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Effect of resveratrol on peritoneal macrophages in rats with severe acute pancreatitis

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Abstract. *Objective*: The literature on resveratrol in severe acute pancreatitis (SAP) is limited though it has been widely studied in infections and trauma. The aim of this study was to investigate the inhibitory effect of resveratrol on inflammatory responses in a rat model of SAP.
Methods: Male Sprague-Dawley (SD) rats were randomly divided into 3 groups: SAP group, resveratrol group and control group. 4.0% sodium taurocholate was injected into the pancreatic duct to induce SAP. In the resveratrol group, resveratrol (10 mg/kg) was injected through penal vein 5 min after SAP was induced. The peritoneal macrophages of the rats were collected 3, 6 and 12 h after stimulus and then incubated for 24 h. The expression of nuclear factor kappa B (NF-κB) and inducible nitric oxide synthase (iNOS) in peritoneal macrophages was measured. The levels of tumor necrosis factor alpha (TNF-α), interleukin-1 (IL-1) and nitric oxide (NO) in culture medium of peritoneal macrophages and serum of rats were evaluated.
Results: Histological examination of pancreas indicated that the damage in the SAP group was more severe than that in the resveratrol group. The expression of NF-κB and iNOS in peritoneal macrophages was significantly higher in the SAP group than in the resveratrol group. The concentrations of TNF-α, IL-1 and NO in culture medium and serum were significantly elevated in the SAP group when compared with the resveratrol group.
Conclusions: The inhibiting effect on the inflammatory response and the decreased expression of TNF-α, IL-1 and NO in peritoneal macrophages suggest resveratrol as a novel anti-inflammatory agent for reducing the severity of SAP.

Key words: Pancreatitis – Macrophages – Resveratrol

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Introduction

The most serious life-threatening complication in the progressive stage of SAP is systemic inflammatory response syndrome (SIRS) [1–5]. SIRS might result in multiple organ failure [6–9], in which lung dysfunction is the major cause of death in patients with SAP. Macrophages are the main inflammatory cells that modulate the progression of SAP [10–16]. The activation of macrophages can lead to the release of a number of inflammatory mediators, including TNF-a, IL-1, interleukin-6 (IL-6), interleukin-8 (IL-8), platelet-activating factor (PAF) and NO. These cytokines exhibit multiple biological activities, many of which show synergism, and ultimately lead to the initiation and progress of SAP [17, 18]. For example, the excessive release of TNF-α can lead to irregular metabolism, renal and lung dysfunction, although low level of TNF-α is known to promote the elimination of pathogens by leukocytes and has a positive effect on inflammatory injury [19]. On the other hand, the increased levels of inflammatory mediators in serum in the early stage of SAP, such as IL-1, can reflect partly the severity of SAP and the occurrence of complications [20, 21].

Since the activation of macrophages is tightly related to the severity of SAP, it is reasonable to conclude that the severity of SAP might be decreased by suppressing the function of macrophages. Recently, Leriol [22] showed that resveratrol, a natural polyphenol from grapes, at the concentrations of 1–10 μM, inhibited the extracellar production of reactive oxygen intermediates (ROIs) by peritoneal macrophages significantly and dose-dependently, which suggested a possible application of resveratrol in the treatment of diseases involving macrophage hyper-responsiveness. Huang [23] suggested that resveratrol could inhibit the production of TNF-α, IL-6 in serum to prevent the initiation and continual aggravation of SAP and the effective dose was more than 10 mg/kg. In the present study, we investigated the suppressive effect of resveratrol on cytokines secreted by peritoneal macrophages in a rat model of SAP.

Material and methods

Materials

Male Sprague-Dawley (SD) rats 12 to 14 weeks old weighing 250–300 g were used in the study. The animals were obtained from the Experimental Animal Center of Xi'an Jiaotong University. They were kept in regular 12 h light-dark cycle and air-conditioned (21–25 °C) and all rats were allowed free access to water and standard laboratory chow. Before the operations, the animals were fasted for 12 h and only allowed free access to water. All animal protocols were approved by Xi'an Jiaotong University Institutional Animal Care and Use Committee.

