

EFFECTS OF SALVIA MILTIORRHIZAE ON THE KIDNEY OF RATS WITH SEVERE ACUTE
PANCREATITIS AND OBSTRUCTIVE JAUNDICE

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Abstract

Background: Severe acute pancreatitis (SAP) and obstructive jaundice (OJ) are frequent recurring diseases that bring about huge threat to human health. Some reports have demonstrated that *Salvia miltiorrhizae* can protect multiple organs of SAP and OJ model animals or patients, but their related mechanisms were not clear. In this study, we observed the effects of *Salvia miltiorrhizae* injection on apoptosis and NF- κ B expression in kidney and explored the protective effect and mechanism of *Salvia miltiorrhizae* on the kidney of SAP or OJ rats. The results obtained will provide a theoretical basis for clinical application of *Salvia miltiorrhizae*.

Material and Methods: A total of 288 rats were used for SAP - and OJ-associated experiments. The mortality rates of rats, the contents of serum BUN and CREA, the expression levels of Bax, NF- κ B proteins and the apoptosis index were observed, respectively.

Results: The pathological changes in the kidney of SAP or OJ rats in treated group were mitigated to varying degrees. At 6 and 12 hours after operation in SAP rats or on 21 and 28 days after operation in OJ rats, the contents of serum CREA in treated group were significantly lower than those in model control group; At 3 and 6 hours after operation, the staining intensity of Bax protein of kidney in treated group was significantly lower than that in model control group; on 14 days after operation, the apoptosis index in the kidney of OJ rats in treated group was significantly lower than that in model control group.

Conclusion: *Salvia miltiorrhizae* can exert protective effects on the kidney of SAP and OJ rats.

Key words: severe acute pancreatitis; obstructive jaundice; *Salvia miltiorrhizae*; traditional Chinese medicine; rats; kidney; apoptosis; Bax; NF- κ B

Introduction

Severe acute pancreatitis (SAP) and obstructive jaundice (OJ) are frequently occurring diseases that bring about a huge threat to human health (Chiutu, et al., 2006; Kucuk, et al.,2003; Padillo, et al.,2005; Petejova, et al.,2013). The aggravation of SAP and OJ are mainly due to the dysfunction of multiple organs and remote organ injury (Assimakopoulos, et al.,2008; Grintzalis, et al.,2009; Ling, et al.,2009; Yang, et al.,2013; Zhou, et al.,2014), the kidney is one of the most frequently affected organs (Lin, et al.,2011; Li, et al.,2010; Li, et al.,2015).

SAP is believed to be major risk factor leading to acute kidney injury (AKI) among critically ill patients (Zhou et al., 2015). It has been reported that, once SAP patients are complicated with acute renal failure, the mortality rate of

these diseases can reach more than 70% (Zhou et al., 2015). AKI is an important cause of morbidity and mortality in SAP (Kumar et al., 2015). SAP usually results in acute renal failure. Obstructive Jaundice (OJ), a frequently observed condition caused by obstruction of the common bile duct or its flow and seen in many clinical situations, may end up with serious complications like sepsis, immune depression, gastrointestinal hemorrhage, and hepatic and renal failures (Assimakopoulos et al., 2008; Uslu et al., 2005). OJ is not only associated with high morbidity and mortality, but also a significant risk of developing acute renal failure (ARF). The report of Martínez-Cecilia D et al demonstrated that oxidative stress and renal dysfunction patients with OJ has a correlation (Martínez-Cecilia et al., 2016).

The main active ingredients of *salvia miltiorrhizae* include tanshinone I, IIA and IIB as well as isotanshinone I and IIA, which are able to protect endothelial cells, fight against inflammation as well as prevent lipid peroxidation and calcium overload (Ou, et al.,2012; Zhang, et al.,2010). Liu Fan et al have investigated the effect of sodium tanshinon II A silate injection on acute pancreatitis (AP). The patients with AP in observation group were treated with sodium tanshinon II A silate injection. The recovery time of body temperature, stomachache, jaundice and the hospitalization time in observation group were significantly shorter than those in control group ($P < 0.05$). Liu Fan et al think that Sodium tanshinon II A silate can significantly improve clinical symptoms, recover the function of the pancreas, reduce pancreatic inflammation and increase the effect of treatment in patients with AP (Liu et al., 2015). A similar clinical trial was carried out by Tao MG which explore the effects of sodium tanshinon II A silate on patients with AP. Their study confirmed that the combination of sodium tanshinone II A silate with comprehensive therapy can promote the rehabilitation of AP (Tao et al.,2013). The sodium tanshinon II A had remarkable effects in SAP rats with lung injury, which might be associated with changing cytokines levels and attenuating infiltration of lung inflammatory cells (Liu et al.,2015).

Some reports have demonstrated that *salvia miltiorrhizae* has some protective effects on the lungs of rats with SAP which may be related apoptosis. *Salviae Miltiorrhizae* in early stage of SAP can inhibit the over-expression of iNOS mRNA to ameliorate the injury of the pancreas, lung and kidney (Zhang et al., 2005).

Salviae Miltiorrhizae can effectively reduce the mortality and complications of SAP. Those reports also indicated that, in SAP, *salvia miltiorrhizae* can significantly enhance the scavenging of oxygen free radicals and inhibit excessive inflammatory reaction in kidney, thereby maintaining the normal morphology, structure and function of the kidney (Wang, et al., 2013; Zhang et al., 2006; Zhang et al., 2009). The renal microcirculation obstacle is the important factors for inducing renal injury in OJ. *Salviae miltiorrhizae* shows a protective effect on kidney against the injury of OJ model rats (Qin et al.,2013).*Salvia miltiorrhizae* may protect renal function of patients with OJ by inhibiting inflammatory mediator and improving blood dynamics (Peng et al.,2001). This study is a part of a series experiments about “Therapeutic effects and mechanisms of *Salviae miltiorrhizae* on injuries of multiple organs in SAP or OJ rats”. In recent years, the corresponding author Zhang and his coadjutants have published some papers about the injuries and *Salviae miltiorrhizae* treatment of multiple organs in SAP or OJ rats. These related papers were reported in different journals included intestinal mucosa (Zhang et al., 2010), mesenteric lymph node (Zhang et al., 2009), lung (Zhang et al., 2009; Zhang et al., 2009), heart (Zhang et al., 2009), liver (Zhang et al., 2009), spleen and thymus (Zhang et al., 2009). All papers were used the same materials and methods (animal grouping, preparation of animal models and associated therapeutic regimen, statistical analysis). From a series experiments, we found that the conclusion were: (1) *salviae miltiorrhizae* have potential therapeutic effect on SAP and OJ through inhibiting production of inflammatory mediators, improving micro-circulational function and attenuating multi-organ pathological impairment; (2) *salviae miltiorrhizae* treatment can lead to induction or restrain of Bax protein expression and change of apoptosis index in some organs in SAP or OJ rats, consisting certain protective effect on multi-organ pathology. However, this modulation of Bax protein expression and apoptosis is feeble, furthermore, apoptosis just contribute secondarily to multi-organ pathological alteration; (3) *salviae miltiorrhizae*-induced depressed expressional levels of NF- κ B in liver and intestine also makes for certain therapeutic effect on SAP and OJ; (4) *salviae miltiorrhizae* treatment degrades ICAM-1

