SIRT1 REPAIRS DEEP VEIN THROMBOSIS INDUCED BY OXIDATIVE STRESS THROUGH INHIBITING PRO-APOPTOTIC PATHWAY, INFLAMMATORY RESPONSE PATHWAY AND ACTIVATING ANTI-APOPTOTIC PATHWAY

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ABSTRACT

Objective*: To analyze the role of silencing information regulator-1 (SIRT1) in repairing oxidative stress-induced deep vein thrombosis by inhibiting the pro-apoptotic pathway and inflammatory response pathway and activating the anti-apoptotic pathway.*

Methods: *72 healthy female C57BL/6 mice (clean grade) were randomly selected and divided into a sham operation group and model group, with 36 mice in each group. The deep vein thrombosis model was established, and the histopathological changes of inferior vena cava in mice were observed. The levels of malondialdehyde (MAD), superoxide dismutase (SOD), and the expression of SIRT1 in each group were measured. Human umbilical vein endothelial cells (HUVECs) were selected and cultured to determine the changes of cell activity and apoptosis in each group. The expression of apoptosis-related proteins in each group was measured. The expression of SIRT1, extracellular protein kinase (ERK), mitogen-activated protein kinase p38 (p38), nuclear transcription factor-κB (NF-κB), protein kinase B (Akt), glycogen synthesis kinase 3β (GSKβ), P-selection glycoprotein ligand 1 (PSGL-1), thrombomodulin (TM), von Willebrand factor (vWF), and tissue plasminogen activator (t-PA) was detected.*

Results: *In the sham operation group, the wall of inferior vena cava was regular and flat, the intima, muscularis, and tunica externa were intact, the endothelial cells were arranged regularly, the nuclei were normal, and there was no inflammatory cell infiltration and thrombosis. In the model group, the wall thickness of inferior vena cava was uneven, the volume of endothelial cells and the size of nuclei were different, inflammatory cell infiltration was observed, and a large amount of thrombosis was observed in the lumen. Compared with the sham operation group, SOD and SIRT expression levels in the inferior vena cava of the model group were significantly decreased, while MDA expression levels were obviously increased (P<0.01). Compared with the control group, the cell activity and expression levels of SIRT1 and Akt were remarkably decreased in the hydrogen peroxide group, and the apoptosis rate and expression levels of Bcl-2, Caspase-3, PSGL-1, TM, vWF, t-PA, ERK, p38, NF-κB, and GSK3β were obviously increased in the hydrogen peroxide group (P<0.05). Compared with the hydrogen peroxide group, the cell activity and the expression levels of SIRT1 and Akt were significantly increased in the SIRT1 group, and the apoptosis rate, the expression levels of Bcl-2, Caspase-3, PSGL-1, TM, vWF, t-PA, ERK, p38, NF-κB, and GSK3β were obviously decreased in the SIRT1 group (P<0.05).*

Conclusion: *SIRT1 was involved in the process of deep vein thrombosis by activating the PI3K/Akt/GSK3β signaling pathway and inhibiting the MAKs and NF-κB signaling pathways, suggesting that SIRT1 might repair oxidative stress-induced deep vein thrombosis by inhibiting the pro-apoptotic pathway and inflammatory response pathway and activating the anti-apoptotic pathway.*

Keywords: *SIRT1, pro-apoptotic pathway, inflammatory response pathway, anti-apoptotic pathway, oxidative stress, deep vein thrombosis injury.*

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Introduction

Deep venous thrombosis (DVT) refers to abnormal clotting of blood in the deep venous cavity and is a common and frequently-occurring disease in cardiovascular diseases. Detachment of thrombus can cause pulmonary artery embolism, hemodynamic instability, and even endanger the patient's life. Deep vein thrombosis and pulmonary artery embolism, collectively known as venous thromboembolism, is one of the leading causes of perioperative death in orthopedics as well as an important cause of unexpected death in hospitals[(1)]. In recent years, studies have confirmed that venous

injury, venous blood stasis, and hypercoagulability are the pathological basis of deep vein thrombosis[2]. At present, the diagnostic method of deep vein thrombosis still relies on high coagulation state or surgical history, clinical manifestation, vascular ultrasound and angiography, hematological examination, and other methods. However, the above methods have certain hysteresis, are difficult to predict and diagnose early, and often develop into pulmonary artery embolism or even venous thromboembolism, which has a serious impact on patients' lives and health. The pathogenesis of DVT is complex and involves multiple systems and factors, often including heredity and acquisition. Its pathophysiological basis often involves multiple roles such as vascular endothelial cells, platelets, coagulation factors, and fibrinolysis systems[3].

