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Study on the Molecular Mechanism of Traditional Medical Postural Intervention Therapy in the Treatment of Sepsis ARDS

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Abstract: Acute respiratory distress syndrome caused by sepsis has become a clinical syndrome that seriously threatens the life of severe patients. Taking cecal ligation and perforation leading to Escherichia coli peritonitis and septic ARDS rats as the model, this paper discusses the molecular pathogenesis of ARDS and the effect of prone position on RIPK3 signal pathway, in order to put forward a possible new way for the prevention and treatment of septic ARDS. A rat model of ARDS caused by sepsis was established. The supine position was maintained for 3 hours in the sham operation group; The operation group was given placebo or RIPK3 inhibitor 30 minutes before modeling; The supine position was maintained for 3h after modeling in the operation + supine position group, and the prone position was maintained for 3h after modeling in the operation + prone position group. Three hours after modeling, blood was collected from inferior vena cava, and the supernatant was taken for preservation after low-temperature centrifugation. After taking blood, take lung tissue for standby. The wet dry ratio of the left lung was measured, and the pathological conditions were observed by HE staining after partial right lung fixation. The expression of RIPK3 protein was detected by immunohistochemistry. The expression of RIPK3 protein was detected by WB in some right lung tissues. The expression level of RIPK3 protein in ARDS model rats is high. RIPK3 inhibitor can effectively reduce the expression level of RIPK3 protein in ARDS model rats, and the therapeutic effect of prone position is better than that of supine position.

1. Introduction

Nowadays, sepsis ARDS seriously threatens human life and is a serious lung injury syndrome. At present, there is no specific treatment method, and no drug can achieve gratifying new progress in

treatment. This is related to the complexity of its pathogenesis and the heterogeneity of pathophysiological changes. The TCM clinical syndrome differentiation of sepsis ARDS includes phlegm heat interaction, lung and kidney insufficiency, qi stagnation and blood stasis, and Yin evil and blood stasis. From the perspective of the overall concept of TCM and syndrome differentiation and treatment, the treatment adheres to the combination of syndrome differentiation and disease differentiation, draws on modern medical research methods, and adheres to the combination of traditional Chinese and Western medicine to reduce the suffering of patients and highlight the great potential of Chinese medicine. From the perspective of long-term illness, blood stasis and lung collateral damage, sepsis ARDS is characterized by external attack of evil and toxin, orthogonal struggle between evil and toxin, heat and toxin, invasion of the lung, loss of clearing of the lung, and adverse Qi mechanism, such as fever, shortness of breath, pulse number and other clinical signs; The lung is out of control, the Qi and blood are disadvantageous, the Qi is stagnant and the blood is stagnant, the fire and heat are abundant, and the blood stasis is stopped. There are clinical manifestations such as thin lips and nails, blue face, and dark tongue. Therefore, we reasonably assume that for patients with sepsis ARDS, changing their body position (in this study, we choose prone position) for treatment can have the functions of purging the lungs and relieving asthma, promoting water and promoting blood circulation and removing blood stasis. It can help the lungs to eliminate the disorder, reverse the disorder of Qi, improve hypoxemia, improve clinical symptoms, and play a positive role in controlling the disease process. The common ways of administration of traditional Chinese medicine include nutrition pipeline administration, enema, intravenous drip, etc. occasionally, it is combined with oral administration. The patients with sepsis ARDS are in critical condition, and the routine oral administration is difficult to apply, but the treatment in prone position is simple and easy.

Necroptosis is an important form of regulating necrotic cell death. It does not depend on the regulation of caspase and is mediated by receptor interacting serine / threonine protein kinase-3 (RIPK3) and mixed lineage kinase domain like protein (MLKL). Necrotic apoptosis can enhance inflammatory response and play an important role in ARDS [1]. Michael et al. [2] showed that plasma RIPK3 levels were associated with ARDS in sepsis and trauma populations, and RIPK3 increased significantly in serum and lung tissue of ARDS model mice. Pan L et al. [3] explored the role of necroptosis in the rat model of acute respiratory distress syndrome (ARDS) induced by oleic acid (OA). The results showed that RIPK3 was highly expressed in the lung tissue of OA induced ARDS rats. The expression of RIPK3 decreased after corresponding treatment. It is suggested that inhibiting RIPK3 mediated necrotic apoptosis may be one of the effective strategies for the treatment of ARDS, but at present, the mechanism of RIPK3 in ARDS is still unclear.