expression in lung of SAP or OJ rats; (5) salviae miltiorrhizae can inhibit hepatic TLR4 expression in OJ rats to facilitate the recovery of hepatic function and pathology; (6) with low price, broad pharmacological action, confirmed curative effect, little side effect and great use-able, salviae miltiorrhizae is indeed worth generalizing for its adjunctive therapeutic effect on SAP and OJ; (7) applying tissue microarray technology to the pathological detection of SAP and OJ is timesaving, laborsaving, high-efficient, finely representative, and deserving generalization.

In this study, we observed the effects of Salvia miltiorrhizae injection on apoptosis and NF- κ B expression in kidney and explored the protective effect and mechanism of Salvia miltiorrhizae on the kidney of SAP or OJ rats. The results obtained will provide a theoretical basis for clinical application of these drugs. And the datas related to apoptosis and NF- κ B expression in kidney have not been published in anywhere.

Materials and methods

Materials

Total 288 healthy male SD rats of clean grade, weighing between 270 and 330 g, were provided by the Laboratory Animal Research Center of the Zhejiang University of Traditional Chinese Medicine(China); sodium taurocholate and sodium pentobarbital were purchased from Sigma Corporation, USA; Salvia miltiorrhizae injection (each 10-ml vial contains active components equivalent to 15 g of the original medicine) was purchased from Chiatai Qingchunbao Pharmaceutical Co., Ltd(China); anti-NF- κ B P65 and anti-Bax antibodies were purchased from Santa Cruz Biotechnology, Inc.(USA).

Methods

Animal grouping: 108 rats were used for SAP-associated experiments and randomly divided into sham operation group, model control group and Salvia miltiorrhizae treated group (n=36), which were further randomly subdivided into 3 h, 6 h, and 12 h groups (n=12) according to time points after operation; another 180 rats were utilized for OJ-associated experiments and randomly divided into sham operation group, model control group and Salvia miltiorrhizae treated group (n=60), which were further randomly subdivided into 7 d, 14 d, 21 d and 28 d groups (n=15) according to time duration after operation.

Preparation of animal models and associated therapeutic regimen: The related research was reported in recent article (Peng, et al.,2001; Qin, et al.,2013; Zhang, et al.,2009; Zhang, et al., 2009). Briefly, the steps of SAP preparation were just as follows: Sodium taurocholate (3.5%, 0.1 mL/100 g) was transfused into the bile duct through the direction of the papilla at a flow rate of 0.2 mL/min. Fifteen minutes after successful operation, a single dose of Salvia miltiorrhizae injection (0.4 ml/100 g body weight) was given via femoral vein to rats in the treated groups while equal volume of physiological saline solution was used in the sham operation and the model control group. Continuous infusion of physiological saline solution using a microinjection pump was then maintained until the end of the 3-hour, 6-hour and 12-hour observation period in the corresponding groups. This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health.

The Committee on the Ethics of Animal Experiments of Zhejiang Cancer Hospital was secured for our research reported. Zhejiang Cancer Institutional Animal Care and Use Committee (IACUC) specifically approved this study. All authors abided the related rules of Committee on the Ethics of Animal Experiments of Zhejiang Cancer Hospital when this study began. All surgery was performed under sodium pentobarbital anesthesia, and all efforts were made to minimize suffering.

Specimen collection: At the corresponding time points after operation, SAP or OJ rats were anesthetized with 2.5% sodium pentobarbital and killed. Blood samples and kidney tissue specimens were then collected, respectively.

Immunohistochemical staining of Bax, NF- κ B P65 proteins in the kidneys: The Envision two-step method was used to detect the expression levels of Bax, NF- κ B P65 proteins in the kidneys. The evaluation criteria were as follows: (1)the staining intensity was evaluated according to the extent of cell coloration: “-” represents negative staining; “+” represents mild staining, positively stained cells showed a yellow pigment; “++” represents moderate staining, positively stained cells showed a brown pigment; “+++” represents intense staining, positively stained cells showed a dark brown pigment, each of which was scored as 0, 1, 2 and 3 points, respectively, during statistical analysis; (2)the evaluation criteria of the positive staining rate: none of positive cells (-); the percentage of positive cells was less than 25% (+); the percentage of positive cells ranged between 26% and 50% (++); and the percentage of positive cells was more than 50% (+++), each of which was scored as 0, 1, 2 and 3 points, respectively, during statistical analysis.

Immunohistochemical staining of Bax, NF- κ B P65 proteins and TUNEL staining in the kidneys: After the tissue microarray sections of kidney, with a core size of 1.5 mm in diameter, were prepared, Bax, NF- κ B p65 immunostaining and TUNEL staining were conducted, respectively. The Envision two-step method was used to detect the expression levels of Bax, NF- κ B p65 proteins in the kidney. See related articles (Qin et al., 2013; Zhang et al., 2009).

Tunel staining in the kidneys: The steps of DNA nick in situ end-labeling (TUNEL) staining were as follows: Baking sections under 60°C for 30 minutes, routine deparaffinage, Milli-Q wash for 5 minutes. Processing tissue with Protease K (10ug/ul) under room temperature for 15 minutes, PBS washes for 5 minutes. Using 3% H₂O₂ solution to block endogenous peroxydase for 5 minutes, PBS wash for 5 minutes×twice. Adding 30ul reaction solution in freezing condition (TdT Enzyme: Labeling Safe Buffer=1:10), 37°C incubation for 90 minutes, PBS wash for 5 minutes×twice. Adding 50ul Anti-FITC HRP Conjugate, 37°C incubation for 30 minutes, PBS wash for 5 minutes×twice. DAB coloration, Milli-Q wash to terminate coloration. Hematoxylin counterstain, water wash and wash fully with water after differentiation till return blue; routine dehydration and transparency; neutral gum mounting. The apoptotic index was calculated. Apoptotic index=apoptotic cell count/ total cell count×100%.

