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Original Research Article

Ulinastatin Reduces T Cell Apoptosis in Rats with Severe Acute Pancreatitis

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Abstract

Purpose: *To investigate the immunoregulative effects of ulinastatin (UTI) on T lymphocytes apoptosis in rats with severe acute pancreatitis (SAP) and to elucidate its underlying molecular mechanism.*
Methods: *Thirty six Wistar rats were randomly divided into 3 groups (n =12): sham, SAP model and UTI-treated group. SAP model was established by intrapancreatobiliary duct injection of 5% sodium taurocholate. A bolus of 10000 U/kg UTI was intravenously injected after SAP establishment. T cell apoptosis was determined by Annexin-V/PI double-staining. Oxidative stress was evaluated by examining changes in the levels of reactive oxygen species (ROS). Total superoxide dismutase (SOD) in serum was tested by hydroxylamine colorimetric assay, and malondialdehyde levels were examined by thiobarbituric acid assay. Mitochondrial function was evaluated by analyzing mitochondrial membrane potential (MMP) and mitochondrial permeability transition pore (MPTP).*
Results: *We found CD_4^+ T cells (32.10±2.87% vs. 45.22±4.38%, P<0.01) and CD_4^+/CD_8^+ T cells in SAP rats significantly decreased compared with sham group (1.15±0.12 vs. 2.23±0.12%, P<0.01), while the percent of the apoptotic CD_4^+ and (17.70±2.10 vs. 3.82±0.50%, P<0.01) CD_8^+ T lymphocytes was highly increased (2.78±0.45 vs. 1.97±0.36%, P<0.01 compared with sham group). After UTI treatment, the apoptosis of CD_4^+ T lymphocytes significantly decreased compared with SAP group (8.58±1.09 vs. 17.70±2.10%, P<0.01), while the percent of CD_4^+ T and CD_4^+/CD_8^+ lymphocytes significantly enhanced (P<0.01). ROS (mean fluorescence intensity): 5107±430 vs. 12904±840, P<0.01) and MDA levels (4.41±0.32 vs. 7.25±0.57nmol/ml, P<0.01) in serum in UTI-treated group were decreased compared with SAP group. SOD activity was enhanced after UTI treatment (59.72±5.45 vs. 48.32±3.81nmol/ml, P<0.01). Mitochondrial function assays showed that MMP (17.30±1.60 vs. 46.94±3.49%, P<0.01) and MPT (30.14±2.46 vs. 51.31±3.23%, P<0.01) were inhibited by UTI.*
Conclusion: *UTI reduces T lymphocytes apoptosis and improves immunological function in SAP rats, possibly via enhancing the scavenging capacity of oxygen free radical and attenuating the influence of oxidative stress.*

Key words: *Ulinastatin, T cell, Apoptosis, Severe acute pancreatitis, Mitochondrion.*

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INTRODUCTION

Severe acute pancreatitis (SAP) is a systematic disease with ongoing immune disorder. In the early stage, it is characterized by overdue inflammatory reaction, with its major clinical manifestation of systematic immune syndrome (SIRS) and the functional damage of related organs like cardiovascular, lungs and kidney [1]. In the late stage, the severe suppression of immune function is the fundamental cause of infection [2], which is manifested by disproportion of CD4/CD8, T helper cell (Th1/Th2) shift, the anergy of lymphocytes and most prominently, the excessive apoptosis of lymphocytes. The

excessive apoptosis of lymphocytes is the critical link that causes the dysfunction of immune system. Large amount of scavenging by the apoptosis of lymphocytes attenuates the number of lymphocytes with biological activity.

It has been demonstrated that oxidative stress (OS) is closely related to lymphocyte apoptosis [3]. As the fundamental organelles to determine the destiny of cells [4], mitochondria are the target of the ROS injury and an important origin of ROS as well [5].

Ulinastatin (UTI) is the glycoprotein separated and purified from the urine of healthy adult men. As an endogenous protective mechanism, UTI can antagonize stress and attenuate organ injury. Researchers have demonstrated that UTI has a strong enzyme inhibitory activity [6], enabling it to inactivate multiple enzymes. Furthermore, UTI has anti-inflammatory activity and can directly inhibit inflammatory mediators and inflammatory cytokines such as TNF-α, IL-1 and IL-8 [7]. Therefore, UTI is widely applied to pancreatitis therapy due to its ability to reduce many critical factors including the pancreative activity and the release of inflammatory mediators [8].

