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Influence of mesenchymal stem cells on respiratory distress syndrome in newborn swines *via* the JAK-STAT signaling pathway

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Abstract. – OBJECTIVE: Acute respiratory distress syndrome (ARDS) threatens humans' health worldwide, causing huge labor and economic cost investment. This study aims to explore whether mesenchymal stem cells (MSCs) affect RDS in newborn swines *via* the Janus kinase-signal transducers and activators of transcription (JAK-STAT) signaling pathway by the establishment of the model of the disease.

MATERIALS AND METHODS: The phosphorylation of the JAK-STAT signal transduction proteins was first detected *via* Western blotting to verify the regulatory effect of MSCs on RDS in newborn swines through the JAK-STAT signaling pathway. Then, the Reverse Transcription-Polymerase Chain Reaction (RT-PCR) was utilized to analyze the influences of the injection of MSCs into the blood of newborn model RDS swines on inflammatory factors *in vivo*. To further demonstrate the signal transduction function put forwarded, the RT-PCR and enzyme-linked immunosorbent assay (ELISA) were adopted to analyze the influences of the JAK-STAT signaling pathway inhibitor on the expression of the signature proteins of RDS in newborn swines and the changes in the inflammatory factors.

RESULTS: MSCs induced the phosphorylation of JAK and STAT, and they activated the JAK-STAT signal transduction of RDS in newborn swines. Compared with those in normal saline group, the interleukin (IL)-2, IL-6, IL-8, and tumor necrosis factor-α (TNF-α) expression levels in MSC group were increased, namely, MSCs substantially promoted their expression levels ($p<0.05$), but those of IL-10 and IL-13 were significantly decreased ($p<0.05$).

CONCLUSIONS: The inhibitor of the JAK-STAT signaling pathway can suppress the therapeutic effect of MSCs on RDS in newborn swines.

Key Words:
Mesenchymal stem cells, JAK-STAT signaling pathway, Respiratory distress syndrome in newborn swine.

Introduction

Acute respiratory distress syndrome (ARDS) has a high incidence rate and mortality rate around the world and has caused huge labor and financial costs[1-3]. The widely reported data show that there are about 150 thousand cases of ARDS in the United States alone[4]. The announced mortality rate among ARDS patients is somewhere from 10% to 90%. The high incidence rate and difficult diagnosis of ARDS are mainly due to the heterogeneity of underlying disease processes and the inhomogeneity of the treatment as well as the failure to confirm that ARDS patients are recognized[4].

It has been proven that mesenchymal stem cells (MSCs) have therapeutic effects on many diseases and a large potential to be tapped[5]. However, some studies[5-7] reported that the feature of these cells contributes less to their therapeutic effect. On the contrary, the secretion of various MSC growth factors and cytokines seems to provide a potential tissue healing mechanism[8].

The Janus kinase-signal transducers and activators of transcription (JAK-STAT) signal transduction is closely related to the binding of cytokines or growth factors to their corresponding receptors[9,10]. The activation of the signaling pathway keeps them close in space and promotes the change in conformation, thus separating the structural domains of their kinases from the inhibitory ones[11]. In mammal animals, the sub-receptors of JAK-STAT include JAK1, JAK2, JAK3, TYK2, STAT1, STAT2, STAT3, STAT4, STAT5a, STAT5b, and STAT6[12,13]. Both interleukin (IL)-6 and IL-10 are effective STAT3 activators in bone marrow cells, but the former mainly exerts a pro-inflammatory effect, while the main effect of the latter is to suppress inflammations.

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This study corroborated the regulatory effect of MSCs on RDS in newborn swines *via* the JAK-STAT signaling pathway by the establishment of newborn swine models of RDS. Then, the influences of the injection of MSCs into the blood of swines as the models of RDS in newborn swines on inflammatory factors *in vivo* were analyzed. Moreover, to further prove the proposed signaling function, the influences of the JAK-STAT signaling pathway inhibitor on the expression of the signature proteins of RDS in newborn swines and the changes in the inflammatory factors were analyzed, and whether MSCs affected RDS in newborn swines *via* the JAK-STAT signaling pathway was investigated.