Experimental parameters observed

Observations of mortality rate and pathological changes: At various time points after operation, the mortality rates of rats in each group were recorded. Besides, the gross pathological changes and the pathological changes under light microscopy were also examined. At various time points after operation, two SAP or OJ rats were randomly selected from each group and mercifully killed to take kidney specimens, which were subsequently subjected to observation of ultrastructure changes under transmission electron microscopy. Determination of the contents of serum BUN and CREA: The contents of serum BUN and CREA were determined using an automatic biochemical analyzer according to the instructions provided with the kits. After the tissue microarray sections of kidney, with a core size of 1.5 mm in diameter, were prepared, Bax,NF- κ B P65 immunostaining and TUNEL staining were conducted, respectively.

Statistical analysis

The compiled data were first input into the Excel sheet, and then read into SPSS15.0 for further analysis. Normal data were expressed as means (standard deviation) while non-normal data were expressed as medians (interquartile

range). Analysis of variance and pairwise comparisons were used for normal data, whereas non-normal data were subjected to non-parametric test, among which Kruskal-Wallis H test was used for pairwise comparisons and Mann-Whitney U test for multiple comparisons. Yates' chi-square test (χ^2) was used for intergroup comparisons of mortality rates.

Results

SAP-associated experiments

Comparison of mortality rate: One and five rats died in the model control groups respectively at 3 and 12 hours after operation, respectively; three died in the treated group at 12 hours after operation; and no rats died in the remaining groups. There was no marked difference in mortality rate between the time points at 3 and 6 hours after operation. At 12 hours after operation, only the mortality rate in the model control group was significantly higher than that in the sham operation group ($P < 0.05$), and no marked difference in mortality rate was observed among other groups ($P > 0.05$) (Zhang et al., 2009; Zhang et al., 2009). See Table 1.

Table 1: Comparison of mortality rate of SAP-associated groups

Time	N(case)	Sham-operated group	Model control group	Treated group
3h	12	12	11	12
6h	12	12	12	12
12h	12	12	7*	9

Note: Compared with sham-operated group, * $P < 0.05$

Comparison of the content of serum BUN: At all-time points after operation, the contents of serum BUN in model control group and Salvia miltiorrhizae treated group were significantly higher than those in sham-operated group ($P < 0.001$); there was no significant difference between Salvia miltiorrhizae treated group and model control group ($P > 0.05$). The results were shown in Table 2.

Table 2: Comparison of serum BUN and CREA of SAP groups ($M(Q_R)$)

Index	Time	Sham-operated group	Model control group	Treated group
BUN	3h	5.7(1.0)	11.2(1.9)	10.4(3.3) **
	6h	6.1(0.7)	13.4(4.0)	11.4(3.2) **
	12h	6.1(0.8)	17.0(6.1)	12.9(4.1) **
CREA	3h	24.8±8.0	48.6±14.6**	40.8±8.7*
	6h	29.9±3.2	55.5±15.1**	39.8±14.1 +
	12h	28.0±2.5	54.6±10.3**	38.70±13.5* +

Note: Compared with sham-operated group, * $P < 0.01$, ** $P < 0.001$; Compared with model control group, + $P < 0.01$, ++ $P < 0.001$

Comparison of the content of serum CREA: At all-time points after operation, the contents of serum CREA in model control group were significantly higher than those in sham-operated group ($P < 0.001$); At 3 and 12 hours after operation, the contents in Salvia miltiorrhizae treated group were significantly higher than those in sham-operated group ($P < 0.01$); At 6 and 12 hours after operation, the contents in model control group were significantly higher than those in Salvia miltiorrhizae treated group ($P < 0.01$). The results were shown in Table 1.

Pathological changes in kidney

Gross pathological changes: In sham-operated group, the kidney showed normal morphology and color at all-time points after operation. In model control group, at 3 hours after operation, no obvious gross pathological changes were seen; At 6 and 12 hours after operation, renal swelling and capsular tension were viewed, red spots were scattered on the surface of renal capsule in some rats, and intrapelvic urine became slightly bloody in severe cases. In treated group, at 6 and 12 hours after operation, the gross pathological changes in kidney were milder than those in model control group.

Pathological changes under light microscopy: In sham-operated group, the kidney was basically normal at all-time points after operation; the hyperplasia and edema could be seen in few glomerular cells and renal tubular epithelial cells, respectively. In model control group, the boundaries of renal tubular epithelium were obscure, and the lumen of renal tubules became narrow; protein casts and interstitial edema were seen; the hyperplasia and edema could be seen in extremely few glomerular cells and renal tubular epithelial cells, respectively; glomerular capillary congestion, scattered necrosis of renal tubular epithelial cells, interstitial edema and inflammatory cell infiltration can be seen in very few rats; the above-mentioned pathological changes were aggravated with the prolongation of time. In treated group, the above-mentioned pathological changes were, to varying degrees, milder than those in model control group. See Figure 1-4.

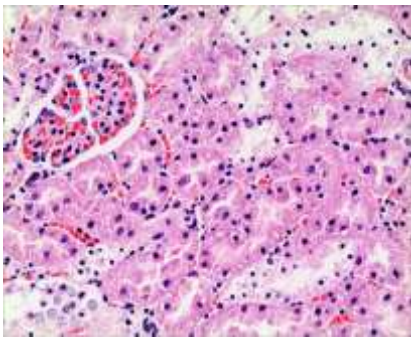


Figure 1: Model control group-12h (Significantly degeneration and necrosis of renal tubular epithelial cells) HE×200

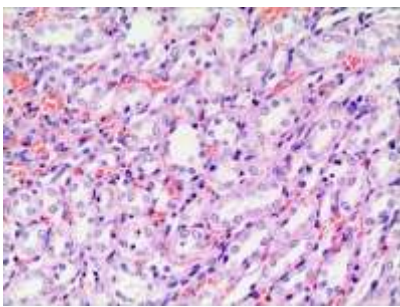


Figure 2: Treated group-12h (Renal tissue structure was clear, glomerular lesions were not obvious, and renal tubular epithelial cells were mild degeneration and necrosis) HE×200