Resveratrol was purchased from Oses, Inc (Xi'an, China). DMEM and sodium taurocholate were purchased from Sigma, Inc (America). NF-κB consensus oligonucleotide and single base pair mutant were purchased from Promega. China). [32] P-ATP was purchased from Free Biotech, China. The rabbit-anti-rat iNOS antibody, the SABC kits and the diaminobenzidine (DAB) reaction kits were purchased from Wuhan Borsd Biological Engineering Co, Ltd (China). TNF-α and IL-1 ELISA kits were purchased from Beijing Zhongshan Golden Bridge Biotechnology Co, Ltd (China). NO detection kit was purchased from Nanjing Jiancheng Biological Engineering Co, Ltd (China). All other reagents were obtained from commercial sources.

Isolation and incubation of peritoneal macrophages

Fifty-four rats were randomly divided into control group, SAP group and resveratrol group, with 18 animals in one group. 2% pentobarbital sodium (0.1 ml/100 g) was administered by intraperitoneal route for anesthesia. Abdomen was opened through a midline epigastric incision. In control group, only a sham laparotomy was performed. In SAP group, 4.0% sodium taurocholate (0.1 ml/100 g) was injected into pancreatic duct to induce SAP in rats, and the injection lasted for one minute. In resveratrol group, the rats were injected with resveratrol of 10 mg/kg through penal vein 5 min after SAP was induced. One-third of rats in each group were killed 3, 6 and 12 h after stimulus, respectively. Abdominal cavities were lavaged with 10 ml DMEM three times, and then the DMEM was collected aseptically and centrifuged at 2000 rpm for 15 min at 4 °C. The supernatants were discarded and the pellets were immersed in 10 ml DMEM culture medium and incubated in a humidified incubator containing 5% carbon dioxide and 95% oxygen at 37 °C. After 2 h, the supernatants were discarded and the adherent cells were washed three times with 10 ml of PBS. The adherent cells were examined by using Giemsa staining to calculate the percentage of peritoneal macrophages. The percentages of peritoneal macrophages in adherent cells in our experiment were all above 95%. These cells were also stained with trypan blue for the assessment of viability. The percentages of living cells in adherent cells in our experiment were all above 90%. Then the peritoneal macrophages in all groups were incubated. After 24 h, adherent cells were collected and then fixed with alcohol-ether solution, while the culture medium was centrifuged at 2000 rpm for 5 min and the supernatants were stored at –20 °C for later use.

Electrophoretic mobility shift assay (EMSA)

Nuclear proteins from peritoneal macrophages were prepared as described previously [24]. For binding reactions, nuclear extracts (10 μg of protein) were incubated in a 25-μL total reaction volume containing 10 mM Tris, pH 7.5, 100 mM NaCl, 1 mM EDTA, 4% glycerol, 80 μg/ml sonicated sperm DNA. Double-stranded -^{32}P labeled NF-κB oligonucleotide probe (5'-AGTTGAGGGTTTCCCAGGC-3') was added to the mixture after preincubation for 10 minutes at 4 °C, and the reaction mixture was then incubated for 20 minutes at room temperature. Samples were loaded on 6% polyacrylamide gels in low-ionic strength 0.25 × TBE buffer (22.3 mmol/L Tris, 22.2 mmol/L borate, 0.5 mmol/L EDTA) and run at 150 V/cm with cooling. The gels were dried and analyzed by autoradiography.

Immunohistochemical detection of iNOS

The expression of iNOS in peritoneal macrophages of rat was detected by immunohistochemistry with the streptavidin-biotin-peroxidase complex (SABC) kit. Briefly, peritoneal macrophages were treated with 3% hydrogen peroxide to block the endogenous peroxide and incubated with normal goat serum to reduce the nonspecific antibody binding. After washing with PBS (0.01 mol/L), the cells were incubated with rabbit-anti-rat iNOS polyclonal antibody (diluted 1:50) at 37 °C for 1.5 h. Then the cells were washed in PBS, and were exposed to secondary goat-anti-rabbit IgG, following by incubation with the streptavidin-biotin-peroxidase complex. The reaction products of peroxidase were visualized by incubation with DAB. PBS was used to substitute for primary antibody for negative control.

The average gray values of positive cells in these groups were assessed by using an image analysis system under the same magnification (400 × microscope). Since the grey value of positive cells is negatively correlated with the content of iNOS antibody, namely, the content of iNOS in peritoneal macrophages, when we set the results of grey value × percentage of negative cells as ANTI^{-1}, the value of ANTI can positively reflect the content of iNOS in peritoneal macrophages.