Materials and Methods

Main Reagents and Consumables

Ribonucleic acid (RNA) isolation kit was purchased from Qiagen (Duesseldorf, Germany), protease inhibitor mixture from Roche (Basel, Switzerland), anti-phosphorylated STAT (pSTAT) antibody and anti-pJAK antibody from Cell Signaling Technology (Danvers, MA, USA), SYBR Green Kit from Bio-Rad (Hercules, CA, USA), and chemiluminescent substrates from Pierce Biotechnology (Rockford, IL, USA).

Source of Laboratory Animals

A total of 40 newborn swines aged 3-5 d (with the mean weight of 3.0 kg, breed: Yorkshire-Duroc hybrid white swines) were purchased from Huazhong University of Science & Technology Animal Laboratory Center. These swines were used to establish the newborn swine model of RDS. This study was approved by the Animal Ethics Committee of the People's Hospital of Zhangqiu Area Animal Center.

Establishment of the Model of RDS in Newborn Swines

The newborn swines were intra-pulmonary perfused with warm isotonic saline solution (30 mL/kg) repeatedly to induce acute pulmonary inflammations until the arterial partial pressure of oxygen (PaO_2)/fraction of inspired oxygen (FiO_2) ratio was less than 200 mmHg. Then, under anesthesia *via* the intravenous injection of Pentobarbital, the swines were placed on a mechanical ventilator for volume-controlled ventilation (10 mL/kg) to maintain the RR required for 35-40 torr of PaO_2, namely, the positive end-expiratory pressure was 3 cm H_2O and the mean FiO_2 (69.6±6.2)%. The pulmonary parameters (peak airway pressure, plateau airway pressure, static compliance, and airway resistance to inspiratory flow) were measured. When the oxygenation index (PaO_2/FiO_2 ratio) was less than 150 mmHg, and the dynamic lung compliance was decreased by more than 50% for over 1 h, the model of ARDS was established successfully.

Preparation of MSCs

MSCs were prepared with reference to the literature[14]. Namely, the bone marrow of homologous swines was taken to separate the marrow cells. Afterwards, the cells were cultured in the α-MEM containing 10% FBS for 2-3 d, followed by medium change and passage culture twice. Finally, the MSCs were obtained when the cell density was over 80%.

Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

The RNA isolation kit was used to extract the total RNA in blood, and the above-mentioned quantitative RT-PCR (qRT-PCR) scheme was performed[15]. In brief, the PCR was conducted using the MyiQ with a SYBR Green kit. The initial denaturalization was operated at 95°C for 3 min, followed by 10 cycles of amplification at 95°C, denaturalization at 55°C for another 10 s, and 30 s of annealing and extension. The dissociation curve was analyzed to examine single amplicon. The MyiQ analysis software was utilized to identify intersections, with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as the reference gene for calculation. The primers detected are as follows: IL-2: sense: CAGTAACCTCAACTCCTACCACA and anti-sense: CCCATTTAACATGAGAGCCACAT, IL-8: sense: ATGACTTCCAAGCTGGCCGTGGCT and anti-sense: TCTCAGCCCTCTTCAAAAACTTCT, IL-6: sense: CTCAGCCCTCTTCGGCAAAT and anti-sense: TGCCCAGTGGCACAGGTTTCT, tumor necrosis factor-α (TNF-α): sense: CTCCTCACCCACACCATCAGCCGCA and anti-sense: ATAGATGGGCTCATACCAGGGCTTG, IL-10: sense: CACCTACTTCCCAGCCAACC and anti-sense: TCAGCAGAGACTCACTCAGCAAC and IL-13: sense: CACCTGCCTGGCGGCTGCCTCC and anti-sense: AGCTGAGACCTTGTGCGGGCA, with GAPDH: sense: AGAAGGCTGGGGCTCATTTG and anti-sense: GGGGCCATCCACAGTCTTC.