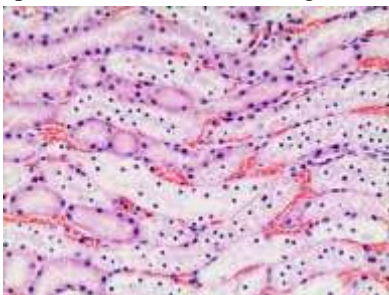


Figure 3: Model control group-12h (Obvious degeneration and necrosis of renal tubular epithelial cells) HE×200

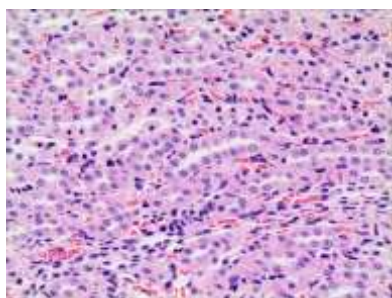


Figure 4: Treated group-12h (Renal tissue structure was clear, glomerular lesions were not obvious, and renal tubular epithelial cells were mild degeneration and necrosis) HE×200

Pathological changes under electron microscopy: In sham-operated group, the kidney showed normal morphology and structure, and vacuolation was seen in few mitochondria. In model control group, the microvilli in proximal tubules were arranged loosely and shed to form casts; dissolution, necrosis and vacuolation of epithelial cells, dilation and disorderly arrangement of rough endoplasmic reticulum, vacuolation and degeneration of mitochondria, and increased lysosomes were seen; interstitial edema and red blood cell exudation into lumen in renal tubules were more obvious. In treated group, vacuolation and degeneration of glomerular capillary epithelial cells, increased lysosomes, and shedding of the microvilli in partial proximal tubules were viewed.

Comparison of the pathological severity score in kidney: The pathological severity score were based on those described in the literature (Zhang et al., 2007). At all-time points after operation, the pathological score in kidney in model control group and Salvia miltiorrhizae treated group were significantly higher than those in sham-operated group (P<0.05); At 6 hours after operation, the score in Salvia miltiorrhizae treated group was significantly lower than that in model control group (P<0.05).

Pathological examination of the kidney

Comparison of the staining intensity of Bax protein of kidney: The positive signals for Bax protein were localized in the cytoplasm of renal tubular epithelial cells. At all-time points after operation, the staining intensity of Bax protein of kidney in model control group was significantly greater than that in sham-operated group (P<0.01); there was no significant difference between sham-operated group and Salvia miltiorrhizae treated group (P>0.05); At 3 and 6 hours after operation, the staining intensity in model control group was significantly greater than that in Salvia miltiorrhizae treated group (P<0.01). The results were shown in Table 3, Figure 5-7.

Table 3: Comparison of different pathological indexes of SAP groups ($M(Q_R)$)

Index	Time	Sham-operated group	Model control group	Treated group
pathological score	3h	0.0(1.0)	1.0(0.0) *	1.0(0.0) *
	6h	0.0(1.0)	1.0(1.0)*	1.0(0.0) *+
	12h	0.0(1.0)	2.0(1.0) *	1.0(0.0) *
Bax staining intensity	3h	0.0(0.5)	1.0(2.0) **	0.0(1.0) ++
	6h	0.0(0.5)	1.0(1.0)**	0.0(1.0) ++
	12h	0.0(0.0)	1.0(0.0) **	0.0(1.0)
product of Bax staining intensity and positive staining rate	3h	0.0(0.5)	1.0(2.0) **	0.0(1.5)
	6h	0.0(0.5)	2.0(2.5)**	0.0(2.0) ++
	12h	0.0(0.0)	2.0(0.0) **	0.0(1.0) ++
apoptotic index	3h	0.0(0.0)	0.0(0.0)	0.0(0.0)

	6h	0.0(0.0)	0.0(0.03)	0.0(0.0)
	12h	0.0(0.0)	0.0(0.0)	0.0(0.0)
NF-κB staining intensity	3h	0.0(0.0)	0.0(1.0)	0.0(0.0)
	6h	0.0(0.0)	0.5(1.5)	0.0(1.0)
	12h	0.0(0.5)	1.0(2.0) **	0.0(1.0)
product of NF-κB staining intensity and positive staining rate	3h	0.0(0.0)	0.0(2.0)	0.0(0.0)
	6h	0.0(0.0)	1.0(2.0) **	0.0(1.5)
	12h	0.0(0.5)	2.0(4.0) **	0.0(2.0)

Note: Compared with sham-operated group, *P<0.01, **P<0.001; Compared with model control group, ⁺P<0.01, ⁺⁺P<0.001

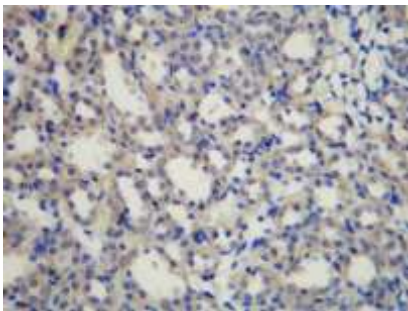


Figure 5: Sham-operated group-6h Expression (+) Bax×200

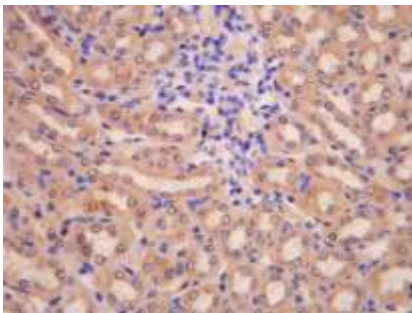


Figure 6: Model control group-6h Expression (+++) Bax×200

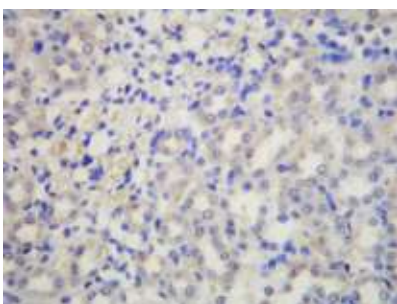


Figure 7: Treated group -6h Expression (–) Bax×200

Comparison of the product of the staining intensity and the positive staining rate of Bax protein of kidney: At all-time points after operation, the products of the staining intensity and the positive staining rate of Bax protein of kidney in model control group were significantly higher than those in sham-operated group (P<0.01); there was no significant difference between sham-operated group and *Salvia miltiorrhizae* treated group (P>0.05); At 6 and 12 hours after operation, the products in *Salvia miltiorrhizae* treated group were significantly higher than those in model control group (P<0.01). The results were shown in Table 3.