As an important structure of vascular intima, venous endothelial cells are not only the bridge of molecular transmission between blood and tissue fluid, as well as tissue cells, but also secrete some important factors, which can play an anticoagulant or pro-coagulant role by maintaining the integrity of the vascular wall, regulating vasoconstriction, and expressing adhesion molecules and cytokines[4]. Studies have found that endothelial cell injury may be the initiating factor of deep vein thrombosis, while oxidative stress is one of the important factors of endothelial cell injury, which can cause vascular wall damage and thus cause thrombosis[5]. Reactive oxygen species (ROS) are directly involved in the pathological process of endothelial cell oxidative damage. Silencing information regulator-1 (SIRT1) is a kind of class III histone acetylation enzyme, which plays an important regulatory role in many biological processes such as gene repair, metabolism, and oxidative stress. Some studies have suggested that SIRT1 plays an important role in venous thrombotic diseases, but its mechanism of action remains unclear[6]. This study mainly investigated and analyzed the changes of SIRT1 in oxidative stress injury of deep venous endothelial cells and its mechanism of action.

Data and methods

Experimental animal

72 healthy female C57BL/6 mice (clean grade, provided by Shanghai Ruitai Mosi Biotechnology Co., Ltd., Production License SCXK (Shanghai) 2016-0001) were randomly selected, with body weight of (22±2) g.

Human umbilical vein endothelial cells (HUVECs) were purchased from Shanghai Hongshun Biotechnology Co., Ltd.

Main instruments and reagents

The main instruments and reagents were the following: high pressure sterilizer (Shanghai Shenan Medical Device Factory, Model: LDZM-80KCS-III); optical microscope (Shanghai Optical Instrument Factory, Model: SG-51); thermostatic water bath (Changzhou Jintan Youlian Instrument Research Institute, Model: HH-600); real-time quantitative fluorescence PCR instrument (Jinan Guangyao Medical Equipment Co., Ltd., Model: CFX384); SIRT1 antibody (Shanghai Shifeng Biotechnology Co., Ltd.); NF-κB antibody (Wuhan Feien Biotechnology Co., Ltd.); PI3K antibody (Wuhan Biorbyt Biotechnology Co., Ltd.); Akt antibody (Shanghai Thermo Fisher Scientific Co., Ltd.); GSK3β antibody (Shanghai Boyan Biotechnology Co., Ltd.); and hydrogen peroxide (Guangdong Hengjian Pharmaceutical Co., Ltd., Batch number: 44023919, specification: 500mL).

Grouping

Mice were divided into a sham operation group and model group, with 36 mice in each group. All mice were adaptively fed for 1 week.

Establishment of deep venous thrombosis model: after successful anesthesia, mice in the sham operation group were fixed on the test table with silk thread, and the abdominal skin was prepared. Between 1.5 to 2 cm was cut along the median line of the abdomen, and the skin, subcutaneous tissue, abdominal muscle, peritoneum, and abdominal cavity were cut in turn. Only the inferior vena cava was exposed without any other treatment, and the abdomen was closed layer by layer. The vital signs of mice were observed and the mice were deliberately kept warm. After successful anesthesia, the abdominal skin of mice in the model group was prepared, and the inferior vena cava was exposed close to the junction of left renal vein and inferior vena cava. The inferior vena cava was exfoliated with surgical microscopic retractors.

A 30 g pre-bent needle parallel to the longitudinal axis of the inferior vena cava was placed on the surface of the inferior vena cava. The needle was knotted with the inferior vena cava, and the needle was removed along the longitudinal axis of the inferior vena cava and cephalic side to prevent the inferior vena cava wall injury and bleeding. After

the operation, the abdominal cavity was rinsed with warm saline, and the mice were confirmed to have stable respiratory circulation, and the abdomen was closed layer by layer. The vital signs of mice were observed and the mice were deliberately kept warm.