Western Blotting

The proteins were isolated *via* Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE), and they were subjected to electrotransfer onto Immobilon-P membranes and incubation with the diluted primary antibody solution (pSTAT, total STAT, pJAK, and total JAK) at 4°C overnight. The secondary antibodies rejoined with 10 ng/mL horseradish peroxidase were used to detect the washed membranes. Finally, the signals were developed using SuperSignal chemiluminescent substrates, and an imager was employed for imaging.

Detection of Inflammatory Factors Via Enzyme-Linked Immunosorbent Assay (ELISA)

The concentration of the nutritional factors in the cell medium and whole tissues/cell extracts (including IL-2, IL-10, IL-8, IL-6, TNF-α, and IL-13) was measured using a kit. The tissues or cells frozen quickly were homogenized in the ice-cold lysis solution (added with normal saline containing 0.1% Triton X-100 and 2 mM EDTA). The tissue homogenate was clarified, diluted into 1 mg protein/mL, and stored at -80°C.

Statistical Analysis

Data were analyzed using GraphPad 5.0 (La Jolla, CA, USA) and expressed as mean ± SD (Standard Deviation). The unpaired *t*-test was conducted for the comparisons between the two groups. p-values < 0.05 were considered statistically significant.

Results

Influence of MSCs on the JAK-STAT Signal Transduction in RDS in Newborn Swines

To verify the regulatory effect of MSCs on RDS in newborn swines *via* the JAK-STAT signaling pathway, MSCs were injected into the blood of the swines as the model of RDS in newborn swines through their ear veins, and the phosphorylation of the JAK-STAT signaling proteins was detected *via* Western blotting. The results showed that MSCs induced the phosphorylation of JAK and STAT (Figure 1A). Additionally, it was detected at different time after the injection of MSCs (Figure 1B and 1C). Ac-