Comparison of the apoptosis index in kidney: The apoptosis cells were mainly renal tubular epithelial cells. At

all-time points after operation, no significant difference in the apoptosis index in kidney was noted between sham-operated group and model control group, between sham-operated group and Salvia miltiorrhizae treated group as well as between model control group and Salvia miltiorrhizae treated group ($P>0.05$). The results were shown in Table 3, Figure 8-10.

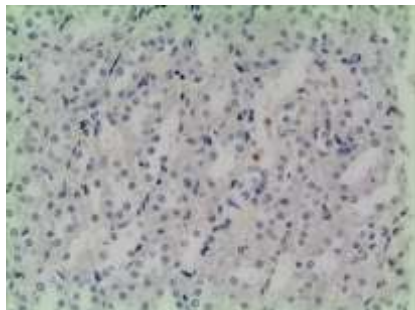


Figure 8: Sham-operated group-6h (Few apoptotic cells were seen, apoptotic cells were renal tubular epithelial cells especially obvious in the proximal tubules) HE \times 200

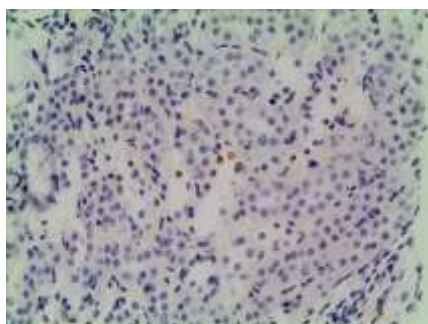


Figure 9: Model control group-6h (Many apoptotic cells were seen, apoptotic cells were renal tubular epithelial cells especially obvious in the proximal tubules) TUNEL \times 200

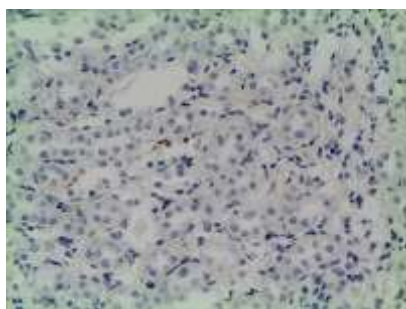


Figure 10: Treated group-6h (Few apoptotic cells were seen apoptotic cells were renal tubular epithelial cells especially obvious in the proximal tubules) TUNEL \times 200

Comparison of the staining intensity of NF- κ B p65 protein of kidney: The positive signals for NF- κ B p65 protein were mainly localized in the cytoplasm of renal tubular epithelial cells. Besides, very few positive signals were seen in the cytoplasm of glomerular mesangial cells. At 12 hours after operation, the staining intensity of NF- κ B p65 protein of kidney in model control group was significantly greater than that in sham-operated group ($P<0.01$); At all-time points after operation, no significant difference in the staining intensity was noted between Salvia miltiorrhizae treated group and sham-operated group as well as between Salvia miltiorrhizae treated group and model control group ($P>0.05$). The results were shown in Table 3. See Figure 11-13.

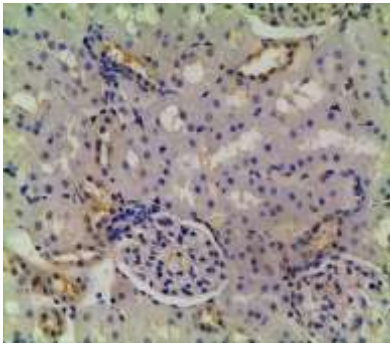


Figure 11: Sham-operated group-12h Expression (+) NF-KB×200

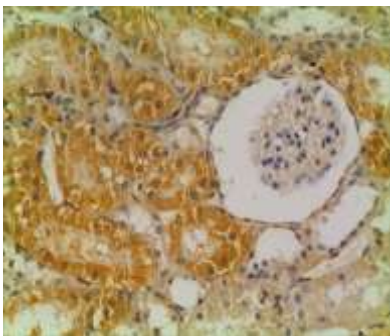


Figure 12: Model control group-12h Expression (+++) NF-KB×200

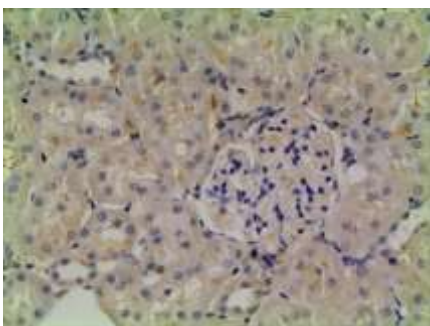


Figure 13: Treated group-12h Expression (+) NF-KB×200

Comparison of the product of the staining intensity and the positive staining rate of NF-κBp65 protein of kidney: At 6 and 12 hours after operation, the products of the staining intensity and the positive staining rate of NF-κBp65 protein of kidney in model control group were significantly higher than those in sham-operated group ($P < 0.01$); At all-time points after operation, there was no significant difference between *Salvia miltiorrhizae* treated group and sham-operated group as well as between *Salvia miltiorrhizae* treated group and model control group ($P > 0.05$). The results were shown in Table 3.

OJ-associated experiments

Comparison of mortality rate: 2, 4, 4 and 7 rats died in the model control groups on 7, 14, 21 and 28 days after operation, respectively; and 3, 2 and 4 rats died in the treated groups on 14, 21 and 28 days after operation. The mortality rates on 7 days after operation showed no marked difference among three experimental groups; On 14 and 21 days after operation, the mortality rates in the sham operation groups were significantly lower than those in the model control groups ($P = 0.032$); On 28 days after operation, the mortality rate in the sham operation group was lower than those in both the model control group ($P = 0.006$) and the treated group ($P = 0.032$), and the difference was statistically significant. See Table 4.

Table 4: Comparison of mortality rate of OJ-associated groups

Time	N(case)	Sham-operated group	Model control group	Treated group
7d	15	15	13	15
14d	15	15	11*	12
21d	15	15	11*	13
28d	15	15 ⁺	8**	11

Note: Compared with sham-operated group, *P<0.05, **P<0.01; Compared with treated group, ⁺P<0.05

Comparison of the content of serum BUN: On 7 and 28 days after operation, the contents of serum BUN in model control group were significantly higher than those in sham-operated group (P<0.01); On 7 days after operation, the content in Salvia miltiorrhizae treated group was significantly higher than that in sham-operated group (P<0.01); At all-time points after operation, there was no significant difference between model control group and Salvia miltiorrhizae treated group (P<0.01). The results were shown in Table 5.