Cell culture

The HUVECs' cells were cultured in cell culture medium with 10% fetal bovine serum and 5% CO_2 at 37°C, and 1 mL of trypsin was added for digestion. After the cells contracted at the edge, the culture medium containing 10% fetal bovine serum was added to terminate digestion, and centrifugation was performed to remove the supernatant. The medium was used for resuspension, and the cell concentration was adjusted to 1×10^5/mL and inoculated into a 6-well plate. The cells were placed in a CO_2 incubator for culture, observed under a microscope, and the culture medium was replaced in time. When the cells grew to the fusion state, they were subcultured, and when the cells were passed to the second generation, they were used for experimental intervention.

The cells were cultured to 10000/mL and inoculated to a 96-well plate with 100 μL per well. The cells were stimulated with different concentrations of hydrogen peroxide (0 μmol/L, 20 μmol/L, 40 μmol/L, 80 μmol/L, 100 μmol/L, 200 μmol/L, and 400 μmol/L) and incubated for 4 h, 12 h, and 24 h, respectively. Multiple tests were carried out to reduce errors. According to the results, the optimum concentration and time were 200 μmol/L and 24 h. Four repeated wells were set in each group.

After selecting the most appropriate hydrogen peroxide concentration and time, the cells were divided into the control group, hydrogen peroxide group, and SIRT1 group. The SIRT1 group was pretreated with different concentrations of SIRT1 (1 μmol/L, 10 μmol/L, 30 μmol/L) for 2 h, and the results showed that 30 μmol/L was the most appropriate concentration of SIRT1 for hydrogen peroxide induced protection. Four repeated wells were set in each group.

Observation indexes

Eighteen mice were selected from each group, inferior vena cava tissues were taken, and paraffin sections were routinely prepared. Histopathological changes of inferior vena cava in each group were observed by HE staining.

Eighteen mice were selected from each group. The inferior vena cava tissues were taken and centrifuged and supernatant was obtained. The levels of malondialdehyde (MAD) were measured by the thiobarbituric acid method. The activity of superoxide dismutase (SOD) was determined by the xanthine oxidase method.

The expression level of SIRT1 in each group was determined by a western blot assay. An MTT assay was used to determine the changes of cell activity in each group. Flow cytometry was used to detect the apoptosis of each group. Western blotting was used to determine the expression of apoptosis-related proteins in each group. The expression of SIRT1, extracellular regulatory protein kinase (ERK), mitogen activated protein kinase p38, nuclear factor κB (NF-κB), protein kinase B (Akt), glycogen synthesis kinase 3β (GSK3β), P-selectin glycoprotein ligand 1 (PSGL-1), thrombomodulin (TM), von Willebrand factor (vWF), and tissue plasminogen activator (t-PA) was detected by western blot and real-time quantitative fluorescence PCR.

Statistical method

The SPSS22.0 software package was used for statistical data analysis. The counting data were expressed as percentages, and an χ^2 test was used for comparison. For the measurement data, the intergroup comparison was carried out by an independent sample t test; a paired sample t test was used for comparison before and after treatment; analysis of variance for repeated measurement was applied for comparison of the same index at different time points; an independent sample t test was used for inter-group difference comparison at each time point; and an LSD-t test was applied for time difference comparison of each group.

Data comparison results $P<0.05$ indicated the difference was statistically significant.

Results

Histopathological changes of inferior vena cava in each group of mice

In the sham operation group, the wall of inferior vena cava was regular and flat, the intima, muscularis, and tunica externa were intact, the endothelial cells were arranged regularly, the nuclei were normal, and there was no inflammatory cell infiltration and thrombosis. In the model group, the wall thickness of inferior vena cava was uneven, the volume of endothelial cells and the size of nuclei were different, inflammatory cell infiltration was observed, and a large amount of thrombosis was observed in the lumen. The results were shown in Figure 1.

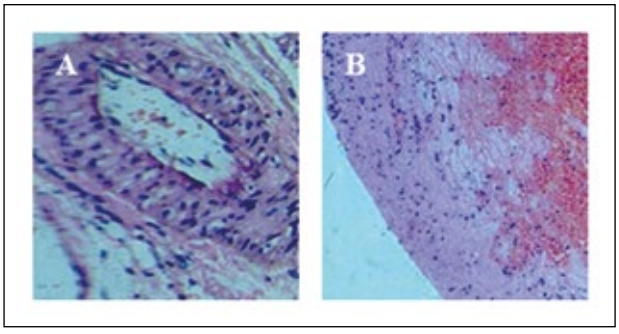

Figure 1: Histopathological changes of inferior vena cava tissues.
Figure A: sham operation group; Figure B: model group.