TNF-α, IL-1 and NO Measurements

5 ml of blood sample was collected from inferior vena cava of rat and was centrifuged at 3000 rpm for 10 min at 4 °C. The serum in upper layer, together with the culture medium of peritoneal macrophages described previously, was used for the following experiments: the concentrations of TNF-α and IL-1 were measured by ELISA kits, while the level of NO was measured by NO detection kit.

Histological examination

Pancreas tissues of the rats were removed at 3, 6 and 12 h after the induction of SAP and was fixed with 10% buffered formalin. Then the specimens were embedded in paraffin and 3 μm thick sections were cut and stained with hematoxylin and eosin (H E). A single blinded pathologist examined the pancreas specimens and the histological characters were judged in term of the "Schmidt" criterion [25].

Statistical analysis

All data are presented as mean ± SEM. Statistical analysis of data was performed with one-way analysis of variance (ANOVA) using SPSS11.5. $P < 0.05$ was considered statistically significant.

Results

NF-κB activation in peritoneal macrophages

The results show that resveratrol decreased the NF-κB activation in peritoneal macrophages. Resveratrol led in peritoneal macrophages to significantly lower levels of NF-κB activation at 3 ($P < 0.01$), 6 ($P < 0.01$) and 12 ($P < 0.01$) h after the induction of SAP, respectively (Fig.1, 2).

Measurement of the levels of iNOS in peritoneal macrophages

Among the three groups, the amount of iNOS in peritoneal macrophages was the lowest in control group, while the value of ANTI^{-1} in control group was the largest. Compared

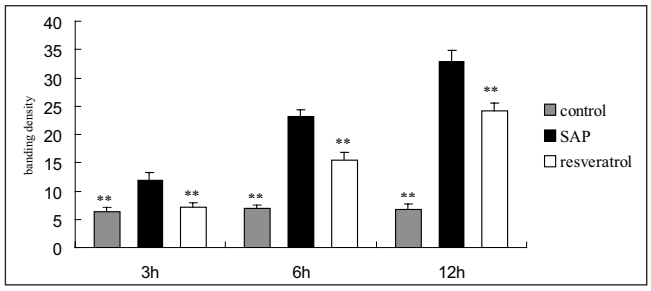

Fig. 1. The NF-κB activation in peritoneal macrophages at 3, 6 and 12 h after induction of SAP. $^{**}P$ <0.01 as compared with SAP group. Data are means ± SEM (n = 6) of binding of oligonucleotide probe.

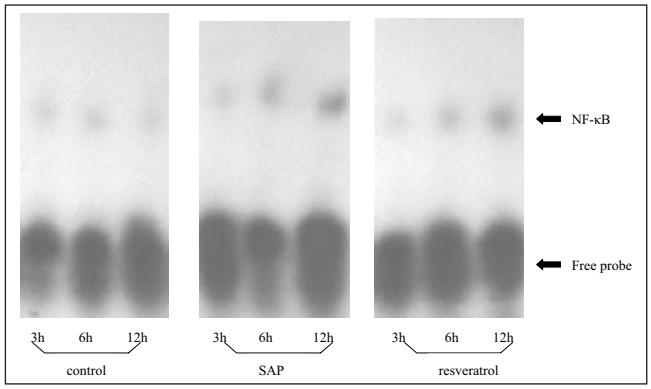

Fig. 2. The NF-κB activation in different groups after the induction of SAP. Data from EMSA gels.

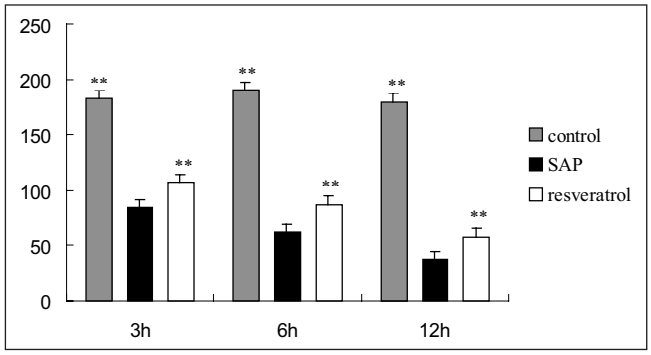

Fig. 3. The iNOS value of $ANTI^{-1}$ (arbitrary unit) in peritoneal macrophages at 3, 6 and 12 h after induction of SAP. $^{**}P$<0.01 as compared with SAP group. Data are means ± SEM (n = 6) of proportion of iNOS-negative staining cells.

with SAP group, resveratrol group had low levels of iNOS as the value of $ANTI^{-1}$ in was high at 3 ($P < 0.01$), 6 ($P < 0.01$) and 12 ($P < 0.01$) h, respectively (Fig. 3, 4).