Table 5: Comparison of BUN and CREA of OJ groups ($M(Q_R)$)

Index	Time	Sham-operated group	Model control group	Treated group
BUN	7d	6.1(1.5)	8.6(1.7)**	7.9(1.6)**
	14d	7.4(2.0)	8.1(1.8)	7.2(2.2)
	21d	6.8(1.6)	7.1(1.4)	7.4(1.1)
	28d	7.5(1.5)	11.2(5.3)**	8.6(3.1)
CREA	7d	29.3±7.1	42.9±12.9**	37.7±6.2**
	14d	32.3±12.8	50.3±8.6**	43.4±5.9**
	21d	27.5±5.1	52.7±4.9**	40.5±7.1**++
	28d	27.9±5.4	53.3±3.2**	42.8±7.2**++

Note : Compared with sham-operated group, *P<0.05,**P<0.01;Compared with model control group, ⁺P<0.05,⁺⁺P<0.01

Comparison of the content of serum CREA: At all-time points after operation, the contents of serum CREA in model control group and Salvia miltiorrhizae treated group were significantly higher than those in sham-operated group (P<0.01); On 21 and 28 days after operation, the contents in Salvia miltiorrhizae treated group were significantly lower than those in model control group (P<0.05). The results were shown in Table 5.

Pathological changes in kidney

Gross pathological changes: In sham-operated group, no obvious abnormality was seen at all-time points after operation. In model control group, the pathological changes were aggravated with the prolongation of time; diffuse yellow staining of renal capsule, cortex and medulla was seen; On 21 and 28 days after operation, renal cortex in nearly half of rats showed brownish-black color; At all-time points after operation, the kidney showed no changes in size and texture. On 7 and 14 days after operation, no obvious difference was seen between treated group and model control group; On 21 and 28 days after operation, renal cortex in 80% of rats in treated group showed dark brown color, which was lighter than that in model control group; renal medulla showed yellow color; the kidney showed no changes in size and texture. Pathological changes under light microscopy: In sham-operated group, no obvious changes were seen in kidney, and hyperplasia of glomerular cells and edema renal tubular epithelial cells could be seen occasionally. In

model control group, on 7 days after operation, hyperplasia of glomerular capillary endothelial cells, epithelial cells, mesangial cells and basement membrane was viewed, capillary basement membrane became widened, and swelling and necrosis of proximal tubular epithelium were observed in some rats; At other time points (14, 21 and 28 days) after operation, swelling and necrosis of proximal or distal tubules, bile pigment deposition, bile pigment casts within the lumen of renal tubules, patchy necrosis and bile pigment deposition in renal tubules, and inflammatory cell infiltration as well as hyperplasia and edema of connective tissue in renal interstitium were seen; these pathological changes were aggravated with the prolongation of time. In treated group, the pathological changes, mainly manifested as hyperplasia of glomerular capillary endothelial cells, epithelial cells, mesangial cells and basement membrane, were milder than those in model control group; the pathological changes present in model control group were observed only in some rats in treated group. See Figure 14-17.

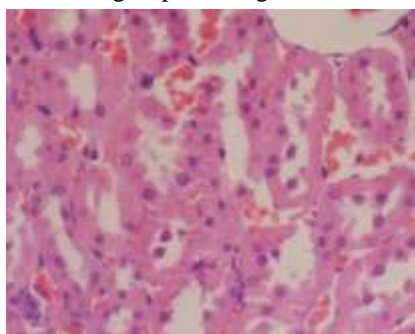


Figure 14: Model control group-28d (Patchy necrosis of renal tubular epithelial cells, interstitial edema) HE×400

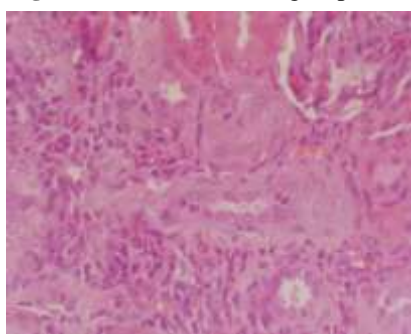


Figure 15: Treated group-28d (Proliferation of interstitial fibrous and vascular of the kidney) HE×400

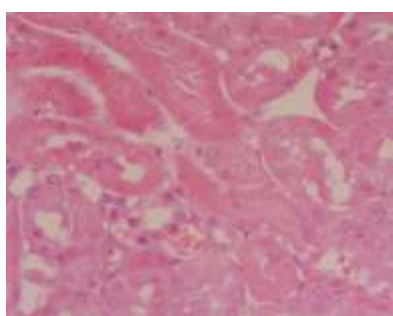


Figure 16: Model control group-21d (Tubular epithelial cells were necrosis) HE×400

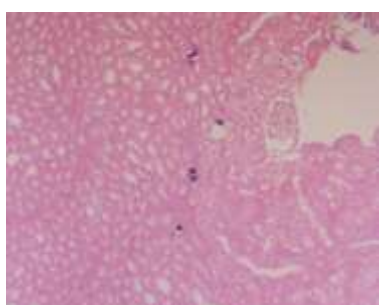


Figure 17: Treated group-21d (Scattered calcified foci in the renal tubular or blood vessel were seen) HE×100

Pathological changes under the electron microscope: In sham-operated group, the kidney showed normal morphology and structure at all-time points after operation. In model control group, the pathological changes were aggravated with the prolongation of time; bile pigment disposition and red blood cell exudation were seen in proximal tubules; the microvilli in proximal tubules were arranged disorderly and partially shed; obvious inflammatory cell exudation, vacuolar degeneration of epithelial cells, increased number of apoptotic cells, dilation of rough endoplasmic reticulum, mitochondrial swelling and vacuolation, and obscure cristae and outer membrane of the mitochondria were seen; protein exudation was seen in distal tubules. In treated group, the ultrastructure of the kidney was changed to varying degrees; inflammatory cell infiltration in proximal tubules, apoptosis of epithelial cells, disorderly arrangement of the microvilli, epithelial necrosis in few proximal and distal tubules, and bile pigment deposition within or outside the lumen were seen. See Figure 18-23.

