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Dexamethasone protects the glycocalyx on the kidney microvascular endothelium during severe acute pancreatitis*

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Abstract: Objective: This study demonstrated that dexamethasone (DEX) protects the endothelial glycocalyx from damage induced by the inflammatory stimulus tumor necrosis factor-α (TNF-α) during severe acute pancreatitis (SAP), and improves the renal microcirculation. Methods: Ninety mice were evenly divided into 3 groups (Sham, SAP, and SAP+DEX). The SAP mice model was established by ligature of pancreatic duct and intraperitoneal injection of cerulein. Renal perfusion and function, and morphological changes of the glycocalyx were evaluated by laser Doppler velocimetry, electron microscopy, and histopathology (hematoxylin and eosin (H&E) staining), respectively. Serum levels of syndecan-1 and TNF-α were assessed by enzyme-linked immunosorbent assay (ELISA). The protective effects of dexamethasone on the glycocalyx and renal microcirculation were evaluated. Results: Significantly high levels of serum TNF-α were detected 3 h after the onset of SAP. These levels might induce degradation of the glycocalyx and kidney hypoperfusion, resulting in kidney microcirculation dysfunction. The application of dexamethasone reduced the degradation of the glycocalyx and improved perfusion of kidney. Conclusions: Dexamethasone protects the endothelial glycocalyx from inflammatory degradation possibly initiated by TNF-α during SAP. This is might be a significant discovery that helps to prevent tissue edema and hypoperfusion in the future.

Key words: Severe acute pancreatitis (SAP); Acute kidney injury (AKI); Glycocalyx; Dexamethasone; Tumor necrosis factor-α (TNF-α)
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1 Introduction

Severe acute pancreatitis (SAP) is one of the most common critical diseases, and has an overall mortality rate of up to 39% (Lankisch et al., 2015). Multiple organ dysfunction is the primary cause of death from SAP, and the acute kidney injury (AKI) is the most common and fatal complication. The incidence of AKI in SAP is as high as 69% (Zhou et al., 2015), which increases mortality by about 10-fold (Kes et al., 1996). The pathogenesis of AKI is still unclear, but malfunction of the microcirculation is considered the main possible mechanism. Injury to the endothelium and increased microvascular permeability resulting from inflammation are the major causes of AKI during SAP (Verma and Molitoris, 2015).

The glycocalyx is a single protective layer that resides on the vascular luminal endothelial surface

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and is composed of proteoglycans and glycosaminoglycans (van Golen et al., 2012). Proteoglycans serve as the core of the glycocalyx. Among various proteoglycan species, syndecan and glypican are specifically connected to the endothelium via a particular anchor (Rosenberg et al., 1997). The glycocalyx has been demonstrated to prevent accumulation of platelets and leukocytes at the vascular wall, and to regulate vascular permeability (Chappell et al., 2010). Accordingly, the integrity of glycocalyx plays a crucial role in the regulation of the inflammation and kidney microcirculation (Ince, 2014). The glycocalyx is known to be degraded by ischemic and inflammatory stress. Recent studies demonstrated that the degradation of the glycocalyx is possibly associated with the release of tumor necrosis factor-α (TNF-α), which is associated with the production of proinflammatory mediators (Nieuwdorp et al., 2009; Glasner et al., 2017). Glucocorticoids, a class of steroids secreted by the adrenal cortex, have specific anti-inflammatory effects. Recent evidence shows that hydrocortisone may protect the glycocalyx from degradation induced by ischemia-reperfusion, which may improve the microcirculation (Gao et al., 2015). Therefore, we hypothesized that TNF-α is released in SAP, and the acute inflammatory response adversely affect the glycocalyx on the kidney vascular endothelium, leading to kidney microcirculation dysfunction.

This study investigated whether the release of TNF-α causes shedding of endothelial glycocalyx and aimed to clarify the relationship between glycocalyx damage and kidney microcirculation dysfunction in the early stage of SAP. We then explored if this action can be prevented by dexamethasone pre-treatment.

2 Materials and methods

2.1 Animals

Male C57BL/6 mice (8–10 weeks of age, weighing 22–25 g) were purchased from the Animal Resource Center of Zhejiang University School of Medicine, Hangzhou, China. All studies were conducted with the approval of Institutional Animal Care and Use Committee at the Zhejiang University. All experimental animals received appropriate anesthesia and analgesia.