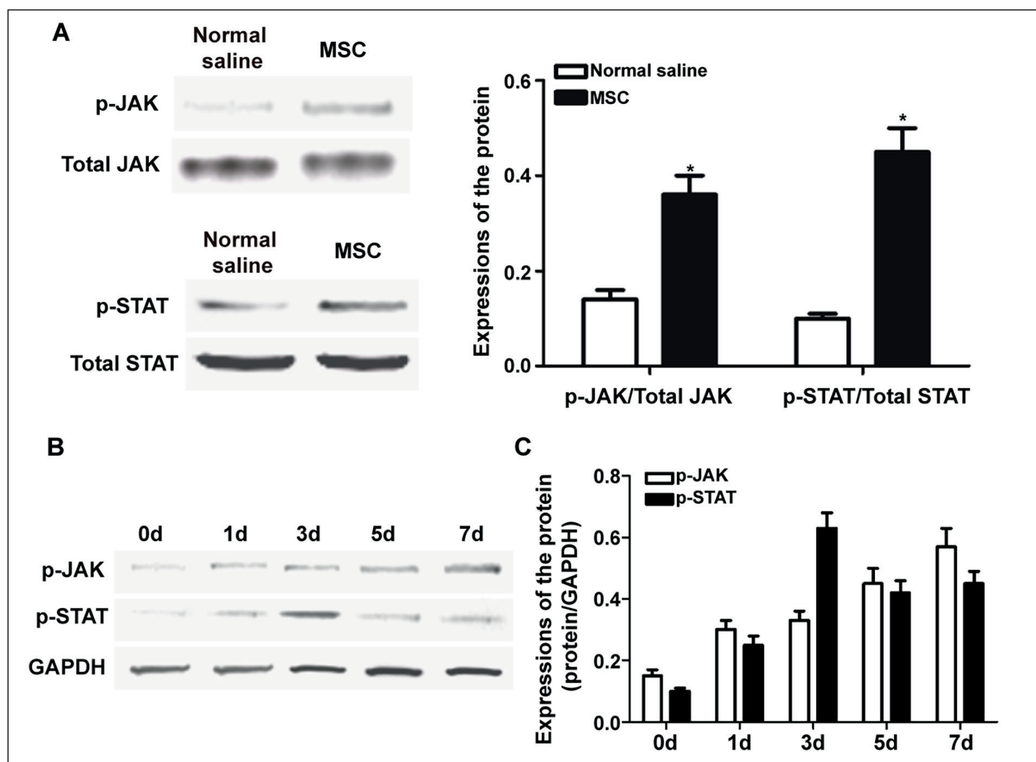

Figure 1. MSC stimulates the JAK-STAT signal transduction in RDS in newborn swines. ***A,*** The influences on the phosphorylation of JAK and STAT detected at 3, 5, and 7 d after the injection of MSCs *via* Western blotting. ***B,*** Expressions of the pJAK and pSTAT proteins at 0, 1, 3, 5, and 7 d after the injection of MSCs detected *via* Western blotting. ***C,*** Expressions of the pJAK and pSTAT genes at 0, 1, 3, 5, and 7 d after the injection of MSCs detected *via* RT-PCR.

cording to the results, the pJAK was increased with the prolonging of the action time, and the pSTAT reached the maximum value on the 3rd day. The above results proved that MSCs can activate the JAK-STAT signal transduction in RDS in newborn swines.

Influences of MSC-Mediated STAT S ignal Transduction on Inflammatory Factors In Vivo

The change in the inflammatory factors is one of the most common change features of RDS in newborn swines. Therefore, the influences of the injection of MSCs into the blood of newborn RDS swines on inflammatory factors *in vivo* were analyzed. The results showed that compared with those in normal saline group, the IL-2, IL-8, IL-6, and TNF-α expression levels in MSC group were significantly increased ($p<0.05$), but those of IL-10 and IL-13 were significantly decreased ($p<0.05$) (Figure 2).

Influences of the JAK-STAT Signaling Pathway Inhibitor on the MSC Therapeutic Effect on RDS in Newborn Swines

To further verify the proposed functional correlation of signaling cascade, whether the JAK-STAT inhibitor WP1066 could block the therapeutic efficacy of MSCs on RDS in newborn swines was assessed, and the expressions of RDS signature proteins in newborn swines were analyzed. The results revealed that the inhibitor remarkably lowered the MSC-induced increase in the pJAK/total JAK ratio and pSTAT/total STAT ratio. Moreover, the expressions of RDS signature proteins in the blood, such as receptor for advanced glycation end products (RAGE), vascular endothelial growth factor (VEGF), High Mobility Group Box 1 protein (HMGB1), and plasminogen activator inhibitor-1 (PAI-1) were detected. According to the results, compared with normal saline group, MSC group exhibited significant decreases in RAGE, HMGB1, and PAI-1 expressions, and it showed an increase in the VEGF expression (Figure 3B-3E). However, the treatment with MSC+inhibitor WP166 significantly up-regulated the expressions of RAGE, HMGB1, and PAI-1 and lowered the VEGF expression.

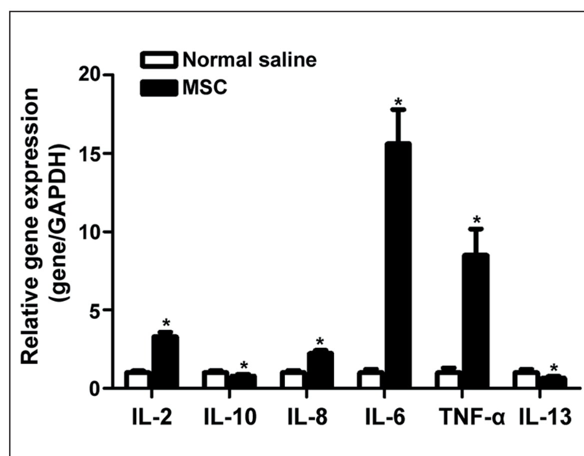

Figure 2. Influences of MSC-mediated STAT signal transduction on inflammatory factors *in vivo*. *$p<0.05$, with significant differences.