Measurements of the levels of TNF-α, IL-1 and NO in culture medium

The results show that resveratrol caused macrophages to secrete less TNF-α, IL-1 and NO. At 3, 6 and 12 h after the induction of SAP resveratrol caused macrophages to secrete significantly lower levels than those in SAP group. The variation of the TNF-α ($P < 0.01$), IL-1 ($P < 0.01$) and NO ($P < 0.01$) secretion by peritoneal macrophages was most evident at 12 h.

Measurement of the levels of TNF-α, IL-1 and NO in serum

The results show that resveratrol decreased the levels of TNF-α, IL-1 and NO in serum. At 3, 6 and 12 h after the induction of SAP resveratrol reduced levels in serum to significantly lower values than those in SAP group. The variance of the levels of TNF-α ($P < 0.01$), IL-1 ($P < 0.01$)and NO ($P < 0.01$) was most evident at 12 h. (Fig. 6).

Histological examination of pancreas

Injection of sodium taurocholate into pancreatic duct caused edema, inflammation, hemorrhage and necrosis changes in the pancreas of rats. We performed pathologic assessment of pancreas tissue according to the scoring criteria of Schmidt and found that in control group almost the entire normal glandular architecture was preserved, while in SAP group, various pathologic manifestations such as moderate edema, substantial inflammatory infiltration, moderate hemorrhage and acinar cell necrosis could be observed in the pancreas tissues of rats. However, these histological changes in resveratrol group were obviously alleviated when compared with the SAP group. The "Schmidt" criterion in resveratrol group at 3 ($P < 0.05$), 6 ($P < 0.01$) and 12 ($P < 0.01$) h after the induction of SAP is shown in Fig. 7.

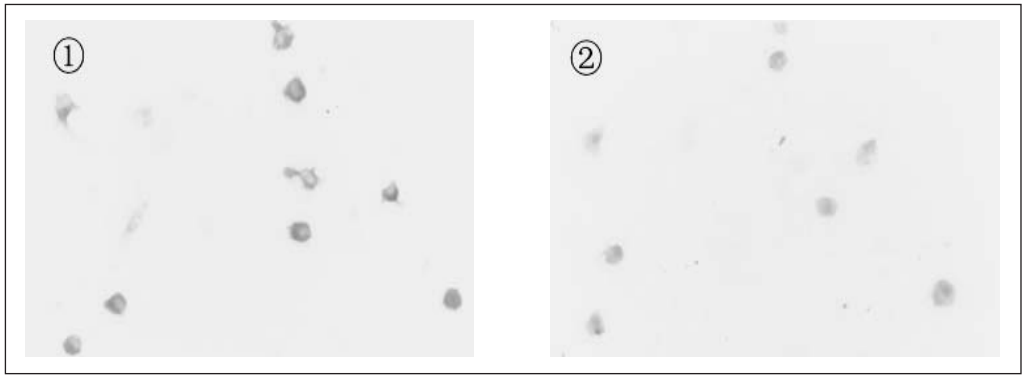

Fig. 4. The iNOS expression in peritoneal macrophages.×400. ① peritoneal macrophages in SAP group at 12 h interval; ② peritoneal macrophages in RESV group at 12 h interval. Cell were stained with anti-iNOS antibody and visualized with DAB-peroxidase after incubation with secondary antibody.