2.2 Model of SAP and the experimental group

The mice were anaesthetized by intraperitoneal injection of 1% (10 mL/kg) sodium pentobarbital. A 1-cm midline laparotomy was carried out, the pancreatic duct was ligated with a 6-0 suture from its distal end, and then the abdomen was closed. After the mice recovered and ambulated postoperatively, six doses of cerulein (Sigma Aldrich, St. Louis, MO, USA) were intraperitoneally injected at a rate of 100 mg/(kg·h). The mice were resuscitated by injecting saline (20 mL/kg) immediately after SAP model was accomplished, and then were carefully monitored for physical activity, response to stimulation, and other signs. The mice were divided into three groups with 30 in each group as follow: (1) Sham group, the mice underwent the same surgical procedures and fluid resuscitation but without ligature and injection of cerulein; (2) SAP group, the mice underwent ligature, cerulein injection, and resuscitation; (3) SAP+DEX group, the mice were treated with dexamethasone (2.5 mg/kg) after SAP model accomplished. Dexamethasone was purchased from Sigma-Aldrich (Missouri, USA, catalog: D1756).

2.3 Assessment of renal function

Blood samples were obtained from post-global veins at 3, 6, 12, 24, and 48 h after the procedure, with six mice assigned to each time point. Blood serum creatinine (SCr) levels were assayed in the laboratory of the School of Medicine, the Second Affiliated Hospital of Zhejiang University, for assessment of renal function.

2.4 Histopathology

The pancreas and kidneys were removed from mice at 3, 6, 12, 24, and 48 h after the procedure, and fixed in 10% formaldehyde for 24 h, and then embedded in paraffin. They were cut into 5-μm-thick sections, which were then stained with hematoxylin and eosin (H&E). Morphological changes were observed with light microscopy by an independent pathologist. The histological assessment of pancreatic injury included observations of edema, acinar necrosis, hemorrhage, fat necrosis, inflammation, and perivascular infiltrate. The severity of injury was scored according to the method of Schmidt et al. (1992). Histological changes of the kidney were scored using a five-point quantitative scale according to the percentage of renal cortical tubular necrosis and

hemorrhage. The changes were ranked as follows: 0, less than 10%; 1, 10%–25%; 2, 26%–50%; 3, 51%–75%; 4, more than 75% (Zheng et al., 2006). All slices were randomly selected for blinded observation.

2.5 Assessment of renal perfusion

Blood flow in the kidney was evaluated by laser Doppler velocimetry (Moor Instruments, Axminister, UK) at different time points. A 633-nm infrared light was used to detect the blood flow through frequency shifts of photons caused by moving erythrocytes in microvasculature. The mean blood flow of the kidney was documented using Moor LDI V5.2 software and expressed in perfusion units in relation to an internal standard calibration of the device (Singh et al., 2009).

2.6 Serum concentrations of TNF-α and syndecan-1

Serum samples were collected from post-global vein blood at 3, 6, 12, 24, and 48 h after the procedure. TNF-α and syndecan-1 were measured with the enzyme-linked immunosorbent assay (ELISA) according to the manufacture's guidelines (TNF-α, Mouse TNF-α ELISA Kit, BioLegend, California, USA, catalog: 430907; syndecan-1, mouse CD138/SDC1 ELISA Kit, Mlbio, Shanghai, China, catalog: ml037982). The concentrations of TNF-α and syndecan-1 were calculated with the help of a standard curve derived using the kit instructions. Values were expressed as picogram per milliliter (pg/mL).

2.7 Electron microscopy

Renal tissue for electron microscopy was cut into three to four pieces of about 1 mm^3 each and fixed in 2.5% glutaraldehyde for one night. Then samples were washed with 0.1 mol/L phosphate buffer (pH 7.0) and fixed with 1% (0.01 g/mL) osmium tetroxide for 2 h, dehydrated in graded ethanols, and embedded in Spurr's resin. Ultrathin sections (70-nm) were stained separately with uranyl acetate and lead citrate for 6 min. Samples were examined by transmission electron microscopy (H-7650, Hitachi High-Tech, Tokyo, Japan). The thickness of the renal endothelial glycocalyx was measured, and the mean thickness of eight positions in each sample was considered as the final thickness.