Influences of the JAK-STAT Signaling Pathway Inhibitor on the Inflammatory Factors in the Swine Blood of the Models of RDS in Newborn Swines

Furthermore, the influences of the JAK-STAT signaling pathway inhibitor on the inflammatory factors were explored. The results manifested that the inhibitor remarkably reduced the expressions of pro-inflammatory factors (IL-2, IL-6, IL-8, and TNF-α) ($p<0.05$), but promoted those of anti-inflammatory factors (IL-10 and IL-13) ($p<0.05$).

Discussion

In this study, the influence of MSCs on RDS in newborn swines *via* the JAK-STAT signaling pathway was investigated. The results proved that MSCs could repair damage in the disease through the signaling pathway. The JAK-STAT signaling pathway inhibitor markedly suppressed the repair function of MSCs for RDS in newborn swines.

With the remarkable immunoregulatory property, MSCs can be used for non-autologous or xenogeneic stem cell therapies[16]. It has been proven that the swine MSCs can be applied to repair their tissues, without causing adverse inflammations to host tissues[17]. The potent immunomodulatory effects of MSCs seem to non-specifically target the immune system cells, and some of them may be mediated by the STAT signal transduction. For example, MSC-derived IL-6 inhibits the differentiation of bone marrow progenitor cells into mature dendritic cells[18], thereby weakening their stimulation effects on the resting natural killer cells and damaging antigens as well as presenting them to T cells.