It has also been suggested that UTI improves immune function [9]. Given the therapeutical value of UTI and ROS production [10], we assumed that UTI may exert immunoinhibitory effects on SAP. In the present study, we investigated the immunoregulatory effects of UTI on T lymphocytes apoptosis in SAP rat model and elucidated the underlying molecular mechanism.

MATERIALS AND METHODS

Animals

Thirty six Wistar rats (250-300g, 3 months old, 18 males and 18 females), were purchased from Animal Laboratory Centre, Academy of Military Medical Sciences of PLA (license: SCXK 2009-003). Animals were kept in clean separate cages with free access to food and water, where room temperature remained between 20℃ to 29℃ in the dry aeration atmosphere, for 12 hours under fluorescent illumination to maintain the diurnal cycle. All the animal experiments were performed in accordance with the guidelines for animal research from the National Animal Center of China (No.95-38, revised 1995). All experimental protocols were approved by the Animal Care and Use Committee of Tianjin Medical University and were conducted according to the Helsinki Declaration.

Animal grouping and establishment of SAP model

Rats were randomly divided into 3 groups (n=12) after one week of adaptive feed: (1) sham-operated control group; (2) SAP group; (3) UTI-treated group. Animals were fasted for 12 hours before operation, with free access to water. Each rat was intraperitoneally administered with 10% chloral hydrate according to the weight that is, 300mg/kg. Abdominal skin preparation and disinfection was given after anesthesia. Then central abdominal incision below the xiphoid process was made under aseptic operation. The lower margin of the liver, the duodenum, the pancreatic duct and the partial pancreas were exposed. Puncture was carried out using disposable IV catheter through the lateral wall on the duodenum where the two cholecystopancreatic ducts form an opening. Part of the needle core was withdrawn immediately after insertion into the duodenal cavity. Then the trocar enters approximately 0.5 cm along the opening of the cholecystopancreatic duct and the needle core was completely pulled out. Cholecystopancreatic duct in the hilar region was clamped with a microclip to prevent the sodium taurocholate from injecting into the liver and the end of the intubation was connected to the syringe pump.

In the SAP model group and UTI-treated group, animals were injected with 5% sodium taurocholate at the speed of 0.1 ml/min according to the weight that is, 1ml/kg. Five minuites after injection, the intubation and artery occlusion were removed and the pin hole was wiped on the duodenal wall with 100% alcohol to make it contract to prevent the outflow of the intestinal contents. In the sham group, injection of sodium taurocholate was excluded. Other operations are the same as the other two groups. After operation, animals in each group recieved the interval subcutaneous injection of 5% glucose and sodium oxide of 50ml/kg. Following the development of the SAP model, animals in the UTI-treated group received injection of UTI through the tail vein of 10000 U/kg. All the animals were taken postoperative insulation and incision care to avoid asphyxia death caused by the rats' scratching and biting the suture or their vomit.

Separation of spleen lymphocytes

Suspensions of spleen T cells were cultured at a density of 2×10^5/well in RPMI 1640 medium supplemented with 5% heat-inactivated fetal bovine serum, 2 mM L-glutamine, 100 U/ml

penicillin and 100 µg/ml streptomycin in a humidified incubator, at 37°C with 5% CO_2.
MagCellect rat $CD3^+$ T cell isolation kit (R&DSystems, Minneapolis, MN, USA) was used according to the manufacturer's recommendation to isolate splenic T cells (C3H).Then, these were stained with FITC-conjugated anti-CD4 or PE-conjugated anti-CD8 antibody (BD Biosciences PharMingen).

Analysis of T cell apoptosis by flow cytometry

T cells were harvested and resuspended in binding buffer. The cell suspension was incubated with FITC Annexin-V (BD Biosciences, USA) and propidium iodide (PI) for 15 min at room temperature in dark followed by flow cytometry analysis. The percentage of cells positive for Annexin-V, PI alone and in combination was calculated by dot plot analysis using Cell Quest Pro software (FCM-500, Beckman).

Determination of malondialdehyde (MDA) production and superoxide dismutase (SOD) activity

The levels of SOD and MDA, special markers for reactive oxygen species (ROS), were determined in the sera of mice. The MDA level and SOD activity were analyzed using specific reagents according to the protocols provided by the manufacturer (Nanjing Jiancheng Bioengineering Institute, China). Briefly, thiobarbituric acid was used as substrate for the detection of MDA, and the xanthine oxidase method was used for the detection of SOD activity.