2.8 Statistical analysis

Analysis of variance (ANOVA) and Tukey's test were used to assess the differences among groups. All data analysis was performed using GraphPad Prism 6.0 (GraphPad Software for Science, San Diego, CA, USA). A value of $P<0.05$ was considered statistically significant.

3 Results

3.1 Establishment of the mouse SAP model

In the Sham group, the pancreas had a normal histopathologic appearance without inflammatory cell infiltration (Fig. 1a). After pancreatic duct ligature and cerulein injection in the SAP and SAP+DEX groups, the destruction and reduction of the pancreatic acinus were apparent. Other morphological changes included stroma edema, inflammatory cell infiltration, and hemorrhage (Fig. 1b). In addition, the severity score of the pancreas in the Sham group was obviously lower than those in the SAP and SAP+DEX groups ($P<0.01$; Fig. 1c). These results demonstrated that the mice SAP model was established successfully.

3.2 Effect of dexamethasone on renal injury during SAP

The SCr of mice in the SAP group was markedly increased 6 h after onset of SAP compared to that in the Sham group ($P<0.01$), and reached a peak at 12 h. Interestingly, although the SCr increased in the SAP+DEX group, it was obviously lower than that in the SAP group ($P<0.05$; Fig. 2e). Next, we compared the pathological changes among the three groups. The renal sections in the SAP group exhibited significant injuries (Fig. 2c) compared to those in the Sham group (Fig. 2a), such as degeneration of tubules, luminal congestion, and intratubular cast formation. In contrast, the renal sections from SAP+DEX groups showed a marked reduction in renal injury (Fig. 2b). Furthermore, the tubular damage scores were significantly higher in the SAP group than in the Sham group, but significantly lower after administration of dexamethasone (Fig. 2d). Based on these observations, we demonstrated that dexamethasone could protect the kidneys from injury induced by SAP.

3.3 Effect of dexamethasone on renal perfusion

We examined perfusion in a time course manner in three groups using laser Doppler velocimetry.

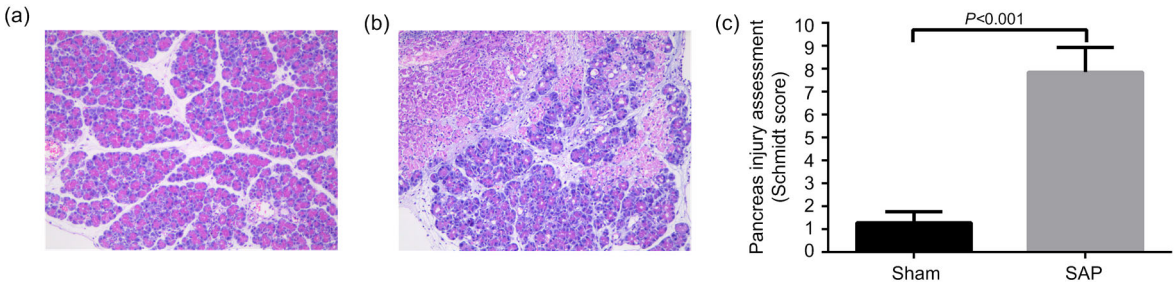

Fig. 1 Pathological changes in the pancreas in SAP mice

The SAP mouse model was successfully established by ligature of pancreatic duct and intraperitoneal injection of cerulein, and confirmed by histological analysis following staining of pancreas tissue with hematoxylin and eosin (H&E; magnification: 20×). (a) Sham group, normal pancreatic structures without necrosis and inflammatory cell infiltrations. (b) SAP group, extensive necrosis accompanied by interstitial tissue edema, inflammatory infiltration, and hemorrhages in the pancreas. (c) Pancreas injury scores (Schmidt score) were significantly higher in the SAP group than in the Sham group (P<0.001, n=8). Data were expressed as mean±standard deviation