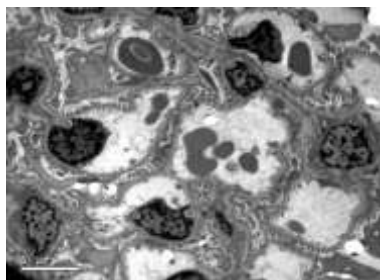


Figure 18: Sham-operated group-28d (Normal glomerulus) Electron microscope×2550

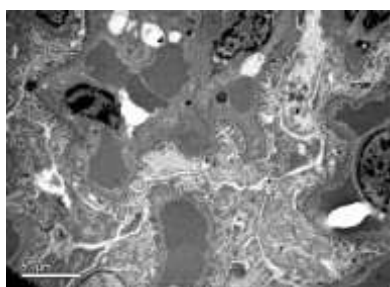


Figure 19: Model control group-21d (Exudation of red blood cells in the glomerulus) Electron microscope×2550

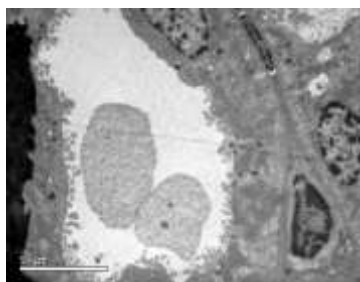


Figure 20: Model control group-21d (Exudation of proteins in distal tubules) Electron microscope×3700

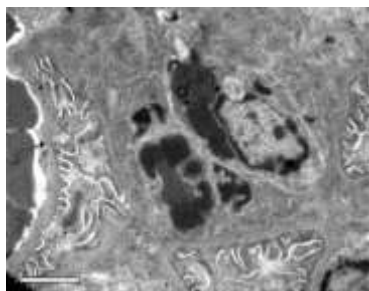


Figure 2: Model control group-28d (Bile pigment deposition in the glomerulus) Electron microscope×6200

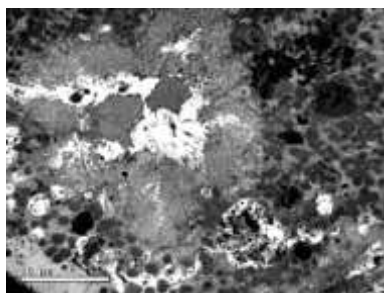


Figure 22: Model control group-28d (The microvilli in proximal tubules were arranged disorderly and partially shed, and inflammatory exudation was obvious) Electron microscope×1850

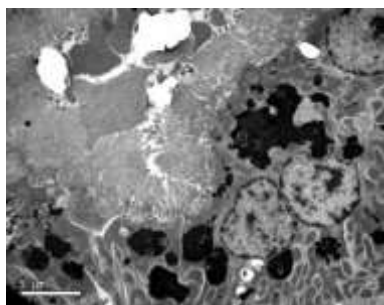


Figure 23: Treated group-28d (The microvilli in proximal tubules were arranged disorderly, and bile pigment was deposited within or outside the lumen) Electron microscope×3700

Comparison of the pathological scores in kidney: The comparison was performed based on the pathological severity score that we established according to experimental needs (see Table 6 for details). At all-time points after operation, the pathological scores in kidney in model control group and *Salvia miltiorrhizae* treated group were significantly higher than those in sham-operated group ($P>0.05$); there was no significant difference between *Salvia miltiorrhizae* treated group and model control group ($P>0.05$).

Table 6: The standard of pathological severity score of kidney

score	Standard
1	Hyperplasia of endothelial cells, 0.2 points; hyperplasia of epithelial cells, 0.2 points; hyperplasia of mesangial cells, 0.2 points; hyperplasia of basement membrane, 0.2 points; widening of capillary basement membrane, 0.2 points
2	Epithelial swelling or necrosis in proximal tubules, 0.4 points; bile pigment deposition, 0.4 points; epithelial swelling or necrosis in distal tubules, 0.4 points; bile pigment casts within the lumen, 0.4 points
3	Patchy necrosis in renal tubules + bile pigment deposition
4	Two points of the above-mentioned contents + three points of the above-mentioned contents
5	Four points of the above-mentioned contents + inflammatory cell infiltration/ hyperplasia or edema in the connective tissue

Pathological examination of the kidney

Comparison of the staining intensity of Bax protein of kidney: The positive signals for Bax protein were localized in the cytoplasm of renal tubular epithelial cells. on 21 days after operation, the staining intensity of Bax protein of kidney in model control group was significantly higher than that in sham-operated group ($P<0.01$); At all-time points

after operation, no significant difference in the staining intensity was noted between Salvia miltiorrhizae treated group and sham-operated group as well as between Salvia miltiorrhizae treated group and model control group (P>0.05). The results were shown in Table 7. See Figure 24-29.

Table 7: Comparison of different pathological indexes of OJ groups ($M(Q_R)$)

Index	Time	Sham-operated group	Model control group	Treated group
pathological score	7d	0.0 (0.4)	1.2 (1.0) *	1.0 (1.0) *
	14d	0.0(0.2)	3.0 (2.0) *	3.0 (1.5) *
	21d	0.0 (0.2)	5.0 (0.0) *	5.0 (0.0) *
	28d	0.0(0.2)	5.0 (1.5) *	3.0 (2.0) *
Bax staining intensity	7d	0.0(1.0)	1.0(2.0)	0.0(2.0)
	14d	0.0(1.0)	1.0(2.0)	0.0(1.0)
	21d	0.0(2.0)	2.0(1.0) **	0.0(2.0)
	28d	0.0(1.0)	1.0(2.0)	0.0(1.0)
product of Bax staining intensity and positive staining rate	7d	0.0 (1.0)	1.0+ (2.0)	0.0 (2.0)
	14d	0.0(1.0)	1.0(2.0)	0.0 (1.0)
	21d	0.0 (2.0)	2.0 (3.0)**	0.0 (2.0) ++
	28d	0.0(1.0)	0.5 (1.5)	0.0 (2.0)
apoptotic index	7d	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)
	14d	0.0(0.0)	0.0(0.01) **	0.0 (0.0) ++
	21d	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)
	28d	0.0(0.0)	0.0 (0.0)	0.0 (0.0)
NF-κB staining intensity	7d	0.0 (0.0)	0.0 (1.0)	0.0 (1.0)
	14d	0.0(1.0)	0.0 (1.0)	0.0 (1.0)
	21d	0.0 (1.0)	0.0 (1.0)	0.0 (0.0)
	28d	0.0(0.0)	1.0 (1.5)	0.0 (1.0)
product of NF-κB staining intensity and positive staining rate	7d	0.0 (0.0)	0.0 (1.0)	0.0 (1.0)
	14d	0.0(1.0)	0.0 (2.0)	0.0 (1.5)
	21d	0.0 (2.0)	0.0 (2.0)	0.0 (0.0)
	28d	0.0(0.0)	1.5 (3.5)	0.0 (2.0)