Comparison of SOD and MDA expression levels in inferior vena cava tissues in each group of mice

Compared with the sham operation group, SOD expression levels in the inferior vena cava of the model group were significantly decreased, while MDA expression levels were obviously increased (P<0.01). The results were shown in Table 1.

Groups	Cases	SOD (U/mg)	MDA (μmol/mg)
Sham operation group	18	20.12±1.53	20.33±1.46
Model group	18	2.43±0.46	52.39±3.59
t		46.977	36.192
P		<0.001	<0.001

Table 1: Comparison of SOD and MDA expression levels in inferior vena cava tissues in each group of mice ($\bar{x}\pm s$).

Comparison of SIRT1 expression levels in each group

Compared with the sham operation group, the SIRT1 expression level in the model group was significantly decreased (P<0.05). The results are shown in Figure 2.

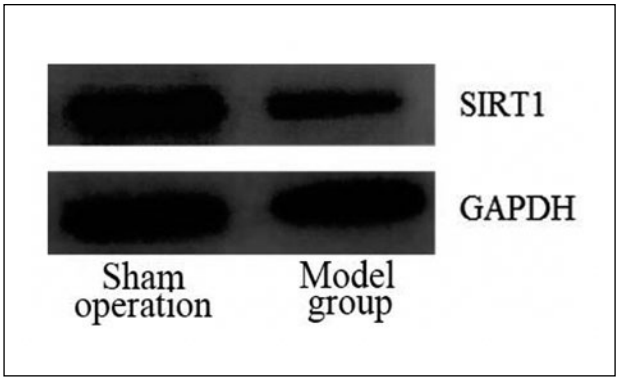

Figure 2: Comparison of SIRT1 expression levels in each group.

Comparison of changes of cell activity in each group

Compared with the control group, the cell activity of the hydrogen peroxide group was significantly decreased (P<0.05). Compared with the hydrogen peroxide group, the cell activity of the SIRT1 group was obviously increased (P<0.05).

Groups	Cell activity
Control group	0.98±0.01
Hydrogen peroxide group	0.47±0.02
SIRT1 group	0.95±0.01
F	1638.00
P	<0.001

Table 2: Comparison of changes of cell activity in each group ($\bar{x}\pm s$).

Comparison of apoptosis in each group

Compared with the control group, the apoptosis rate and expression levels of Bcl-2 and Caspase-3 in the hydrogen peroxide group were significantly increased (P<0.05).

Compared with the hydrogen peroxide group, the expression levels of Bcl-2 and Caspase-3 in the SIRT1 group were obviously decreased (P<0.05). The results are shown in Table 3.

Groups	Cell apoptosis (%)
Control group	0.06±0.02
Hydrogen peroxide group	17.26±1.22
SIRT1 group	0.19±0.08
F	785.50
P	<0.001

Table 3: Comparison of apoptosis in each group ($\bar{x}\pm s$).

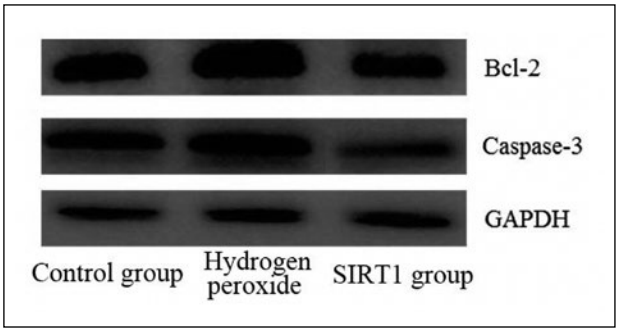

Figure 3: Comparison of apoptosis-related proteins in each group.

Comparison of SIRT1, PSGL-1, TM, vWF, and t-PA expression in each group

Compared with the control group, the expression levels of SIRT1 were remarkably decreased in the hydrogen peroxide group, and the expression levels

of PSGL-1, TM, vWF, and t-PA were obviously increased in the hydrogen peroxide group (P<0.05). Compared with the hydrogen peroxide group, the expression levels of SIRT1 were significantly increased in the SIRT1 group, and the expression levels of PSGL-1, TM, vWF, and t-PA were obviously decreased in the SIRT1 group (P<0.05). The results are shown in Figure 4 and Table 4.