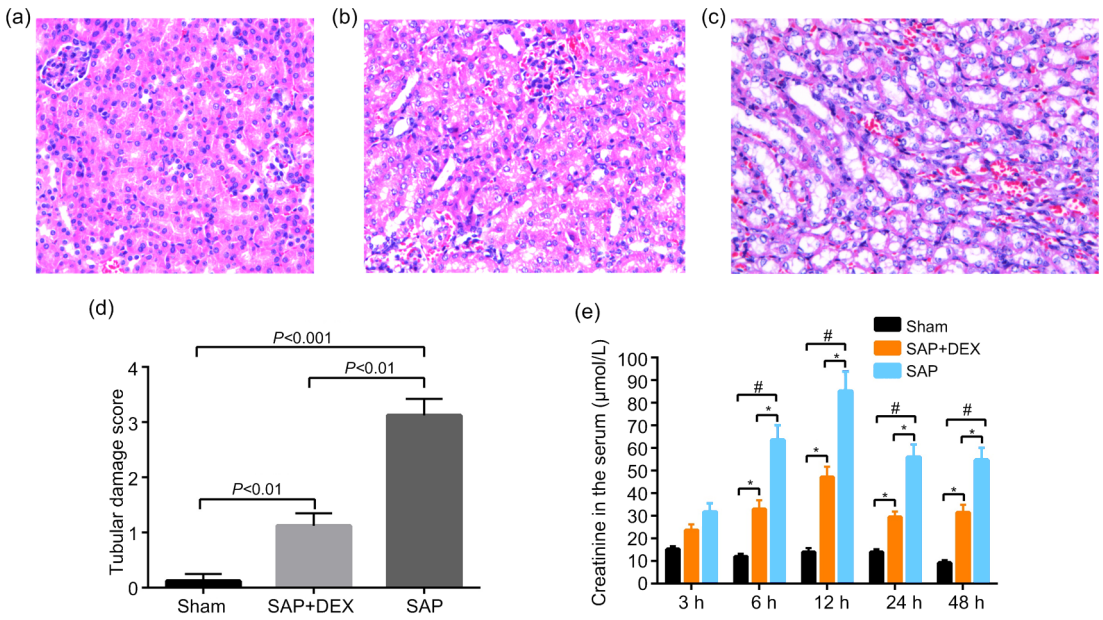

Fig. 2 Histological and laboratory assessment of renal injury in the early stage of SAP

The H&E-staining images (magnification: 20×) showed the evidences of renal tubular damage in accordance with the high levels of serum creatinine in the early stages of SAP, which was reduced by administration of dexamethasone. (a) Sham group, normal kidney structure without tubular necrosis. (b) SAP+DEX group, dexamethasone treatment resulted in an attenuation of kidney injury. (c) SAP group, several injurious changes were observed on the renal tubulars during SAP. (d) The tubular damage score in the mice of SAP group was significantly higher than that in the Sham mice (P<0.001, n=8), and the score was reduced by dexamethasone treatment (P<0.01, n=8). (e) Serum creatinine levels indicated similar elevations at each time point from 6 to 48 h after onset of SAP ($^{\#}P$<0.01, n=8), which were attenuated by administration of dexamethasone ($^{*}P$<0.05, n=8). Data were expressed as mean±standard deviation

Compared to the Sham group, renal perfusion in mice from SAP group was significantly decreased 6 h after the onset of SAP (P<0.01) and reached a nadir at 24 h. After administration of dexamethasone, the perfusion in SAP+DEX mice was obviously increased compared to that in the SAP group (P<0.05; Fig. 3). This result indicated that the dexamethasone significantly improved renal perfusion during SAP.

3.4 Effect of dexamethasone on renal endothelial glycocalyx during SAP

Electron microscopy was performed to detect the renal microvasculature of the endothelial glycocalyx. Normal integrity of the glycocalyx lining was observed in mice of the Sham group (Fig. 4a), and a mostly intact lining was observed in the SAP+DEX