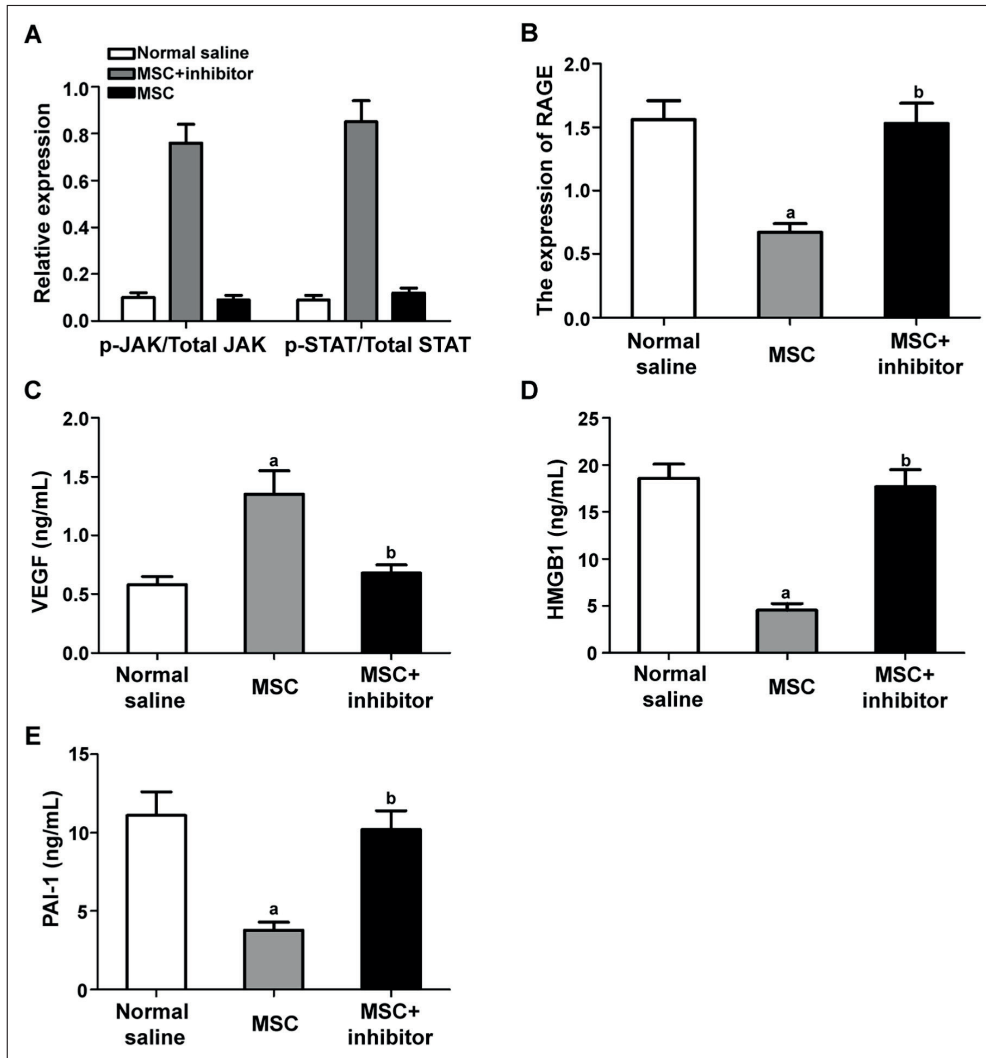

Figure 3. WP1066 inhibits the JAK-STAT signal transduction and eliminates the MSC-mediated therapeutic effect. ***A,*** The inhibitory effect of the JAK-STAT inhibitor WP1066 on the phosphorylation of the JAK-STAT detected *via* RT-PCR. ***B-E,*** Influences of the JAK-STAT inhibitor WP1066 on the expression of RDS signature proteins (RAGE, VEGF, HMGB1, and PAI-1) detected *via* ELISA. a: compared with those in normal saline group, the expressions are significantly decreased in MSC group ($p<0.05$); b: compared with those in MSC group, they are substantially elevated in MSC+inhibitor group ($p<0.05$).

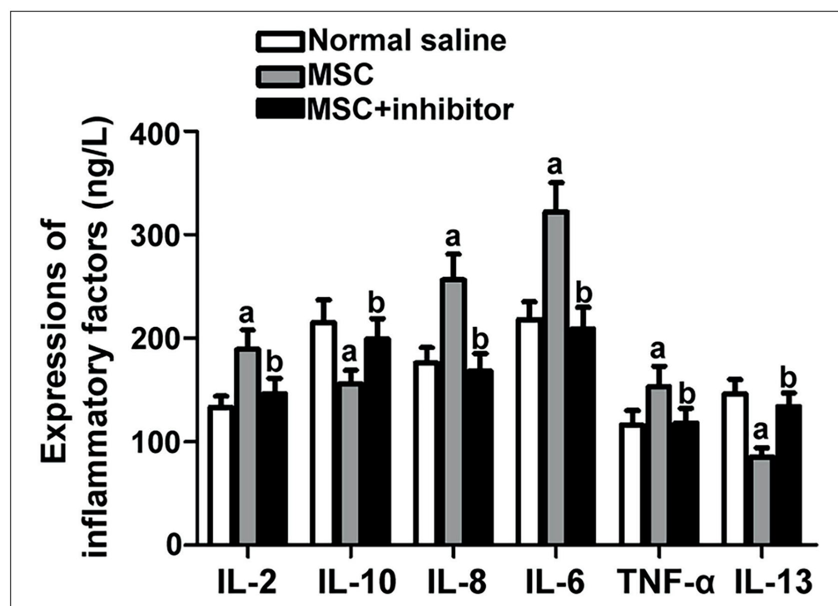

Figure 4. Expressions of inflammatory factors in blood detected *via* ELISA. $^{a}p<0.05$, *vs.* normal saline group, with significant differences and $^{b}p<0.05$, *vs.* normal saline group, with significant differences.

MSC-derived IL-8 has an anti-inflammatory effect since these cytokines can suppress the generation of the inflammatory ones through the STAT signal transduction[19]. We revealed that the nutrition function of MSCs can promote the generation of inflammatory cytokines while inducing the decrease in the anti-inflammatory cytokines. The JAK-STAT signaling inhibitor significantly promoted the increase in the levels of anti-inflammatory cytokines and the decrease in those of IL-6 and the corresponding TNF-α, suggesting that the MSC/IL-6-type cytokine loop is likely to potentially regulate the immune system through the paracrine effect. Therefore, other cells that can actively produce multiple IL-6-type cytokines may similarly help to recover from RDS in newborn swines through the intramuscular injection of the host muscle JAK-STAT signaling.