This study intends to establish a rat model of sepsis ARDS by ligation and perforation of cecum leading to E. coli peritonitis, and to explore the molecular pathogenesis of sepsis ARDS and the effect of postural intervention therapy on RIPK3 signaling pathway, so as to provide a possible new method and approach for the prevention and treatment of sepsis ARDS under the guidance of the theory of "lung injury and collateral injury" in traditional Chinese medicine.

2. Method

2.1. Experimental Animal

30 SD rats, weighing (210 ±20) g, male, were provided by the animal center of Nanchang Medical College for 8-12 weeks. They were kept in the barrier room for 2 weeks and then used for the experiment. Feeding management: Ensure that the rats are sterile when entering the experimental area. The animal center of Nanchang Medical College provided feed and sterilized water for self feeding. The set temperature in the living area of rats is 18 ℃-26 ℃, and the relative humidity of air is 30%-

70%. Ultraviolet lamp air disinfection twice a day, 20 minutes each time. Spray disinfection shall be carried out on the table and ground of animal laboratory regularly. All experimental articles were disinfected. Once a week: disinfect and wipe the indoor walls, roofs and used instruments once. The bedding material in the living area of rats is coarse wood chips, which are replaced twice a week.

2.2. Experimental Grouping

They were divided into 5 groups with 5 rats in each group:
S group: Sham operation group
SP group: Operation + supine position group (placebo)
SI group: Operation + supine position group (RIPK3 inhibitor)
PP group: Operation + prone position group (placebo)
PI group: Operation + prone position group (RIPK3 inhibitor)

2.3. Animal Model Preparation

The rats were intraperitoneally injected with 10% chloral hydrate at an injection dose of 300mg/kg. After anesthesia, the hair on the abdomen of the rats was removed. The rats were placed on the foam board and fixed in a supine position. According to the principle of aseptic operation, the abdomen of the rats was disinfected with iodophor, and then the operation scissors were gently cut from the middle of the abdomen to expose the abdominal cavity of the old rats. The small intestine was pulled out with a cotton swab to find the cecum, and the abdominal cavity was placed on a wet gauze, Then ligate 1/4 of the cecum with surgical suture, then puncture the rear end of the ligated 1/4 cecum with 10ml needle for 3 times, squeeze out the feces with cotton swab and plug it back into the abdominal cavity, take 2ml of 0.9% normal saline to infiltrate the intestinal tube, and then suture the intestinal tube to close the abdominal cavity. In order to prevent animal shock, 50ml/kg of 0.9% normal saline is injected subcutaneously into the abdominal cavity immediately after abdominal closure.

2.4. Detection Index

2.4.1. Determination of Lung Dry Wet Ratio

Separate the left lung of the mouse, remove other superfluous tissues, control the surface moisture with gauze, make weighing records, then put it into the drying oven, bake it at 80℃ for three days, take it out for weighing, bake it for another day, take it out for weighing until the weight is not reduced. The final value is determined as the dry weight, and then the dry weight value is compared with the wet weight value.

2.4.2. HE Staining Was Used To Examine the Pathological Sections of Each Group

Paraffin sections were dewaxed and hydrated→ Hematoxylin stained nuclei→ Eosin stained cytoplasm→ Dehydration seal→ Microscopic examination, image acquisition and analysis.

2.4.3. The Expression of RIPK3 Protein Was Detected By Immunohistochemistry

Section pretreatment→Section antigen repair→Elimination of endogenous peroxidase→Non-specific blocking→Immune reaction→Chromogenic counterstaining.

2.4.4. Western Blot Was Used To Detect the Expression of Ripk3 Protein in Each Group

Ripk3 protein extraction→Determination of RIPK3 protein concentration→Gel

preparation→Loading→Protein electrophoresis→Membrane transfer→Blocking→Incubation of primary Antibody→Incubation of secondary antibody→Exposure and development.

2.5. Statistical Analysis

SPSS 19.0 software was used for statistical analysis. All experiments were repeated three times, and the quantitative results were expressed as mean ± standard deviation (x ± s). One way ANOVA was used for quantitative numerical comparison among multiple groups, and LSD method was used for comparison between two groups. Inspection level α= 0.05, graphpad5.0 software was used for mapping, and imagepro J software was used for gray value analysis.