Mitochondrial membrane potential assay

An reagent, 5,5',6,6'- tetrachloro-1,1',3,3' tetraethylbenzimidazoly-carbocyanine iodide (JC-1), was used to assess mitochondrial potential changes using confocal microscope. Briefly, cells in 24-well plates at a density of 1×10^5 cells/ml cultured at 37C overnight for 24 hrs before addition of 1 µg/ml JC-1 in culture media for 30 min. The cells were examined by confocal microscope (Nikon, Japan). Changes in the fluorescence from red to green indicate the changes in mitochondrial membrane potential; the green/red ratio was calculated from the changes in the intensity of red and green fluorescence assessed using a computer program.

Determination of mitochondrial permeability transition

Mitochondrial permeability transition was determined by MitoProbe transition pore assay kit (Molecular Probes Inc., Eugene, OR, USA), a technique based on calcein acetoxymethyl ester (AM) and CoCl2.

Statistical analysis

The data was analyzed with SPSS18.0 software. The measurement data was expressed as X±S, the multiple mean comparison was evaluated by one way ANOVA, and the comparison between any two means by LSD test. The significant difference was P<0.05.

RESULTS

Ratio of CD_4^+ to CD_8^+ T cell subsets

As shown in Table 1, we found the percents of CD_4^+ T cells (32.10 ± 2.87 % vs. 45.22 ±4.38 %, $p < 0.01$) and CD_4^+/CD_8^+ T cells in SAP rats significantly decreased compared with sham group (1.15 ± 0.12 vs. 2.23 ± 0.12 %, $p < 0.01$)

Table 1: Ratio of CD_4^+ to CD_8^+ T cell subsets in each group (n = 12)

Group	CD_4^+(%)	CD_8^+(%)	CD_4^+/CD_8^+
Sham	45.22±4.38	20.28±2.11	2.23±0.12
SAP	32.10±2.87*	28.41±2.31	1.15±0.12*
Ulinastatin	37.63±3.41#	24.31±2.62	1.66±0.12#

*All the values are given as mean ± SEM; * p < 0.01 vs. control group ; # P < 0.01 vs. SAP group*

Ratio of CD_4^+ to CD_8^+ T cell subset apoptosis

By using annexin V/PI Double Staining, we observed that the percent of apoptotic CD_4^+ T cells and (17.70 ± 2.10 vs. 3.82 ± 0.50 %, $p < 0.01$) apoptotic CD_8^+ T cells in the SAP group greatly increased (2.78 ± 0.45 vs. 1.97 ± 0.36 %, $p < 0.01$) in comparison with sham group. After UTI treatment, the apoptosis of CD_4^+ T lymphocytes in the SAP rats significantly decreased (8.58 ± 1.09 vs. 17.70 ± 2.10 %, $p < 0.01$), but the percent CD_4^+ T and CD_4^+/CD_8^+ lymphocytes markedly enhanced ($p < 0.01$). (Table 2).

ROS Level in spleen lymphocytes

As seen from Table 3, ROS (MFI: 5107±430 vs. 12904±840, P<0.01) and MDA levels (4.41±0.32 vs. 7.25±0.57nmol/ml, P<0.01) in serum in UTI-treated group were decreased. SOD activity was

enhanced after UTI treatment (59.72±5.45 vs. 48.32±3.81 nmol/ml, P<0.01) (Table 4).

Table 2: Apoptosis of CD_4^+、CD_8^+ T cells subsets in each group (n = 12)

Group	CD_4^+ T cells subset apoptosis (%)	CD_8^+ T cells subset apoptosis (%)
Control	3.82±0.50	1.97±0.36
SAP	17.70±2.10*	2.78±0.45*
Ulinastatin	8.58±1.09#	2.46±0.36▲

*All the values are given as mean ± SEM; *p < 0.01 vs. control group; # p < 0.01 vs. SAP group; ▲p > 0.05 vs. SAP group*

Table 3: ROS level in spleen lymphocytes for each group (n = 12)

Group	Mean fluorescence intensity of ROS
Sham	467±48
SAP	12904±840*
Ulinastatin	5107±430#

*All values are given as mean ±SEM; * p < 0.01 vs. control; # p < 0.01 vs. SAP group*

Table 4: Serum SOD activity and MDA level for each group (n=12)

Group	SOD activity (U/ml)`	MDA level (nmol/ml)
Sham	80.68±0.50	2.27±0.26
SAP	48.32±3.81*	7.25±0.57*
Ulinastatin	59.72±5.45#	4.41±0.32#

*All the values are given as mean ± SEM; * p < 0.01 vs. control group; #p < 0.01 vs. SAP group*

Spleen lymphocyte MMP

Mitochondrial function was evaluated by analyzing mitochondrial membrane potential. It showed that MMP (17.30 ± 1.60 vs. 46.94 ± 3.49 %, p < 0.01) (30.14±2.46 vs. 51.31±3.23 %, p < 0.01) was inhibited by UTI (Table 5).