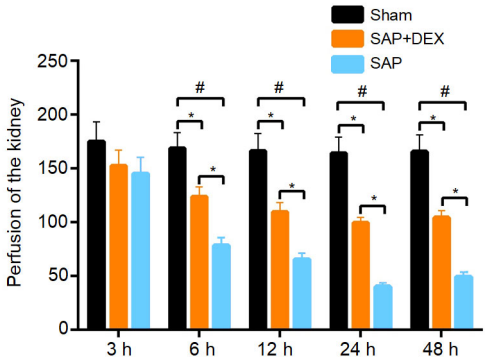

Fig. 3 Changes in kidney perfusion in the early stage of SAP

SAP caused a reduction of perfusion in the kidneys of mice from the SAP and SAP+DEX groups at each time point from 6 to 48 h after onset of SAP compared to those in the Sham group (* P<0.05, $^{\#}$ P<0.01, n=8). The perfusion of the kidney in the mice from the SAP+DEX group was significantly higher than that in mice from the SAP group (* P<0.05, n=8). Data were expressed as mean±standard deviation

group (Fig. 4b). However, a rudimentary glycocalyx was visualized in the SAP group (Fig. 4c). The thickness of the glycocalyx in the SAP group was apparently lower than that in SAP+DEX group (P<0.05; Fig. 4d). Syndecan-1, which is a constituent of the glycocalyx, was positively detected in blood samples from mice in the SAP group, which demonstrated SAP enhanced shedding of glycocalyx. Application of dexamethasone significantly decreased shedding at all time points except 3 h after onset of SAP compared to that of mice in the SAP group (P<0.05; Fig. 5a). These results demonstrated the protective effect of dexamethasone on the renal endothelial glycocalyx during SAP.

3.5 Effect of dexamethasone on TNF-α release

We then examined the effect of dexamethasone on the level of TNF-α in the bloodstream. The concentration of TNF-α started to elevate in the SAP and SAP+DEX groups 3 h after onset of SAP (P<0.01), and peaked at 6 h. Application of dexamethasone significantly decreased the concentration of TNF-α at all time points except 3 h after onset of SAP (P<0.05; Fig. 5b). Reduction of TNF-α following treatment with dexamethasone may be a part of the mechanism protecting the renal endothelial glycocalyx microvasculature and microcirculation during SAP.

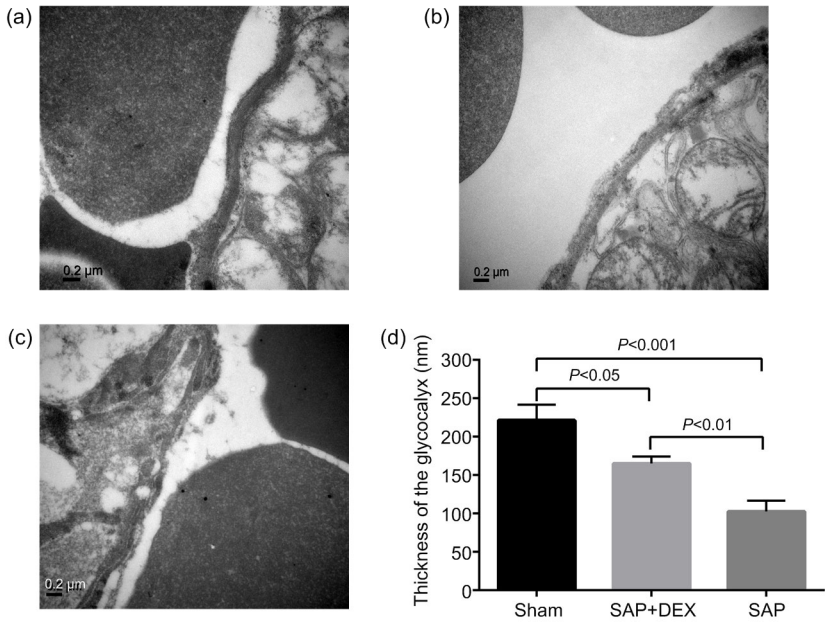

Fig. 4 Degradation of the endothelial glycocalyx in the kidney during SAP

Damage to the glycocalyx was observed by electron microscopic analysis (magnification: 40000×). (a) Sham group, normal glycocalyx lining on the surface of endothelial cells. (b) SAP+DEX group, the glycocalyx was slightly damaged after DEX treatment. (c) SAP group, the glycocalyx was seriously damaged 48 h after onset of SAP. (d) The thickness of the glycocalyx in the mice from SAP group was significantly lower than that in mice from the Sham group (P<0.01, n=8), which showed attenuation after application of dexamethasone (P<0.05, n=8). Data were expressed as mean±standard deviation