3. Results

3.1. 3h after Modeling, the Dry Wet Ratio of Left Lung in Each Group

Compared with the placebo supine group, the dry wet ratio of the inhibitor prone group was increased, and the difference was statistically significant (P<0.05). (See Table 1, Table 2).

3.2. Pathological Conditions of Each Group

Microscopic observation of he stained sections of rat lung specimens showed that the lung structure of sham operation group was clear under microscope. In the operation + supine position placebo group and operation + prone position placebo group, the normal structure of lung tissue was destroyed, alveolar epithelium was diffusely damaged, and pulmonary interstitial hemorrhage was accompanied by a large number of inflammatory cell infiltration. The lung structure of rats in the operation + supine RIPK3 inhibitor group is relatively clear, but there is still some diffuse injury of alveolar epithelium, while the lung structure of rats in the operation + supine RIPK3 inhibitor group is clear, suggesting that RIPK3 inhibitor has a certain therapeutic effect on ARDS model rats, and the therapeutic effect of prone position is better than that of supine position (see Figure 1).

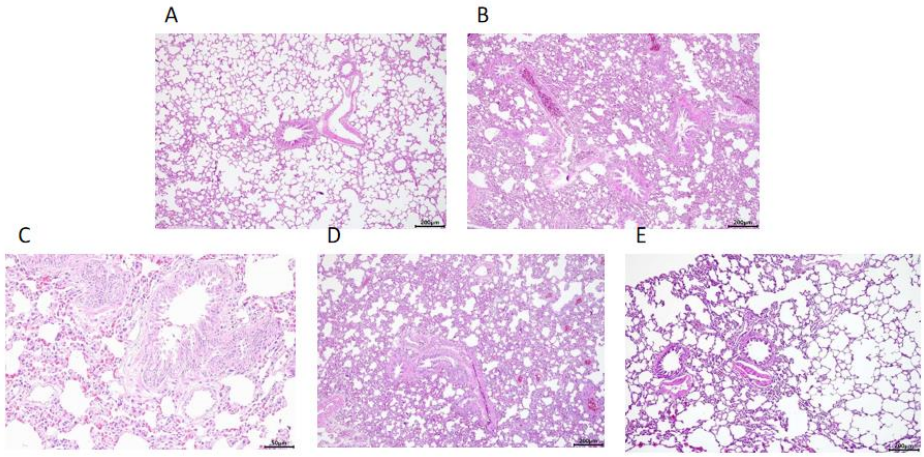
Table 1: Analysis of dry wet ratio data

group	n	mean value	standard deviation	Standard error	95% CI		minimum value	maximum value
					Confidence lower limit	Confidence upper limit		
S	5	21.3171	0.8774	0.3924	20.2275	22.4066	20.2817	22.4244
PP	5	20.8361	0.9369	0.4190	19.6727	21.9994	19.9478	22.0351
SP	5	20.1502	0.8183	0.3659	19.1341	21.1663	19.0501	21.2229
PI	5	21.8716	1.2005	0.5369	20.3809	23.3623	19.7907	22.8375
SI	5	20.6672	1.775	0.7941	18.4624	22.8720	17.7322	22.2696
total	25	20.9684	1.2285	0.2457	20.4613	21.4755	17.7322	22.8375

Table 2: Multiple comparisons between groups

(I) group	(J) group	Mean difference (I-J)	Standard error	Significance	95% CI	
					Confidence lower limit	Confidence upper limit
S	PP	0.4809	0.7436	0.525	-1.0701	2.0321
	SP	1.1668	0.7436	0.132	-0.3842	2.7180
	PI	-0.5545	0.7436	0.464	-2.1057	0.9965
	SI	0.6498	0.7436	0.393	-0.9012	2.2010
PP	S	-0.4809	0.7436	0.525	-2.0321	1.0701
	SP	0.6858	0.7436	0.367	-0.8652	2.2370
	PI	-1.0355	0.7436	0.179	-2.5866	0.5155
	SI	0.1688	0.7436	0.823	-1.3822	1.7200
SP	S	-1.1668	0.7436	0.132	-2.7180	0.3842
	PP	-0.6858	0.7436	0.367	-2.2370	0.8652
	PI	-1.7214*	0.7436	0.031	-3.2725	-0.1703
	SI	-0.5169	0.7436	0.495	-2.0681	1.0341
PI	S	0.5545	0.7436	0.464	-0.9965	2.1057
	PP	1.0355	0.7436	0.179	-0.5155	2.5866
	SP	1.7214*	0.7436	0.031	0.1703	3.2725
	SI	1.2044	0.7436	0.121	-0.3466	2.7555
SI	S	-0.6498	0.7436	0.393	-2.2010	0.9012
	PP	-0.1688	0.7436	0.823	-1.7200	1.3822
	SP	0.5169	0.7436	0.495	-1.0341	2.0681
	PI	-1.2044	0.7436	0.121	-2.7555	0.3466

*The significance level of mean difference is <0.05.