Table 5: The spleen lymphocyte MMP for each group (n = 12)

Group	Lymphocytes with decreased MMP (%)
Sham	3.69±0.45
SAP	46.94±3.49*
Ulinastatin	17.30±1.60#

*All the values are given as mean ± SEM; * p < 0.01 vs. control group; #p < 0.01 vs. SAP group*

Opening level of the spleen lymphocyte MPTP

Mitochondrial function was also evaluated by analyzing by mitochondrial permeability transition pore. As shown in Table 6, compared with SAP group, MPT (30.14 ± 2.46 vs. 51.31 ± 3.23 %, p < 0.01) was inhibited in UTI-treated rats.

Table 6: Opening level of the spleen lymphocyte MPTP in each group (n = 12)

Group	Lymphocytes with abnormal opening of MPTP (%)
Sham	7.69±0.77
SAP	51.31±3.23*
Ulinastatin	30.14±2.46#

*All the values are given as mean ± SEM; * p < 0.01 vs. control group; #p < 0.01 vs. SAP grou*

DISCUSSION

UTI attenuates the abnormal apoptosis of CD4+ T lymphocytes in SAP rats

Lymphocyte apoptosis is closely related immunosuppression [11]. Recent study has proved that immunosuppression is mainly caused by acceleration of lymphocytes apoptosis. Inflammation of the pancreas and its surrounding areas, caused by weakened immunity, is the commonest and severest complication and constitutes the main cause of death in the later progress of SAP, with mortality rate reaching 80% [12].

In our study, the percentage of apoptotic CD_4^+T lymphocytes in the SAP group went up more noticeably than that of apoptotic CD_8^+T lymphocytes, which led to the phenomenon that the percentage of CD_4^+T lymphocytes and the ratio of CD_4^+ and CD_8^+T lymphocytes in SAP group were remarkably lower than that in sham operation control group. It can be concluded that when SAP occurs, the body stimulates the apoptosis of CD_4^+T lymphocytes to down-regulate inflammatory reaction. This can lighten the injury that resulted from excessive inflammatory reaction but will result in low cellular immune function which causes immunosuppression.

UTI, a glycoprotein secreted by the liver [13], can act on several key steps of pancreatic disease, such as activation of pancreatic enzymes and cytokines and release of inflammatory mediators, so it is widely applied in pancreatitis treatment [8]. In 2009, Huang et al demonstrated UTI can noticeably improve the living conditions of sepsis patients [14].

In this study, the proportion of CD_4^+T lymphocytes and ratio of CD_4^+ and CD_8^+T lymphocytes in UTI-treated group has obviously picked up compared with the SAP group, mainly

due to the fact that the percentage of apoptotic CD_4^+T lymphocytes decreased significantly while the proportion of apoptotic CD_8^+T lymphocytes was almost the same with that in the SAP group. Through reducing abnormal apoptosis of CD_4^+T lymphocytes in SAP rats, UTI has a direct effect in improving the state of immunodepression so as to reduce the susceptibility of secondary infection, prevent occurrence of septicopyemia and MODS and lower the mortality rate of SAP.

UTI attenuates oxidative stress level in rats with SAP

During the process of pancreatic damage, ROS can not only peroxidize fatty acids, but also destroy the lipid membrane and lysosomal membrane, causing injury to the gland. Moreover, in the cases of peripancreatic injury, ROS is the key factor which generates cytokines and inflammatory mediator cascade, mediates inflammatory cell infiltration, endothelial dysfunction and microcirculation dysfunction, thereby resulting in organ lesions. ROS level is closely related to the degree of apoptosis the lymphocytes [3].

The following reasons may mainly account for the increase of oxygen free radical in SAP: Bile salts, trypsin and chymotrypsin activate xanthine oxidase, which catalyzes the oxidation of hypoxanthine to xanthine and produce a great number of oxygen free radicals [15]. Inflammatory mediators like thromboxane can engender neutrophil chemotaxis and produce oxygen free radicals through respiratory burst.

