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Expression change and research of the roles of *Sirt-2* in liver tissue of sepsis mice.

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Abstract

Silent information regulator-2 (*SIRT2*) is a recently discovered type of dependence on nicotine amines adenine dinucleotide type III histone acetylation enzyme. However, the expression change and research of *SIRT2* in macrophage remains unclear. Here in this study, we demonstrate that the regulatory effects of *SIRT2* on sepsis and the feasible correlational research. BALB/c mice were used to establish Cecum ligation puncture (CLP)-induced sepsis model. At 3, 6, 12, 24, 48 and 72 h after CLP-induced, the expression of *SIRT2* and *MMP-9* gene were analyzed using Quantitative real time polymerase chain reaction (Q-PCR), the serum of ALT, inflammatory factor and MCP-1 levels were measured by ELISA kits, pathological changes of liver tissue was observed using HE Dyeing and Immuno-histochemical analysis, and the expressions of NF-κB p65 and p38MAPK protein were surveyed using western blotting. Firstly, the expression of *SIRT2* gene and the serum of ALT level in sepsis mice were rapidly increased since 3 h operation and reached peak at 48 h after operation. Next, seep inflammatory cells was surged and the serum of TNF-α, IL-1, IL-6, IL-10 and MCP-1 were observably increased and reached peak at 6 or 12 h after operation in sepsis mice. Lastly, NF-κB p65 and phosphorylation-p38MAPK protein expression, and the *MMP-9* gene expression were rapidly increased and reached peak at 12 h or 6 h, respectively, after operation in sepsis mice. Our data suggested that *SIRT2* can increase inflammatory of sepsis mice through mediation of NF-κB and p38 MAPK pathway.

Keyword:

SIRT2, Sepsis, Inflammatory, p38 MAPK.

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Introduction

Sepsis is caused by systemic inflammatory response syndrome (SIRS), is the common complications of infection, gen/burns and surgical operations, they are closely related with individual factors including age, genetic, if with chronic disease [1]. Sepsis with organ damage and then develop for severe sepsis, even septic shock, multiple organ dysfunction syndrome [2]. Sepsis is a common disease with high mortality. In the United States, each year at least 750000 people suffer from sepsis, and the patients' mortality of sepsis was 34.0%, treatment costs was $22100 per person [3]. In China, the incidence rate of sepsis in Intensive Care Unit (ICU) was 8.68%, the mortality was 48.7%, and per people cost of treatment is about 100000 RMB/person [4]. The incidence of sepsis in the world is increasing year by year, and the mortality is still high.

The pathogenesis of sepsis is very complicated; the infection and inflammation reaction in the development of the occurrence of sepsis plays a vital role [5]. After pathogenic microorganism invasion to the body, the pathogen associated molecular patterns through Toll-like receptors and immune cells such as pattern recognition receptor interactions, activate the transmembrane signal transduction pathways, regulate the expression of inflammatory mediators, which produce a large number of inflammatory related molecules, which triggers the inflammatory response [6]. Monocyte/macrophage as important effector cells plays a key role in the body's natural immune system. Monocyte/macrophage on one hand removed by phagocytosis invasion in the body by a variety of pathogenic microorganisms, on the other hand, it is the main source of the body's inflammatory related molecules, by producing a large number of pro-inflammatory/anti-inflammatory medium, mediated inflammatory reaction [7].

Silent information regulator-2 (*SIRT2*) is a recently discovered type of dependence on nicotine amines adenine dinucleotide type III histone acetylation enzyme, it mainly located in cytoplasm, the recently reported main functions involve involved cell cycle, maintain the stability of the genome, and tumorigenesis [8]. *SIRT2* through acetylated histone H4K16Ac loci, regulate the cell cycle. H4K16Ac loci acetylation

specificity occur between the cell cycle stage of G2 and M, and in this phase, *SIRT2* transfer from cytoplasm to nuclei [9]. H4K16 loci of acetylation level reached to the highest in S phase, this state is an important signal in mammalian DNA repair [10]. *SIRT2* adjust the acetylation level of H4K16 loci, release DNA damage situation in the cell cycle signal, to participate in regulating the cell cycle [11]. Recent studies reveal that *SIRT2* related to the differentiation of fat cells and the neural microglia [12]. *SIRT2* in liver cancer research get more and more people's attention in recent years [13]. At present, the study of *SIRT2* is concentrated in the tumors, metabolic diseases, etc. And the study of sepsis has not yet clear, this experiment adopts the caecal ligation and puncture, making sepsis model, aimed to explores *SIRT2*'s influence on sepsis inflammatory reaction and prognosis, provide a new train of thought for the treatment of sepsis. This study is to explore its role in the pathogenesis of sepsis.