The JAK-STAT signal transduction facilitates the normal functions of the respiratory system[20]. The altered JAK-STAT3 signal transduction is related to myocardial aging[21]. MSCs can activate the JAK-STAT3 signaling *in vitro* to induce many pJAKs and pSTATs. Also, the analysis on the biomarkers of RDS (RAGE, VEGF, HMGB1, and PAI-1) revealed the influence of the JAK-STAT3 signal transduction on RDS in new swines. RAGE in the alveolar epithelium, as the receptors in alveolar epithelial cells, plays an iconic role in the occurrence of pneumonia[22]. Lung VEGF, an important regulator for the alveolar capillary membrane repair, features in the occurrence and development of ARDS[23]. HMGB1 has been proven to have a regulatory effect on the inflammatory factors in ARDS[24]. PAI-1 is closely correlated with the oxygenation index and the onset of ARDS, and the higher its content, the higher the mortality rate of ARDS[25]. Hence, this work showed that MSCs markedly decreased the expressions of RAGE, HMGB1, and PAI-1 and raised that of VEGF, indicating the repair effect of MSCs on RDS in newborn swines.

Besides, MSCs promoted the increase in the expressions of the pro-inflammatory factors and lowered those of the anti-inflammatory factors ($p<0.05$). In the newborn swine model of RDS, the MSCs produced large numbers of pro-inflammatory factors and effectively activated the host JAK-STAT signaling pathway, thus facilitating tissue repair. The JAK-STAT signaling pathway inhibitor markedly reversed the repair function of MSCs, further proving the effect of a signaling pathway in RDS in newborn swines.

Conclusions

We first revealed that MSCs can repair damage in RDS in newborn swines, and demonstrated that such an effect is mainly mediated *via* the JAK-STAT signaling pathway.

Conflict of Interest

The Authors declare that they have no conflict of interests.

References

1) Tasaka S. [Acute lung injury/acute respiratory distress syndrome: progress in diagnosis and treatment. topics: I. Pathogenesis and pathophysiology; 3. Pathogenesis and pathophysiology of ALI/ARDS]. Nihon Naika Gakkai Zasshi 2011; 100: 1529-1535.
2) Davies MW, Sargent PH. Partial liquid ventilation for the prevention of mortality and morbidity in paediatric acute lung injury and acute respiratory distress syndrome. Cochrane Database Syst Rev 2004: D3845.
3) Matthay MA, Zimmerman GA. Acute lung injury and the acute respiratory distress syndrome: four decades of inquiry into pathogenesis and rational management. Am J Respir Cell Mol Biol 2005; 33: 319-327.
4) Bakowitz M, Bruns B, McCunn M. Acute lung injury and the acute respiratory distress syndrome in the injured patient. Scand J Trauma Resusc Emerg Med 2012; 20: 54.
5) Guérit D, Maumus M, Apparailly F, Jorgensen C, Noël D. Therapeutic mesenchymal stem cells in rheumatic diseases: rationale, clinical data and perspectives. Clin Investig 2011; 1: 1269-1277.
6) Hu KK, Dai YC, Li J, Li J, Wu Q. Evaluation of wound healing effects on skin-defect nude mice by using umbilical cord blood-derived mesenchymal stem cells. Injury Extra 2008; 39: 186-187.
7) Wu YQ, Ding YJ. Overexpressed microRNA-615-3p promotes progression of neonatal acute respiratory distress syndrome by inhibiting differentiation of mesenchymal stem cells to alveolar type II epithelial cells. Eur Rev Med Pharmacol Sci 2018; 22: 4625-4633.
8) Patel DM, Shah J, Srivastava AS. Therapeutic potential of mesenchymal stem cells in regenerative medicine. Stem Cells Int 2013; 2013: 496218.
9) Seidel HM, Lamb P, Rosen J. Pharmaceutical intervention in the JAK/STAT signaling pathway. Oncogene 2000; 19: 2645-2656.
10) Rawlings JS, Rosler KM, Harrison DA. The JAK/STAT signaling pathway. J Cell Sci 2004; 117: 1281-1283.
11) Lee WH, Liu FH, Lin JY, Huang SY, Lin H, Liao WJ, Huang HM. JAK pathway induction of c-Myc crit-

ical to IL-5 stimulation of cell proliferation and inhibition of apoptosis. J Cell Biochem 2009; 106: 929-936.