SOD can effectively scavenge superoxide anion radicals produced in the metabolic process, whose activity can indirectly reflect the body's ability to eliminate the oxygen free radicals. MDA is one of the major aldehyde formed via lipid peroxidation, whose contents can reflect the degree of the body's lipid peroxidation and indirectly reflect the severity of oxygen free radicals' attack on cells. Therefore the SOD activity and the MDA contents can reflect the equilibrium condition between the body's oxidation and anti-oxidation.

The present study showed that UTI can resist lipid peroxidation induced by free radicals and their metabolic products. Furthermore, as a high efficiency, broad spectrum enzyme inhibitor, UTI can block the accumulation of neutrophils mediated by protease in lungs, inhibit the activity and the phagocytosis of the lysosomal enzyme in neutrophils, hence reducing the release of inflammatory mediators and oxygen free radicals and restricting the vicious cycle and magnified effect due to the cascade reaction of enzyme, cytokine and oxygen free radical. This study shows that UTI can significantly enhance the scavenging capacity of oxygen free radical, reduce the apoptotic ratio of lymphocytes, and attenuate the influence of oxidative stress on immunologic function in SAP rats through lowering the intracellular ROS production in splenic lymphocytes and the MDA contents in serum, and increasing the activity of SOD in serum as well.

The mitochondrial mechanism of UTI underlying its role of reducing apoptosis of T lymphocytes

Mitochondria are not only the place for tricarboxylic acid cycle, oxidative phosphorylation and fatty acid oxidation, but also a crucial organelle for apoptosis [16]. As a key organelle [4] which could decide a cell's fate--- survival or apoptosis, mitochondrion is both the damage target point and significant source of ROS [5]. Though it is the main place for the generation of ROS, excessive ROS could result in the damage of mitochondrial oxidative stress and respiratory chain dysfunction, which will in reverse stimulate mitochondria to produce more ROS. This vicious circle could gradually lead mitochondria to dysfunction and finally damage cell function and viability [17]. Data shows that mitochondrion is also a vital source for ROS in T lymphocyte. Large quantities of energy is needed for T lymphocyte to activate and proliferate, which could cause over polarization of mitochondrial membrane and further fierce oxidative phosphorylation, and more electrons overflow from the respiratory chain to bring more ROS [18]. The accumulated ROS in mitochondrion could result in its DNA mutation, lipid peroxidation and open-up MPTP, which could finally affect its structure and function. According to some studies, the key factors which could open up MPTP are excessive Ca^{2+} in mitochondria, oxidative stress after massive oxygen radicals, the exhaustion of adenine nucleotide and mitochondrial membrane depolarization [19]. If the vicious cycle between ROS and mitochondria keeps going, cytochrome C will be released to activate the cascade of caspase-9, 8, 3 enzymes, which finally result in lymphocyte apoptosis. During apoptosis, ROS stimulates the opening up of MPTP and can also be produced as products during this stimulation. Hausenloy DJ,et al reported that MPTP may acts as a terminal effect factor regulating apoptosis, both as endogenous self activator and ROS initiation factors [20].

In this experiment, compared with the control group, lymphocytes with dropped mitochondrial membrane potential or abnormally opened MPTP from the SAP group got accelerated remarkably, which suggested that, during SAP, splenic lymphocytic mitochondrial membrane permeability enhanced and potentially decreasing, the mitochondrial apoptosis pathway played an important role in regulating apoptosis. UTI could effectively protect mitochondria through stabilizing mitochondrial membrane structure and enhancing the activity of mitochondrial Na^+-K^+-ATPase enzyme. Other studies found that UTI could lessen mitochondrial damages and inhibit apoptosis induced by mitochondrial pathway, through limiting the expressions of ischemia-reperfusion organic cytochrome C and AIF. During the experiment, it was found that ulinastatin could dramatically lower the ratio of lymphocytes with opened MPTP in SAP rats and the ratio with dropped MMP. It is finally demonstrated that UTI attenuates lymphocytes apoptosis and improves immunological function in SAP rats. It exerts the therapeutic effects through reducing ROS induced by SAP, blocking the vicious cycle between ROS and mitochondrial damage, thereby inhibiting the apoptosis induced by mitochondrial signaling pathways.

CONFLICT OF INTEREST

The authors have declared no conflict of interest.

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