Materials and Methods

Grouping

Mixing Sumianxin, Ketamine and saline by volume ratio of 2:1.5:3.5, 4°C keep aside using 0.05 to 0.06 ml/one for BALB/c mice anesthesia (anesthesia by intraperitoneal injection).

Sepsis modelling

Cecum ligation puncture (CLP) method was used to construct sepsis model (main indicators of model construct success are the modeling survival rate of mice for 20-30%).Using 75% alcohol dip in surgical instruments. Maxing Sumianxin, Ketamine and saline by volume ratio of 2:1.5:3.5, 4°C saved for later use. The anaesthetic abdominal cavity injection by 50-60 μl/mice, about 2 to 4 min later, the mice lose consciousness. After completely anesthesia, mice will be fixed on the operating table, with 75% alcohol disinfection abdomen. Exposed mice abdominal cavity, under xiphoid one finger long refers to the midline of abdomen from top to bottom cut about 2 cm long incision, and cut open abdominal peritoneum along the Hunter's line, reveal the abdominal cavity. Pulling the cecum from the lower left corner of the abdominal cavity, about ligation on the cecum two-thirds distance to caecum, ligation about 2/3 width (not ligation too tight to prevent intestinal necrosis and influence the results). Using 8 needles on the outer edge of the ligation puncture intestinal wall for 2 times, pay attention to avoid loss of capillaries on the intestinal wall, to prevent bleeding from the walls of intestines, squeeze right amount of contents carefully. Using line suture needle to step by step stitch peritoneum and muscle layer (with the "8" suture). Control group (Sham) treatment in mice, just pulling out the cecum and then put the cecum back into the abdominal cavity, do not need the cecum ligation perforation. In this study, animal processing method is according to the ethics standard.

Tissue paraffin embedding

Fresh organization fixed in 4% paraformaldehyde for more than 24 h. Remove tissue from a fixed liquid, repair the purpose tissue to flat with scalpel in the fuming cupboard, put the repaired organization and the corresponding labels in a dehydrated box; Dehydration, put the dehydration box in the hanging basket in the dryer, in turn, gradient alcohol dehydration.75% ethyl alcohol 4 h-85% ethyl alcohol 2 h-90% ethyl alcohol 2 h-95% ethyl alcohol 1 h-absolute ethyl alcohol I 30 min-absolute ethyl alcohol II 30 min-Alcohol benzene 5-10 min-dimethylbenzene I 5-10 min-dimethylbenzene II 5-10 min-wax I 1 h-wax II 1h-wax III 1 h; Embedding: The tissue will be immersed within the organization on the embedding machine wax embedding. Melting wax into the embedding box first, stay before wax solidification and remove tissue from the dehydration box in accordance with the standard embedding the into the embedding box with the requirements and the paste the corresponding label. Cooling in -20°C refrigerator, after wax solidification to remove wax block from the embedding box and repairing the wax block; Slice: Dressed wax block in paraffin wax for microtome section 4 μm thick. Slicing machine will float in 40°C warm water and organization flattening by stand machine, use glass slide to get the organize salvage, and placed into 60°C oven to bake.

HE Dyeing experiment steps

Putting slice in turn into dimethylbenzene I 20 min-dimethylbenzene II 20 min-absolute ethyl alcohol I 10 min-absolute ethyl alcohol II 10 min-95% ethyl alcohol 5 min-90% ethyl alcohol 5 min-80% ethyl alcohol 5 min-70% ethyl alcohol 5 min-distilled water to wash; Sliced into the Harris logwood dye 3-8 min, water washing, 1% hydrochloric acid alcohol differentiation into a few seconds, tap water rinse, 0.6% ammonia return to blue, water flushing; Sliced into the red dye solution dyed 1-3 min; Slice was in turn into the 95% alcohol I 5 min-95% alcohol II 5 min-absolute ethyl alcohol I 5 min-the anhydrous ethanol II 5 min-dimethylbenzene I 5 min-dimethylbenzene II dehydration transparent in 5 min, the slice out of dimethylbenzene for a bit dry, neutral gum sealing piece.