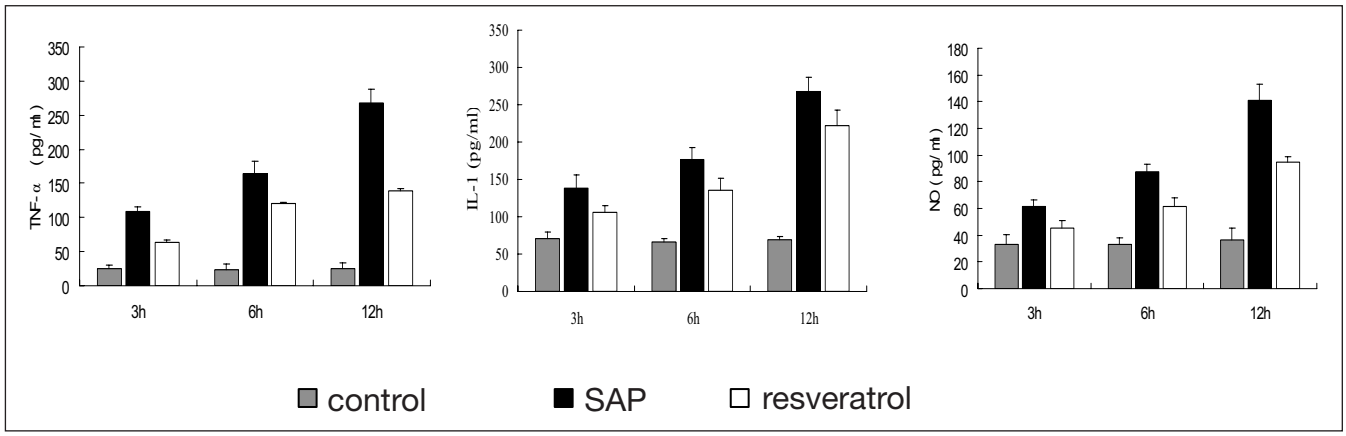

Fig. 5. Time course study of the effect of resveratrol on levels of cytokines in culture medium of peritoneal macrophages. Results are mean ±SEM, $P < 0.05$ (n = 6).

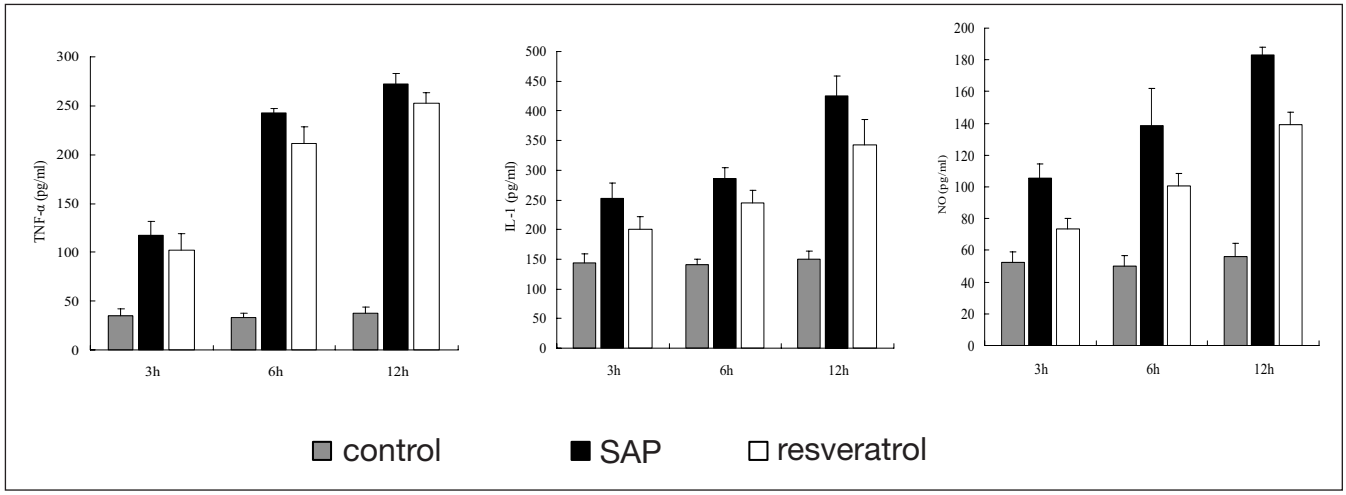

Fig. 6. Time course study of the effect of resveratrol on levels of cytokines in serum. Results are mean ±SEM, $P < 0.05$ (n = 6).

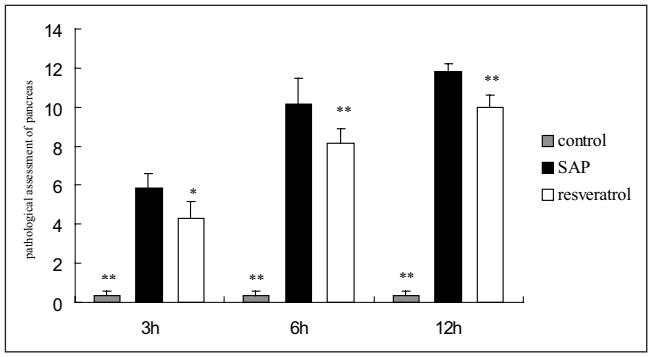

Fig. 7. Pathological assessment of pancreas at 3, 6 and 12 h after induction of SAP. *P<0.05 as compared with SAP group, **P<0.01 as compared with SAP group. Data are means ± SEM (n = 6) for the Schmidt criterion.