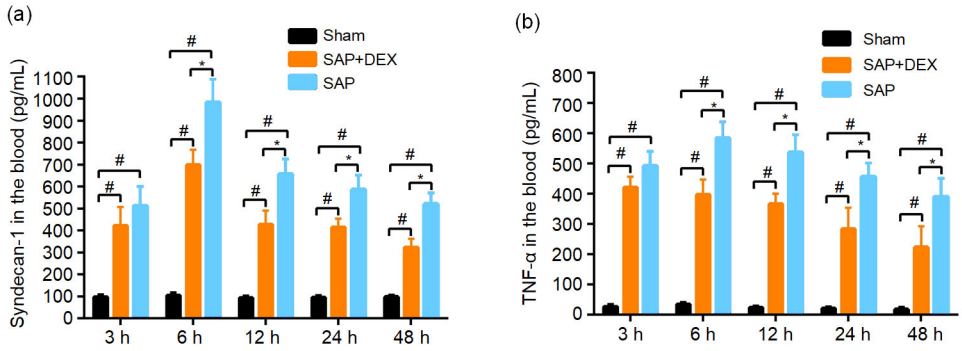

Fig. 5 Serum levels of syndecan-1 and TNF-α in the early stage of SAP

SAP caused elevation of TNF-α in serum, which resulted in shedding of syndecan-1 from the endothelial glycocalyx into the vascular lumen. (a) Levels of syndecan-1 were remarkably increased at each time point from 3 to 48 h after onset of SAP ($^{\#}P<0.01$, $n=8$), and were reduced by applying dexamethasone at each time point from 6 to 48 h after onset of SAP ($^{*}P<0.05$, $n=8$). (b) Levels of TNF-α were remarkably elevated with SAP ($^{\#}P<0.01$, $n=8$), and were reduced by applying dexamethasone ($^{*}P=0.05$, $n=8$). Data were expressed as mean±standard deviation

4 Discussion

AKI is the most common organ dysfunction in SAP patients, and the presence of AKI is known to be an independent risk factor for mortality (Pavlidis et al., 2013). Malfunction of microcirculation plays a key role in AKI during SAP. In the present study, we investigated changes in serum creatinine levels and kidney histology following kidney injury caused by SAP. In addition, we identified renal perfusion and degradation of endothelial glycocalyx in relation to the release of TNF-α, and determined the efficacy of dexamethasone therapy in renal protection during SAP.

We found that kidney injury can be caused in the early stage of SAP. For instance, serum creatinine, the biomarker of kidney injury, became elevated at 6 h after the onset of SAP, and histopathology showed serious tubular necrosis and inflammation. Despite progress in research on mechanisms of AKI, the pathophysiology of the occurrence of AKI is still unclear (Wan et al., 2008). Recent research showed that ischemia and poor perfusion were considered a mechanism responsible for malfunction of renal microcirculation caused by inflammatory and oxidative stress that leads to AKI (Ince, 2014; Ergin et al., 2015). In the present study, we found that the renal perfusion in SAP mice was markedly decreased 6 h after the onset of SAP. These findings suggest that hypoperfusion is a possible reason for microcirculation dysfunction.

The normal healthy luminal side of microvascular endothelium is covered by glycocalyx, which consists of proteoglycans, mainly glypicans and syndecans, anchored on the endothelial membrane and attached with hyaluronic acid and heparan sulphate (van Golen et al., 2014). The integrity of endothelial glycocalyx can prevent adhesion of leukocytes and platelets at the microvascular wall. As a part of maintaining microvascular homeostasis, the glycocalyx regulates vascular permeability and keeps a fluid balance between vascular lumen and interstitial tissue (Curry and Adamson, 2010; Levick and Michel, 2010). Degradation of endothelial glycocalyx may result in interstitial edema, increased permeability of microvasculature, and accumulation of leukocytes and platelets at endothelium. These changes can cause fluid redistribution and tissue hypoperfusion. Henry and Duling (2000) suggested that the degradation of the glycocalyx was related to the release of TNF-α. They found that TNF-α might activate leukocytes to release free radicals that degrade components of the endothelial glycocalyx and lead to shedding of syndecans from glycocalyx into the vascular lumen. These effects were correlated with microcirculation dysfunction and organ injury. We found a significantly higher concentration of TNF-α in serum 3 h after the onset of SAP. Interestingly, we also found increased syndecan-1 serum levels in mice from the SAP group associated with the higher TNF-α concentration. In addition, a rudimentary glycocalyx was observed in mice in the SAP group, and the thickness

of the glycocalyx was markedly lower than that in mice in the Sham group. These findings are in agreement with previous published study (Gao et al., 2015). Our findings demonstrated that an SAP-induced severe inflammatory reaction with TNF-α released as one of the main inflammatory factors might be involved in the shedding of syndecan-1 and degradation of the endothelial glycocalyx in renal vasculature, leading finally to renal hypoperfusion and microcirculation dysfunction.