A. Sham operation group; B. Operation + supine position group (placebo); C. Operation + supine position group (RIPK3 inhibitor); D. Operation + prone position group (placebo); E. Operation + prone position group (RIPK3 inhibitor)

Figure 1: Pathological conditions of each group

3.3. The Expression of RIPK3 Protein Was Detected by Immunohistochemistry

The positive rate of RIPK3 immunohistochemistry in each group was expressed by the integrated optical density (IOD) / effective area of immunohistochemical image gray analysis. The results were 0.0980 ± 0.0062 in the sham operation group, 0.1329 ± 0.0083 in the operation + supine RIPK3 inhibitor group, 0.0981 ± 0.0084 in the operation + supine RIPK3 inhibitor group, 0.1608 ± 0.0167 in the operation + supine placebo group and 0.0898 ± 0.0035 in the operation + supine RIPK3 inhibitor group. The results of multiple comparisons between groups showed that compared with the control group, the expression level of RIPK3 protein in operation + supine placebo group (P = 0.001) and operation + prone placebo group (P = 0) increased significantly, and the difference was statistically significant (P < 0.05), suggesting that the expression level of RIPK3 in ARDS model rats was high. Compared with the operation + supine position placebo group, the expression level of RIPK3 protein in the operation + supine position RIPK3 inhibitor group (P = 0.001) decreased significantly. Compared with the operation + prone position placebo group, the expression level of RIPK3 protein in the operation + prone position RIPK3 inhibitor group (P = 0) decreased significantly, and the difference was statistically significant (P < 0.05), suggesting that RIPK3 inhibitor can effectively inhibit the expression of RIPK3 in ARDS model rats. Compared with the operation + supine RIPK3 inhibitor group, the expression level of RIPK3 protein in the operation + prone RIPK3 inhibitor group decreased, but the difference was not statistically significant (P = 0.32), suggesting that the curative effect of the prone position has certain advantages over the supine position (see Table 3, Table 4, Figure 2).

3.4. The Expression of RIPK3 Protein in Each Group Was Detected by WB

Table 3: Ratio analysis of IOD/Area

group	n	mean value	standard deviation	Standard error	95% CI		minimum value	maximum value
					Confidence lower limit	Confidence upper limit		
S	5	0.0980	0.0062	0.0036	0.0823	0.1136	0.0924	0.1048
SP	5	0.1329	0.0083	0.0048	0.1121	0.1538	0.126	0.1425
SI	5	0.0981	0.0084	0.0048	0.0771	0.1192	0.0914	0.1076
PP	5	0.1608	0.0167	0.0096	0.1191	0.2024	0.1460	0.1790
PI	5	0.0898	0.0035	0.0020	0.0809	0.0986	0.0866	0.0936
total	25	0.1159	0.0290	0.0074	0.0998	0.1320	0.0866	0.1790

The WB positive rate of RIPK3 in each group was expressed by the IOD ratio between RIPK3 group and GAPDH group. The results were 0.5938 ± 0.1817 in sham operation group, 1.9611 ± 0.6078 in operation + supine position placebo group, 1.0616 ± 0.2730 in operation + supine position RIPK3 inhibitor group, 2.3710 ± 0.5867 in operation + prone position placebo group and 0.7261 ± 0.1216 in operation + prone position RIPK3 inhibitor group. The results of multiple comparisons between groups showed that compared with the control group, the expression level of RIPK3 protein in operation + supine placebo group (P = 0.002) and operation + prone placebo group (P = 0) increased significantly, and there was significant difference (P < 0.05), suggesting that the expression level of RIPK3 in ARDS model rats was high. Compared with the operation + supine position placebo group, the expression level of RIPK3 protein in the operation + supine position RIPK3 inhibitor group (P = 0.023) decreased significantly. Compared with the operation + prone position placebo group, the expression level of RIPK3 protein in the operation + prone position RIPK3 inhibitor group (P = 0.001)

decreased significantly (P < 0.05), suggesting that RIPK3 inhibitor can effectively reduce the expression level of RIPK3 in ARDS model rats. Compared with the operation + supine RIPK3 inhibitor group, the expression level of RIPK3 protein in the operation + supine RIPK3 inhibitor group (p=0.339) decreased, but the difference was not statistically significant (P > 0.05), suggesting that the curative effect of the prone position was better than that of the supine position (see Table 5, table 6).