Discussion

TNF-α and IL-1 are mainly produced and released by macrophages in SAP. Excessive release of TNF-α and IL-1 will lead to a series of pathological changes in pancreas and other organs in SAP and the concentrations of TNF-α and IL-1 in serum are positively related to the severity of SAP. Masamune [26] suggested that anti-cytokine therapies against TNF-α, IL-1 and inhibition of macrophage migration showed appropriate effects in experimental animal models of SAP. Moreover, microcirculatory disturbance also plays an important role in the progress of SAP. Excessive NO production and release can initiate and aggravate the microcirculatory failure in SAP through injuring vascular endothelium and increasing vascular permeability [27].

Many studies suggested that transcription factor NF-κB plays a pivotal role in the inflammatory responses of SAP. When macrophages received inflammatory stimulation by SAP, such as TNF-α and IL-6, the NF-κB in cytoplasm would be activated and then translocated to nucleus. As an important factor of delivering intracellular signals, NF-κB modulates the transcription of many inflammatory mediators and furthermore, results in substantive release of inflammatory mediators in SAP, including TNF-α , IL-1 and iNOS [28–30]. In previous studies, the functions of monocytes in circulation and macrophages in organs has been studied extensively. These studies showed existence of a tight correlation between these cells and the failure of pancreas and

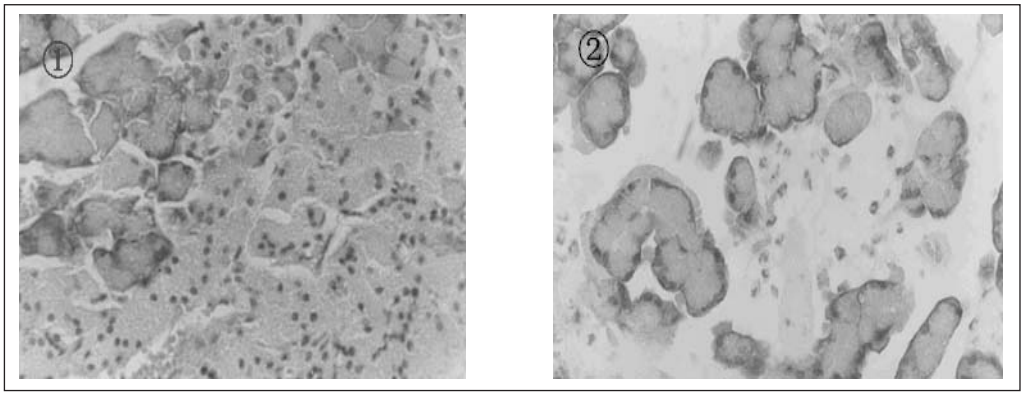

Fig. 8. Histological alterations in pancrease.×400. ① SAP group at 12 h interval ② RESV group at 12 h interval. Z E staining.

distant organs [31–34]. However, the functions of peritoneal macrophages were rarely discussed. In present study, our results clearly indicated that the levels of TNF-α, IL-1 and NO in culture medium of peritoneal macrophages and serum of rats are elevated, along with the upregulation of the expression of NF-κB and iNOS in peritoneal macrophages. We conclude that peritoneal macrophages are involved in the progression of acute pancreatitis.

A series of experiments have demonstrated that resveratrol exhibits a wide range of biological and pharmacological activities both *in vitro* and *in vivo*. Many of the biological activities of resveratrol, like anti-inflammatory, anti-oxidation, chemopreventive effects, and inhibition of platelet aggregation [34–36], indicate a possible effect on SAP. Histological changes in pancreas were consistent with the levels of TNF-α and IL-1 in culture medium and serum in our study, with an obviously milder histological change in pancreas found in resveratrol group, when compared with SAP group.

In conclusion, resveratrol can reduce the severity of pancreatic and systemic inflammatory response in SAP. A possible mechanism might be related to inhibition of the release of TNF-α, IL-1 and NO from peritoneal macrophages by suppressing NF-κB activation. Conceivably, NF-κB activation in macrophages may be of fundamental importance in severity of inflammation and a target in stress inflammation for resveratrol. It is possible that resveratrol may be considered as an agent for reducing the severity of inflammation.

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