Dexamethasone, a class of steroids secreted by the adrenal cortex, is a potent anti-inflammatory agent, used for treating sepsis and septic shock (Rhodes et al., 2017). Previous studies have shown that corticosteroids might attenuate the increase of various chemokines and cytokines and improve vascular permeability (de Leeuw et al., 2016; Barabutis et al., 2017). Zhang et al. (2013) proved that dexamethasone significantly inhibited ischemia-reperfusion-induced TNF-α expression by suppressing PI3K/AKI signaling, which improved perfusion of the kidney and attenuated kidney injury. In our study, administration of dexamethasone in SAP mice significantly suppressed TNF-α expression, possibly as a result of its anti-inflammatory effect. It also reduced the damage to the endothelial glycocalyx, increased perfusion in the renal microcirculation, and attenuated renal injury. Our findings support the hypothesis that dexamethasone attenuates inflammatory reaction and reduces the release of TNF-α, which might be the mechanism by which it reduces degradation of the glycocalyx and prevents kidney injury from SAP.

5 Conclusions

Degradation of the glycocalyx and malfunction of renal microcirculation were found during SAP, which might be associated with increased TNF-α. The effects could be attenuated by application of dexamethasone.

Contributors

Wen-qiao YU and Qing-hui FU performed the experimental research and data analysis, wrote and edited the manuscript. Shui-qiao FU and Shao-yang ZHANG performed the data analysis and wrote the paper. Wei-na LU and Jiang ZHANG performed the establishment of animal models. Zhong-yan LIANG collected and analyzed the data. Yun ZHANG and Ting-bo LIANG contributed to the study design, data analysis, writing and editing of the manuscript. All authors read and approved the final manuscript and, therefore, have full access to all the data in the study and take responsibility for the integrity and security of the data.

Compliance with ethics guidelines

Wen-qiao YU, Shao-yang ZHANG, Shui-qiao FU, Qing-hui FU, Wei-na LU, Jian ZHANG, Zhong-yan LIANG, Yun ZHANG, and Ting-bo LIANG declare that they have no conflict of interest.

All institutional and national guidelines for the care and use of laboratory animals were followed.

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中文概要

题　目：地塞米松可以减少由重症急性胰腺炎引起的肾脏微血管内皮糖萼的损伤

目　的：明确地塞米松可以减少重症急性胰腺炎（SAP）引起的肿瘤坏死因子（TNF-α）的释放，减轻TNF-α导致的肾脏血管内皮糖萼的降解，从而改善肾脏微循环和缓解肾损伤。

创新点：本研究通过小鼠活体研究的方法，建立小鼠重症急性胰腺炎模型，并用地塞米松进行干预对照，采用透射电镜、激光多谱勒和酶联免疫的方法，检测了各组小鼠肾脏血管内皮糖萼的完整性、肾血流灌注和TNF-α表达情况，阐明了地塞米松对内皮糖萼的保护作用。

方　法：通过“胰管结扎+腹腔内雨蛙素注射”的方法建立SAP模型，分别留取各组小鼠的血液和组织标本，采用透射电镜观察内皮糖萼的损伤情况，用酶联免疫检测血清 TNF-α 和糖萼成份多配体聚糖的浓度，并用激光多谱勒检测活体小鼠肾脏的灌注，分析地塞米松对内皮糖萼的保护和改善肾脏灌注的作用。

结　论：SAP可以引起TNF-α的大量释放，并导致内皮糖萼的降解和肾脏灌注下降，而地塞米松可以减少TNF-α的释放，减轻糖萼的降解，改善肾脏血流灌注。

关键词：重症急性胰腺炎；急性肾损伤；糖萼；地塞米松；肿瘤坏死因子α