Immuno-histochemical analysis method

Image pro-plus 6.0 software analysis method of immune-histochemical image: Within each group of each section of at least three times the 200 randomly selected photograph field of vision. As far as possible when you take a photo for tissue full of the entire field of vision, ensure each photo light background are the same. Application of Image-Pro Plus6.0 software to select the same tan as judge all photos positive unified standard, for each photograph analysis, it is concluded to get the accumulation of each photo positive optical density (IOD).

Quantitative real time polymerase chain reaction (Q-PCR) of SIRT2 and MMP-9 expression

Liver tissue samples were froze by liquid nitrogen, and used homogenizer to homogenize with buffer. Total RNA was extracted using TRIzol (Invitrogen). 1 μg of RNA and with One Step RT-PCR Kit (TaKaRa) was used to synthesize cDNA. ABI-7500 RT-PCR system (Applied Biosystems) and the SYBR Green (TaKaRa) were used to perform Q-PCR and analyze the *SIRT2* gene expression. The primers of *SIRT2*, *MMP-9* and *GAPDH* were showed at Table 1.

Table 1. *The primers of SIRT2, MMP-9 and GAPDH.*

Gene	Sequence (F; 5`- 3`)	Sequence (R; 5`- 3`)
SIRT2	CCATGGCAAATTCTTCTGGCGTGT	TAGAGACTTGCACTGCACGGTTGA
MMP-9	GCCTGGGTTCCCAAAAGGAG	GAGCGGAAGTCAGGGATACC
GAPDH	CCTTCATTGACCTCAACTAC	GGAAGGCCATGCCAGTGAGC

Serum levels of alanine transaminase (ALT), inflammatory factor and chemoattractant protein 1 (MCP-1) expression

The serum samples were collected form eye socket and microcentrifuged for 15 min 16,000 g at 4°C. Supernates were collected and used to analyze cytokines and MCP-1 expression. The serum of ALT, tumor necrosis factor-α (TNF-α), interleukin-1 (IL-1), interleukin-6 (IL-6), interleukin-10 (IL-10) and MCP-1 expressions were measured by using Quantikine ELISA kits (R&D Systems) according to the manufacturer's protocol.

Western blotting of NF-κB p65 and p38MAPK

Liver tissue samples were froze by liquid nitrogen, and used homogenizer to homogenize with buffer containing lysed with Radio-Immunoprecipitation Assay (RIPA). Miscible liquids were microcentrifuged for 15 min 16,000 g at 4°C. Protein concentrations were estimated by Pierce BCA protein assay kit (BD Biosciences, San Jose, CA). Equal protein samples were loaded onto 10% SDS–polyacrylamide gel. Separated proteins were transferred into polyvinylidene fluoride (PVDF) membrane (Bio-Rad, Hercules, CA) after electrophoresis. The PVDF membrane was washed with 5% non-fat dry milk in TBS for 1-2 h, incubated with antibodies: NF-κB p65 (dilution 1/2000) and phosphorylation-p38MAPK (dilution 1/1000) overnight at 4°C. The PVDF membrane was incubated with ECL Prime reagents from GE Life Sciences (Piscataway, NJ, USA).

Statistical analysis

Data was analyzed expressed as mean ± SEM using either Student's t test (for two groups) or one-way ANOVA (for more than two groups) and performed using the SPSS 17.0 (IBM SPSS Statistics, IBM corporation, Armonk, NY).

Results

Expression of SIRT2 gene in liver tissue of sepsis mice

To assess the expression of *SIRT2* in sepsis mice, we evaluated the expression of *SIRT2* gene in liver tissue of sepsis mice. In sham group, the expression of *SIRT2* gene was lower and basic similarity in different time (Figure 1A). As expected, the expression of *SIRT2* gene in sepsis mice was rapidly increased since 3 h operation, and reached peak at 48 h after operation, compared with the sham group (Figure 1A).

Serum levels of ALT in liver tissue of sepsis mice

To check into the expression of *SIRT2* on the serum of ALT level, we inspected the serum of ALT level in this study. In sham group, there was no different in the serum of ALT level in different time, which was lower level (Figure 1B). Nevertheless, sepsis markedly induced the serum of ALT level, and reached peak at 48 h after operation, compared with the sham group (Figure 1B).