12) Leu JH, Yan SJ, Lee TF, Chou CM, Chen ST, Hwang PP, Chou CK, Huang CJ. Complete genomic organization and promoter analysis of the round-spotted pufferfish JAK1, JAK2, JAK3, and TYK2 genes. DNA Cell Biol 2000; 19: 431-446.
13) Tanase S, Bawden JW. The immunohistochemical localization of signal-transduction pathway components Jak1, Jak2, Jak3, Tyk2 and STAT-1 during early enamel and dentine formation in rat molars. Arch Oral Biol 1996; 41: 925-940.
14) Peterbauer-Scherb A, van Griensven M, Meinl A, Gabriel C, Redl H, Wolbank S. Isolation of pig bone marrow mesenchymal stem cells suitable for one-step procedures in chondrogenic regeneration. J Tissue Eng Regen Med 2010; 4: 485-490.
15) Shabbir A, Zisa D, Lin H, Mastri M, Roloff G, Suzuki G, Lee T. Activation of host tissue trophic factors through JAK-STAT3 signaling: a mechanism of mesenchymal stem cell-mediated cardiac repair. Am J Physiol Heart Circ Physiol 2010; 299: H1428-H1438.
16) Nenasheva T, Nikolaev A, Diykanov D, Sukhanova A, Tcyganov E, Panteleev A, Bocharova I, Serdyuk Y, Nezlin L, Radaeva T, Adrianov N, Rubtsov Y, Lyadova I. The introduction of mesenchymal stromal cells induces different immunological responses in the lungs of healthy and M. tuberculosis infected mice. PLos One 2017; 12: e178983.
17) Shabbir A, Zisa D, Leiker M, Johnston C, Lin H, Lee T. Muscular dystrophy therapy by nonautologous mesenchymal stem cells: muscle regeneration without immunosuppression and inflammation. Transplantation 2009; 87: 1275-1282.
18) Wehner R, Wehrum D, Bornhauser M, Zhao S, Schakel K, Bachmann MP, Platzbecker U, Ehninger G, Rieber EP, Schmitz M. Mesenchymal stem cells efficiently inhibit the proinflammatory properties of 6-sulfo LacNAc dendritic cells. Haematologica 2009; 94: 1151-1156.
19) Payne NL, Sun G, McDonald C, Layton D, Moussa L, Emerson-Webber A, Veron N, Siatskas C, Herszfeld D, Price J, Bernard CC. Distinct immunomodulatory and migratory mechanisms underpin the therapeutic potential of human mesenchymal stem cells in autoimmune demyelination. Cell Transplant 2013; 22: 1409-1425.
20) Xu C, Li X, Guo P, Wang J. Hypoxia-induced activation of JAK/STAT3 signaling pathway promotes trophoblast cell viability and angiogenesis in preeclampsia. Med Sci Monit 2017; 23: 4909-4917.
21) Kleinbongard P, Schulz R, Heusch G. TNFalpha in myocardial ischemia/reperfusion, remodeling and heart failure. Heart Fail Rev 2011; 16: 49-69.
22) M'Fadyean J. Transformation of the alveolar epithelium in verminous pneumonia in the sheep. 1920. J Comp Pathol 2010; 142: 255-259.
23) Medford AR, Millar AB. Vascular endothelial growth factor (VEGF) in acute lung injury (ALI) and acute respiratory distress syndrome (ARDS): paradox or paradigm? Thorax 2006; 61: 621-626.
24) Ishizaka A, Hashimoto S. High-mobility group box 1: role in ARDS. Crit Care 2005; 9: P178.
25) Wygrecka M, Morty RE, Markart P, Kanse SM, Andreasen PA, Wind T, Guenther A, Preissner KT. Plasminogen activator inhibitor-1 is an inhibitor of factor VII-activating protease in patients with acute respiratory distress syndrome. J Biol Chem 2007; 282: 21671-21682.