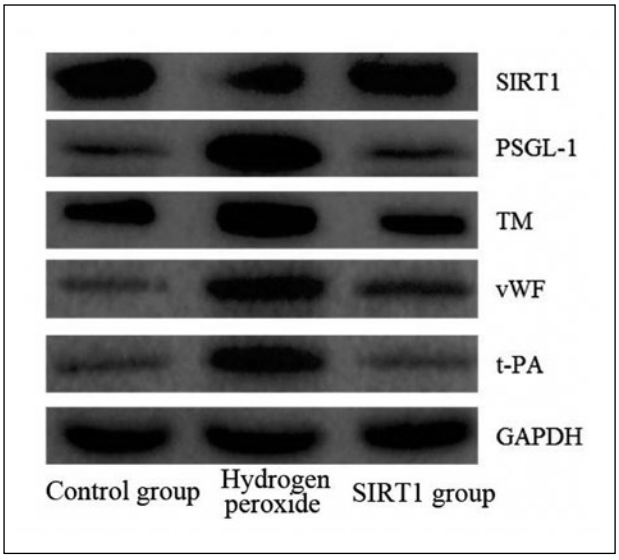

Figure 4: Comparison of SIRT1, PSGL-1, TM, vWF, and t-PA expression in each group.

Groups	SIRT1	PSGL-1	TM	vWF	t-PA
Control group	1.01±0.01	93.52±3.58	168.62±14.73	71.58±5.96	91.28±8.52
Hydrogen peroxide group	0.25±0.03	151.31±25.63	222.29±20.73	199.85±11.63	143.91±15.85
SIRT1 group	0.52±0.06	122.18±12.47	185.19±16.28	98.66±9.73	119.83±11.69
F	169.83	12.14	206.64	18.09	146.83
P	<0.001	0.003	<0.001	0.001	<0.001

Table 4: Comparison of SIRT1, PSGL-1, TM, vWF, and t-PA expression in each group ($\bar{x}$±s).

Comparison of ERK, p38, NF-κB, Akt, and GSK3β expression levels in each group

Compared with the control group, the Akt expression levels were remarkably decreased in the hydrogen peroxide group, and the expression levels of ERK, p38, NF-κB, and GSK3β were obviously increased in the hydrogen peroxide group (P<0.05).

Compared with the hydrogen peroxide group, the Akt expression levels were significantly increased in the SIRT1 group, and the expression levels of ERK, p38, NF-κB, and GSK3β were obviously decreased in the SIRT1 group (P<0.05). The results are shown in Figure 5.

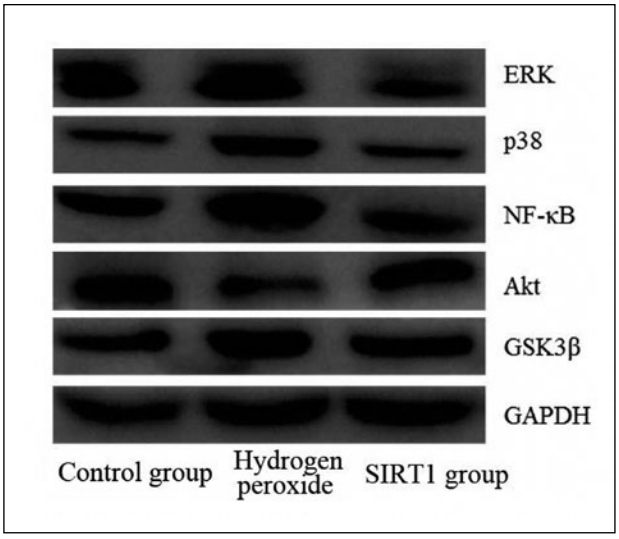

Figure 5: Comparison of ERK, p38, NF-κB, Akt, and GSK3β expression levels in each group.