Pathological changes of liver tissue in sepsis mice

These results of HE Dyeing showed that cellular structure of liver tissue was normal and clearness, these were no seep inflammatory cells of hepatic sinus and portal area in sham group (Figure 1C). Meanwhile, sepsis mice obviously appeared seep inflammatory cells after operation at 3 h, compared with the sham group (Figure 1C).

Serum levels of inflammatory factor in liver tissue of sepsis mice

To investigate the expression of *SIRT2* on inflammatory factor, we measure the serum of TNF-α, IL-1, IL-6 and IL-10 in our study. In sham group, the serum of TNF-α, IL-1, IL-6 and IL-10 maintained lower levels and basic similarity in different time (Figure 1D). However, the serum of TNF-α, IL-1, IL-6 and IL-10 in sepsis mice were rapidly enhanced since 3 h operation, the serum TNF-α level reached peak at 6 h after operation, the serum IL-1 and IL-6 level reached peak at 12 h after operation, and the serum IL-10 level reached peak at 24 h after operation, compared with the sham group (Figures 1D-1G).

Expression of MCP-1 in liver tissue of sepsis mice

To detect the expression of *SIRT2* on the serum of MCP-1 level, we measured the serum of MCP-1 level in this study.

Results demonstrated that the serum of MCP-1 level were very similar and lower level in different time of sham group (Figure 1H). As shown in Figure 1, the serum of MCP-1 level was memorably increased in sepsis mice since 3 h operation and reached peak at 12 h after operation, compared with the sham group (Figure 1H).