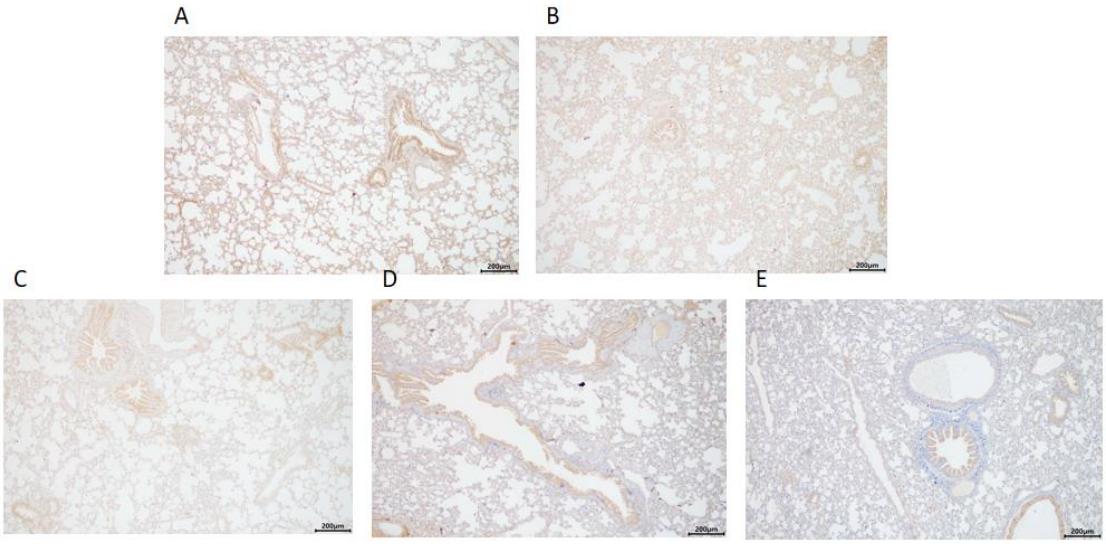
Table 4: Multiple comparisons between groups

(I) group	(J) group	Mean difference (I-J)	Standard error	Significance	95% CI Confidence lower limit	95% CI Confidence upper limit
S	SP	-0.0349*	0.0079	0.001	-0.0527	-0.0172
	SI	-0.0001	0.0079	0.985	-0.0179	0.0175
	PP	-0.0627*	0.0079	0	-0.0805	-0.0450
	PI	0.0081	0.0079	0.329	-0.0095	0.0259
SP	S	0.0349*	0.0079	0.001	0.0172	0.0527
	SI	0.0347*	0.0079	0.001	0.0170	0.0525
	PP	-0.0278*	0.0079	0.006	-0.0455	-0.0100
	PI	0.0431*	0.0079	0	0.0253	0.0608
SI	S	0.0001	0.0079	0.985	-0.0175	0.0179
	SP	-0.0347*	0.0079	0.001	-0.0525	-0.0170
	PP	-0.0626*	0.0079	0	-0.0803	-0.0448
	PI	0.0083	0.0079	0.32	-0.0094	0.0260
PP	S	0.0627*	0.0079	0	0.0450	0.0805
	SP	0.0278*	0.0079	0.006	0.0100	0.0455
	SI	0.0626*	0.0079	0	0.0448	0.0803
	PI	0.0709*	0.0079	0	0.0532	0.0887
PI	S	-0.0081	0.0079	0.329	-0.0259	0.0095
	SP	-0.0431*	0.0079	0	-0.0608	-0.0253
	SI	-0.0083	0.0079	0.32	-0.0260	0.0094
	PP	-0.0709*	0.0079	0	-0.0887	-0.0532

*The significance level of mean difference is <0.05.