Discussion

Deep vein thrombosis (DVT) is a venous reflux disorder, which refers to abnormal clotting of blood in veins and complete or incomplete occlusion of blood vessels. The detachment of the thrombosis causes pulmonary artery embolism. According to statistics, more than 50% of patients with severe deep vein thrombosis develop post-thrombotic syndrome, seriously affecting their quality of life[7]. Pulmonary artery embolism is an acute complication of deep vein formation. When an embolus is formed, the thrombus from the venous system falls off and blocks the main pulmonary artery or other branches, causing acute respiratory and circulatory failure[8]. The pathogenesis of DVT is complex, which may be closely related to vascular endothelial cells, white blood cells, platelets, coagulation factors, and fibrinolysis system. Vascular wall damage, vasoconstriction, blood stasis, platelet adhesion, and coagulation factor cascade are the basic processes of thrombosis.

As the main structure of vascular intima, venous endothelial cells are not only the bridge between blood and tissue fluid, as well as tissue cells, but also secrete some important factors through the autocrine and paracrine pattern. It has been reported that disorder of endothelial cell structure and function plays an important role in the process of deep vein thrombosis[9]. The damage of vascular endothelial cells is the initiating factor in the pathogenesis of deep vein thrombosis. Oxidative stress is one of the important factors that cause endothelial cells to be damaged, which can cause injury to the blood vessel

wall and thus lead to thrombosis. SIRT1 is a NAD-dependent histone deethylase, a novel anti-aging protein that can not only cause histone deacetylation, but also participate in the modification of non-histones. Studies have found that the activation of SIRT1 is closely related to cell proliferation, inflammatory response, oxidative stress, and other pathological processes[10]. In addition, SIRT1 can reduce the formation of arterial thrombosis. In this study, it was found that oxidative stress was involved in the process of deep vein thrombosis, and SIRT1 could inhibit the damage caused by oxidative stress and inhibit cell apoptosis.

Studies have found that in the process of endothelial cell injury and apoptosis, a variety of pro-thrombotic molecules can be secreted and interact with platelets, white blood cells, coagulation and anticoagulation systems, and fibrinolysis and anti-fibrinolysis systems to promote thrombosis[11]. PSGL is a ligand of P-selectin, which can mediate leukocyte adhesion thrombus formation and plays an important role in tumor metastasis and extracellular signal transduction. TM is a transmembrane glycoprotein, mainly located on the surface of cell membranes. When endothelial cells are damaged, TM is abnormally secreted and can be used as an important marker of endothelial cell damage. Some studies have shown that TM can prolong the time of dissolving embolus by u-PA and t-PA to some extent[12]. It has been reported that when endothelial cells are damaged, a large amount of vWF is synthesized and enters the blood, and the change of vWF expression level can serve as a characteristic marker of endothelial cell damage or dysfunction[13]. PSGL-1, TM, vWF, and t-PA are all pro-thrombotic markers. The results of this study showed that SIRT1 could significantly inhibit the expression of pro-thrombotic markers.

It has been reported that hypoxia, infection, and oxidative stress can cause endothelial cell damage and promote the formation of deep vein thrombosis[14]. Mitogen-activated protein kinase (MAPKs) is a serine/threonine kinase that regulates cell proliferation, differentiation, or apoptosis, including ERK and p38. When ERK and p38 are activated, phospholipase A2 is phosphorylated, thus promoting platelet adhesion and aggregation. NF-κB is a protein complex. When NF-κB is activated, inflammatory factors such as TNF-α are significantly increased, triggering a series of chronic inflammatory responses that lead to vascular endothelial cell damage, resulting in disease occurrence[15]. During the thrombus formation process, reactive oxygen species can induce phosphatidylinositol 3-kinase (PI3K) abnormal activation, thus promoting platelet adhesion and aggregation. GSK3β is one of the downstream factors of Akt and plays an important role in regulating cell growth, differentiation, and apoptosis. In this study, ERK, p38, NF-κB, Akt, GSK3β, and other signaling pathways were involved in the process of deep vein thrombosis, and SIRT1 could regulate their expression.

In conclusion, SIRT1 was involved in the process of deep vein thrombosis by activating the PI3K/Akt/GSK3β signaling pathway and inhibiting the MAKs and NF-κB signaling pathways, suggesting that SIRT1 might repair oxidative stress-induced deep vein thrombosis by inhibiting the pro-apoptotic pathway and inflammatory response pathway and activating the anti-apoptotic pathway.

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