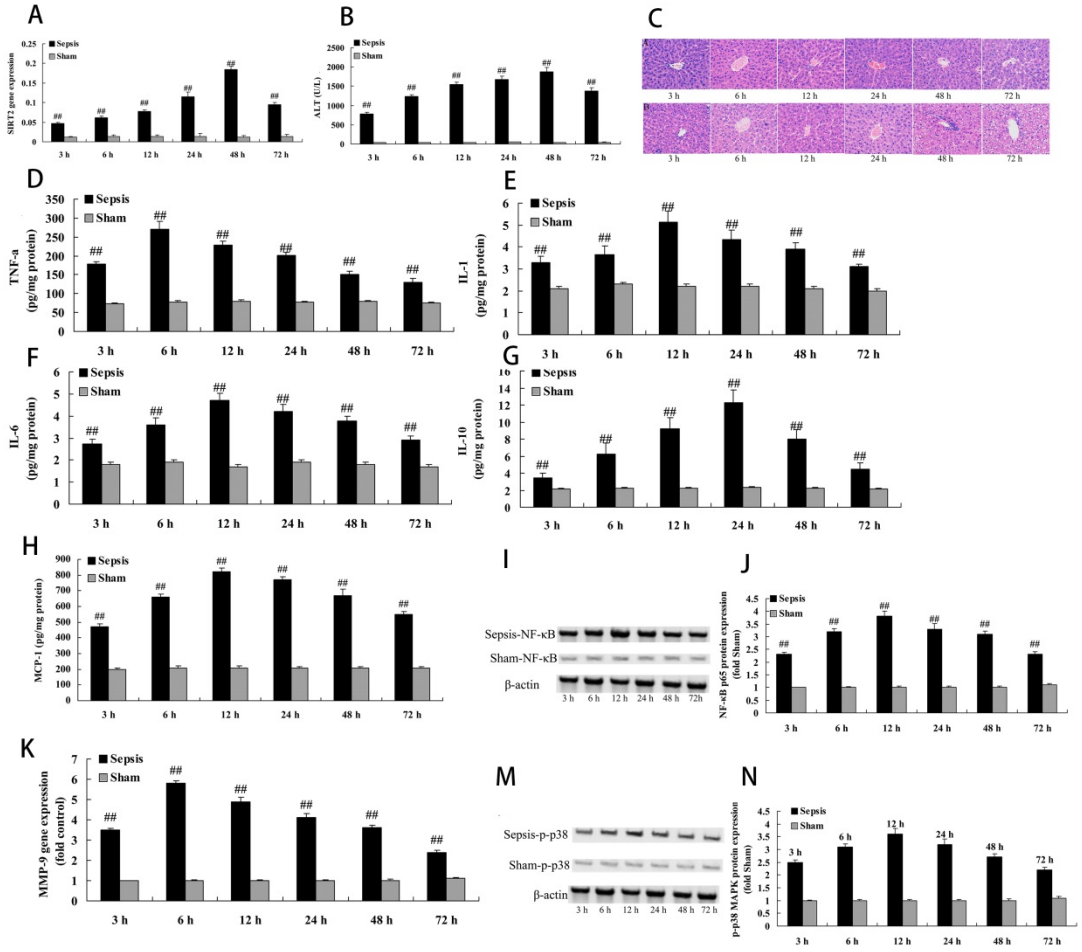

Figure 1. *A) Expression of SIRT2 gene in liver tissue of sepsis mice. ##p<0.01 versus Sham group; B) Serum levels of ALT in liver tissue of sepsis mice. ##p<0.01 versus Sham group. C) Pathological changes of liver tissue in sepsis mice. Pathological changes of liver tissue in Sham group (C). Pathological changes of liver tissue in sepsis mice (C). ##p<0.01 versus Sham group. D-G) Serum levels of inflammatory factor in liver tissue of sepsis mice The serum of TNF-α (D), IL-1 (E), IL-6 (F) and IL-10 (G) in liver tissue of sepsis mice ##p<0.01 versus Sham group. H) Expression of MCP-1 in liver tissue of sepsis mice. ##p<0.01 versus Sham group; I&J) Expression of NF-κB p65 protein in liver tissue of sepsis mice. Western blot analysis was used to the expression of NF-κB p65 protein (I) statistical analysis of NF-κB p65 protein level (J) in liver tissue of sepsis mice. ##p<0.01 versus Sham group; K) Expression of MMP-9 gene in liver tissue of sepsis mice ##p<0.01 versus Sham group; M&N) Expression of p38MAPK protein in liver tissue of sepsis mice. Western blot analysis was used to the expression of p-p38MAPK protein (M) statistical analysis of p-p38MAPK protein level (N) in liver tissue of sepsis mice. ##p<0.01 versus Sham group.*

Expression of NF-κB p65 protein in liver tissue of sepsis mice

We further investigated whether *SIRT2* is involved in NF-κB p65 protein expression. There was no significant difference the NF-κB p65 protein expression between different times of sham group, which was very low level (Figures 1I and 1J). In addition, the NF-κB p65 protein expression was observably improved and reached peak at 12 h after operation in sepsis mice, compared with the sham group (Figures 1I and 1J).

Expression of MMP-9 gene in liver tissue of sepsis mice

We evaluated investigated whether *SIRT2* is involved in *MMP-9* gene expression. We found no significant the *MMP-9*

gene expression in different time of sham group (Figure 1K). However, the *MMP-9* gene expression was quickly improved since 3 h after operation and reached peak at 6 h after operation in sepsis mice, compared with the sham group (Figure 1K).

Expression of p38MAPK protein in liver tissue of sepsis mice

To investigate whether *SIRT2* is involved in p38MAPK protein in liver tissue of sepsis mice, phosphorylation-p38MAPK (p-p38) was analyzed using western blotting. Therefore, western blot analysis indicated that the p-p38 protein expression was low level and basic similarity in different time of sham group (Figure 1M). Predictably, the p-p38 protein expression was markedly promoted and reached peak at 12 h after operation in sepsis mice, compared with the sham group (Figure 1N).

Discussion

Sepsis as systemic inflammatory response syndrome caused by infections is an extremely complex process, there are several mechanisms involved in regulating, involves a large number of changes in gene expression profile [14]. The role of Epigenetic mechanisms gets more and more attention of people in the pathogenesis of sepsis in recent years. Think of epigenetic mediated inflammatory related changes in gene activity plays an important role in the pathogenesis of sepsis [15]. Because of various factors to break the body steady start SIRS in the body, the body produces a large amount of inflammatory medium into the start stage, the strength of this period's inflammatory response is important for the prognosis of sepsis [16]. When the body coming into the second phase change period (adaptation), also named endotoxin tolerance period, with a large amount of pro-inflammatory genes suppressed, and anti-inflammatory gene activation [17]. After safely through this stage, the third stage of stationary phase will come. The strength of the start-up stage decided the duration and prognosis of the phase change stage, change phase stages involved in inhibition of pro-inflammatory genes, anti-inflammatory genes, metabolic activation of genes [18]. In our study, we found that the expression of *SIRT2* gene and the serum of ALT level in sepsis mice was rapidly increased since 3 h operation, and reached peak at 48 h after operation, compared with the sham group.