Table 5: Ratio analysis of IOD

	n	mean value	standard deviation	Standard error	95% CI Confidence lower limit	95% CI Confidence upper limit	minimum value	maximum value
S	5	0.5938	0.1817	0.1049	0.1424	1.0453	0.4488	0.7977
SP	5	1.9611	0.6078	0.3509	0.4511	3.4712	1.3797	2.5924
SI	5	1.0616	0.2730	0.1576	0.3834	1.7398	0.8244	1.3600
PP	5	2.3710	0.5867	0.3387	0.9134	3.8286	1.7224	2.8649
PI	5	0.7261	0.1216	0.0702	0.4238	1.0284	0.6425	0.8657
total	25	1.3427	0.8041	0.2076	0.8974	1.7880	0.4488	2.8649



A. Sham operation group; B. Operation + supine position group (placebo); C. Operation + supine position group (RIPK3 inhibitor); D. Operation + prone position group (placebo); E. Operation + prone position group (RIPK3 inhibitor)

Figure 2: Immunohistochemistry of each group

Table 6: Multiple comparisons between groups

(I) group	(J) group	Mean difference (I-J)	Standard error	Significance	95% CI	
					Confidence lower limit	Confidence upper limit
S	SP	-1.3673*	0.3339	0.002	-2.1112	-0.6233
	SI	-0.4677	0.3339	0.192	-1.2117	0.2762
	PP	-1.7771*	0.3339	0	-2.5211	-1.0331
	PI	-0.1322	0.3339	0.7	-0.8762	0.6117
SP	S	1.3673*	0.3339	0.002	0.6233	2.1112
	SI	0.8995*	0.3339	0.023	0.1556	1.6435
	PP	-0.4098	0.3339	0.248	-1.1538	0.3341
	PI	1.2350*	0.3339	0.004	0.4910	1.9790
SI	S	0.4677	0.3339	0.192	-0.2762	1.2117
	SP	-0.8995*	0.3339	0.023	-1.6435	-0.1556
	PP	-1.3094*	0.3339	0.003	-2.0534	-0.5654
	PI	0.3354	0.3339	0.339	-0.4085	1.0794
PP	S	1.7771*	0.3339	0	1.0331	2.5211
	SP	0.4098	0.3339	0.248	-0.3341	1.1538
	SI	1.3094*	0.3339	0.003	0.5654	2.0534
	PI	1.6449*	0.3339	0.001	0.9009	2.3888
PI	S	0.1322	0.3339	0.7	-0.6117	0.8762
	SP	-1.235*	0.3339	0.004	-1.9790	-0.4910
	SI	-0.3354	0.3339	0.339	-1.0794	0.4085
	PI	-1.6449*	0.3339	0.001	-2.3888	-0.9009

*The significance level of mean difference is <0.05.

4. Discussion

Necroptosis is a form of regulation of necrotic cell death that is independent of caspase regulation and mediated by receptor interacting serine / threonine protein kinase-3 (RIPK3) and mixed lineage kinase domain like protein (MLKL) [4,5]. Recent studies have shown that necroptosis is a key mode of cell death in tissue and animal injury models. Studies have shown that lung injury induced by lipopolysaccharide and erythrocytes can be weakened by inhibiting necrotic apoptosis [6, 7]. This form of cell death may have a special relationship with ARDS. Unlike apoptosis, necroptosis leads to tissue damage by activating cell membrane lysis and driving the release of damage related molecular patterns (DAMPs). Unlike the traditional description of cell necrosis, the induction and execution of necrotic apoptosis are highly regulated [5, 8-12]. Thus, Necroptosis may be a new potential therapeutic target for the prevention or treatment of acute organ dysfunction.

Our research also has shortcomings. Firstly, this study used the septic ARDS rats induced by E. coli peritonitis caused by cecal ligation and perforation as the model. This model can not fully simulate the complex pathophysiological changes of ARDS patients, and it is also necessary to carefully extrapolate the results of this study to ARDS patients. Secondly, this study found that the difference of RIPK3 protein expression in the lung of rats in supine position and prone position was related to ARDS, but a larger study was needed to determine the correlation between plasma and lung RIPK3 concentration. If there was a correlation, the detection of plasma RIPK3 may help to reflect the expression of RIPK3 in lung tissue.

5. Conclusion

Through this experiment, we can draw the following conclusions: the expression level of RIPK3 protein in ARDS model rats is high, RIPK3 inhibitor can effectively reduce the expression level of RIPK3 protein in ARDS model rats, and the therapeutic effect of prone position is better than that of supine position. The study of this biomarker will help to further understand ARDS and open up a new way to prevent and treat ARDS.

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