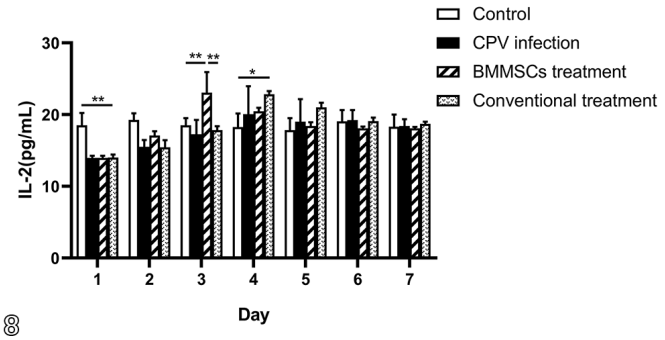

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Fig.8. Concentration of IL-2 in the serum in four groups during 7-day treatment. *P*<0.05 (*), *P*<0.01 (**).

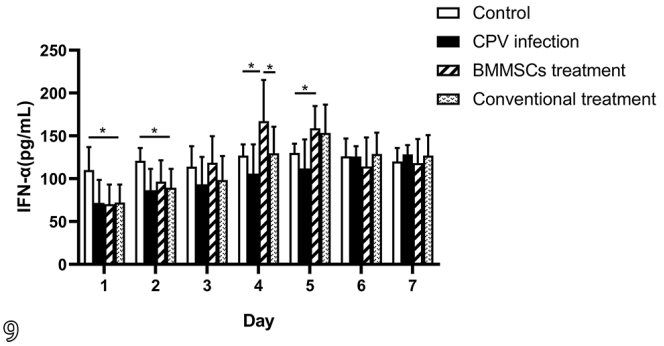

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Fig.9. Concentration of IFN-α in the serum in four groups during 7-day treatment. *P*<0.05 (*), *P*<0.01 (**).

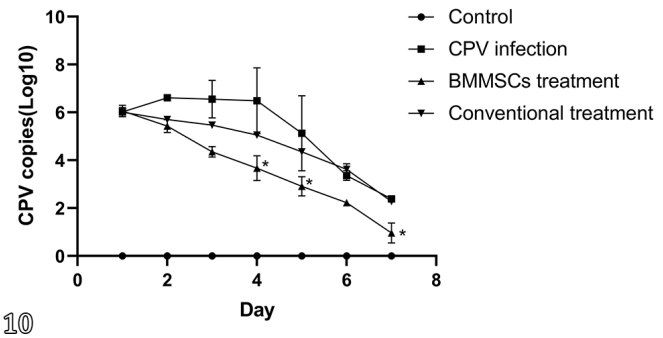

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Fig.10. Expression level of virus in four groups during 7-day treatment. *P*<0.05 (*), *P*<0.01 (**).

Intravenous autologous or allogeneic transplantation of bone marrow-derived MSCs is safe and feasible for the treatment of aplastic anemia in humans since MSCs can function to support hematopoiesis. Our results indicate that intravenous allogeneic transplantation of BMMSCs can safely and effectively treat CPV infection-induced white cell reduction is in line with the previous report by Weiden et al. (1976). In this study, after dogs were infected with CPV, the virus attacked dogs' bone marrow hematopoietic stem cells, causing anemia, leukopenia, and a decrease in platelet count. As the treatment time prolonged, blood routine test indicators were gradually recovered. By detecting changes in blood routine indicators, the severity of CPV infection can be assessed, and the healthy status of the immune system can also be determined, thus providing guidance lines for treating canine parvovirosis. BMMSCs can induce the secretion of cytokines through paracrine action, such as interleukin-6, IL-12, IL-14, IL-7, IL-11, IL-15, IL-8, and some stem cell factors and colony-stimulating factors. These cytokines can promote the proliferation and differentiation of hematopoietic stem cells, thus increasing the number of cells in the blood (Pereira et al. 2019). BMMSCs have immune regulatory functions, which can increase the number of blood cells by regulating the activity and differentiation of immune cells. The direct contact between BMMSCs and immune cells provides the support for cell-cell interactions, thus stimulating the proliferation and differentiation of immune cells, eventually increasing the number of blood cells including leukocytes. These findings reveal the action mechanisms by which BMMSCs increase leukocytes.

Several studies have reported that MSCs can promote the repair of damaged cells, improve the self-repair of the small intestinal epithelial cells, increase the nutrient absorption capacity of the small intestine, and enhance the transport of nutrients (Semont et al. 2010, Watanabe et al. 2014). A growing number of studies suggest that BMMSCs can not differentiate into target cells directly, but rather regulate the healing environment and contribute to tissue repair, which depends on their ability to regulate pathogenic immune responses and release trophic factors. Additionally, BMMSCs can secrete some cytokines by paracrine action to diffuse to adjacent target cells, thus improving the function of target cells, thus significantly alleviating vomiting, diarrhea, and other symptoms in sick dogs (Nagaya et al. 2005, Tögel et al. 2005). Our data showed that the duration of the conventional treatment was significantly longer than that of BMMSC treatment, further suggesting the advantage of BMMSC therapy over pure anti-inflammatory therapy.

This study examined the levels of IL-2 and IFN-α during treatment, and the results showed that the levels of IL-2 and IFN-α were significantly increased after intravenous infusion of allogeneic BMMSCs. IL-2 is a cytokine with a wide range of biological activities, and it is produced by activated T lymphocytes. IL-2 has multiple functions including activating T cells to enhance cytotoxic lymphocyte (CTL)

cytotoxicity, stimulating cells to secrete interferon (IFN) and other cytokines to promote the body's cellular immune response, and activating B lymphocytes to participate in the body's humoral immune response. IFN-α is a cytokine with antiviral, antitumor, and immunomodulatory functions. IFN-α is mainly produced by macrophages and has antiviral, antitumor, and immunomodulatory effects. IFN-α exhibits strong immunomodulatory function, and it can enhance the immune-killing activity of virus-infected cells. IFN-α can also enhance phagocytosis and the cellular activity of macrophages. In this study, BMMSC transplantation substantially increased the levels of IL-2 and IFN-α, which were higher than those in the conventional treatment group. The increased level of IFN-α can control the growth and proliferation of the virus. Besides, IFN-α can directly activate immune cells and inhibit the virus replication process. This explains our observation that the level of the virus in the blood gradually decreased in the course of the treatment. However, we cannot deny the fact that all dogs under recovery may affect the accuracy of the results when comparing indices such as IL-2, IFN, and blood parameters. This leads to the possibility that the differences we observed between the untreated group and conventionally treated groups may be caused by more than just the treatment. Thus, the differences between treatment groups may not fully reflect the effect of the treatment, but rather a combination of treatment and recovery process.

Our study reveals that the application of mesenchymal stem cells to the treatment of canine CPV infection is of theoretical and practical significance for the first time. However, when the number of white blood cells was below the reference ranges (3.0×10^9/L) and continued to decrease, the likelihood of the cure was small. Therefore, in the case of unsatisfactory treatment, we could consider the combination of anti-viral and anti-inflammatory therapies, or adopt other measures such as adjusting the acid-base balance. This study provides a new treatment strategy for canine leukopenia induced by CPV infection. Future studies are suggested to verify our results by using a greater number of dogs.

Authors' contributions.- The authors Yangging Tian and Xuefeng Zhang contributed to the work equally and should be regarded as co-first authors.

Acknowledgments.- This study was supported by a cooperation project between academics and industry: Application and development of canine stem cell series products (No. 2017530117000288).

Conflict of interest statement.- None of the authors have any conflict of interest.

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