Studies have shown that during sepsis, inflammatory cell infiltration, besides macrophage is the main cell of throughout the wound healing process, monocyte chemoattractant protein-1 (MCP-1) abnormally elevated can stimulate macrophages release large amounts of lysosomal enzymes, and by producing oxygen free radicals and pro-inflammatory factor aggravating inflammation of the organization [19]. *SIRT2* agonist resveratrol, inhibit nicotinamide processing nucleus pulposus cells [20]. When the *SIRT2* is activated, the TNF-α induction of nucleus pulposus cells express lower MCP-1 expression; When *SIRT2* is inhibited, the TNF-α induce nucleus pulposus cells' MCP-1 expression increasing [21]. Therefore, confirmed that *SIRT-2* have the function of down-regulate chemokines MCP-1 in TNF-α induce nucleus pulposus cells [21,22]. We have previously shown seep inflammatory cells was surged and the serum of TNF-α, IL-1, IL-6, IL-10 and MCP-1 were observably increased and reached peak at 6 or 12 h after operation in sepsis mice. These results hinted that *SIRT2* might promote inflammatory and MCP-1 in sepsis mice. Our results were the opposite of those of other studies. For example, Lee et al. suggested that *SIRT2* can inhibit lipopolysaccharide-induced inflammation in macrophages [23]. Lin et al. also indicated that *Sirt2* suppresses inflammatory responses and MCP-1 in collagen-induced arthritis [24]. In acute and chronic inflammation, *SIRT-2* suppresses inflammatory responses, but SIRT family and acetylation can unduly inhibits inflammation, increases a mass of immunoreactions in sepsis mice [25-27]. Several study showed that the plentiful expression of *SIRT-2* inducts programmed necrosis through RIP1-RIP3 [20,28], which *SIRT-2* may promotes inflammation and immunoreactions in sepsis mice. The viewpoint and specific mechanisms deserve attention and are explained in further study.

SIRT2 as a group of histone deacetylase with NAD+dependent, participate in a number of acetylation of histone and transcription factors process [29]. Histone acetylation is an important epigenetic mechanisms, it through the control of gene transcription to determine the intercellular and intracellular signal transmission, which will affect the pathophysiologic process of the individual. *SIRT2* will causes the acetylation of P300's K314 K315, K310 loci, after the implementation of P65 gene transcription regulation, and then adjust the activity of NF-κB depends inflammatory genes. In numerous pathways of *SIRT2* inhibit inflammation, most notably is the inhibition of *SIRT2* to the NF-κB signaling pathway [30]. The NF-κB exists in many kinds of cells, and it is the focal point of the cells' multiple signaling pathways, and plays an extremely important role in the occurence and development of inflammation. In the stillness of the cells, the NF-κB normally exists in the form of p65 dimers [14]. Connecting to the inhibitory protein IκB, in a state of inactivation, stay in the cytoplasm, when activated, IκB with phosphorylation, ubiquitination and proteasome degradation, dissociation combine with the NF-κB, after the activated, p65 entered into the nucleus, the switching control of downstream genes including gene transcription of inflammatory factors such as IL-1, TNF-α, IL-8 and, IL-6 [31]. We observed that NF-κB p65 protein expression and the *MMP-9* gene expression were rapidly accumulatived and reached peak at 12 h or 6 h, respectively, after operation in sepsis mice.

SIRT2 inhibition of p38 MAPK signaling pathways-MAPKs are serine/threonine protein kinase in cells, the article consists of three parallel signaling pathways, are respectively ERK signaling pathway, JNK pathway, p38MAPK pathway, participate in a variety of inflammatory factor signal transduction [32]. P38MAPK kinase reaction information transfer process can be described as: After cells stimulation, make MAPK kinase kinase (MAPKKK) activation through certain link [33]. Activated MAPKKK activate MAPK kinase (MAPKK), the latter through double loci p38MAPK

phosphorylation activation [34]. Many stimulus such as cytokines, pathogenic microorganism products, changes in the physical and chemical properties of extracellular fluid, extracellular hypertonic, etc. all can activate p38 MAPK pathway, causing a series of related inflammatory response [35]. Our results identify the p-p38 protein expression of sepsis mice was enhanced and reached peak at 12 h after operation.

In summary, this study demonstrates that *SIRT2* can increase the serum of ALT level, inflammatory response, the *MMP-9* gene expression in sepsis mice. Together, our results identify that *SIRT2* intensifies sepsis through mediation of NF-κB and p38 MAPK pathway. Our study may provide a new molecular way for protection effect of *SIRT2* against sepsis.

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