Note: Compared with sham-operated group, *P<0.05, **P<0.01; Compare with model control group, +P<0.05, ++P<0.01

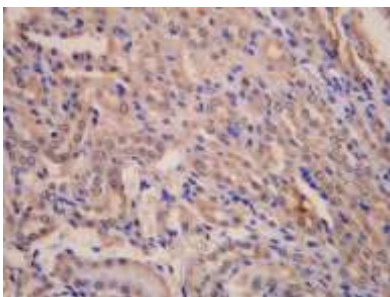


Figure 24: Model control group-21d Expression (+++) Bax×200

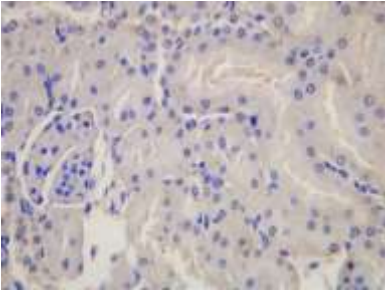


Figure 25: Treated group-21d Expression (+) Bax×400

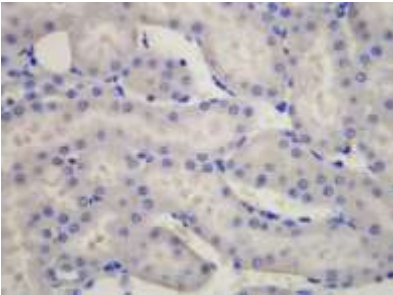


Figure 26: Sham-operated group -21d Expression (+) Bax×400

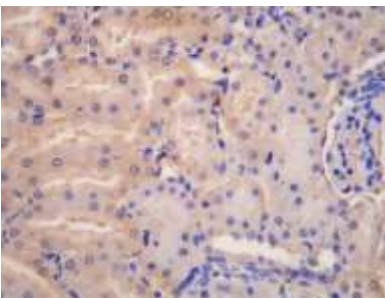


Figure 27: Model control group-28d Expression (+++) Bax×400

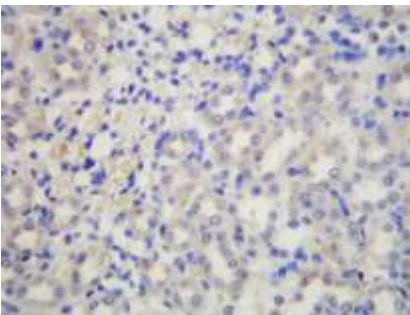


Figure 28: Treated group -28d Expression (+) Bax×200

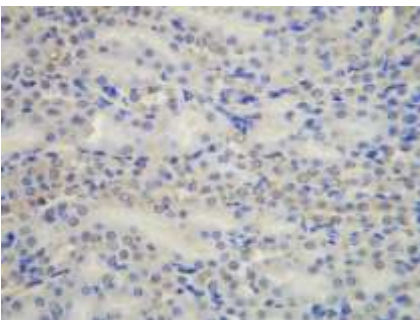


Figure 29: Sham-operated group -28d Expression (+) Bax×200

Comparison of the product of the staining intensity and the positive staining rate of Bax protein of kidney: On 21 days after operation, the product of the staining intensity and the positive staining rate of Bax protein of kidney in model control group was significantly higher than that in sham-operated group ($P < 0.01$); At all-time points after operation, there was no significant difference between sham-operated group and Salvia miltiorrhizae treated group ($P > 0.05$); On 21 days after operation, the product in Salvia miltiorrhizae treated group was significantly lower than that in model control group ($P < 0.01$). The results were shown in Table 7.

Comparison of the apoptosis index in kidney: The apoptosis cells were mainly renal tubular epithelial cells. on 14 days after operation, the apoptosis index in kidney in model control group was significantly higher than that in sham-operated group ($P < 0.01$); At all-time points after operation, no significant difference in the apoptosis index was noted between Salvia miltiorrhizae treated group and sham-operated group ($P > 0.05$); On 14 days after operation, the apoptosis index in Salvia miltiorrhizae treated group was significantly lower than that in model control group ($P < 0.01$). The results were shown in Table 7 See Figure 30-31.

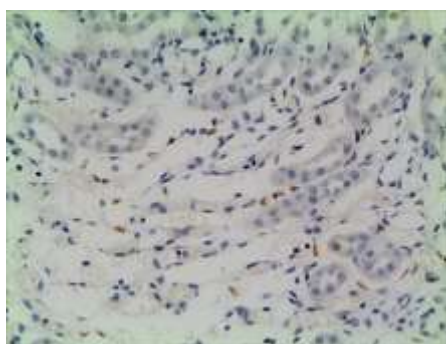


Figure 30: Model control group-14d (Apoptosis of tubular epithelial cells) tunel×200

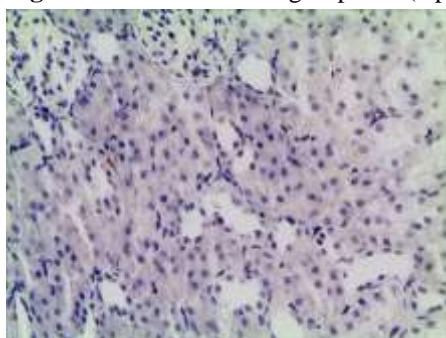


Figure 31: Treated group-14d (Apoptosis of tubular epithelial cells) tunel×200

Comparison of the staining intensity of NF- κ B p65 protein of kidney: The positive signals for NF- κ B p65 protein were mainly localized in the cytoplasm of renal tubular epithelial cells. Besides, very few positive signals were seen in the cytoplasm of glomerular mesangial cells. at all-time points after operation, no significant difference in the staining intensity of NF- κ B p65 protein of kidney was noted between sham-operated group and model control group, between sham-operated group and Salvia miltiorrhizae treated group as well as between model control group and Salvia miltiorrhizae treated group ($P > 0.05$). The results were shown in Table 7. See Figure 32-35.

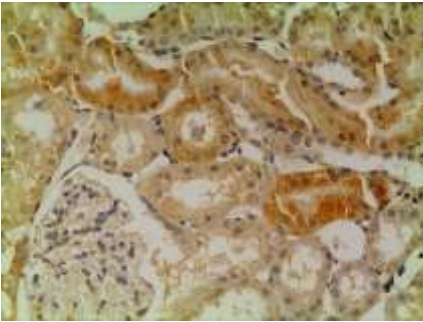


Figure 32: Model control group-28d Expression (+++) NF-KB×200

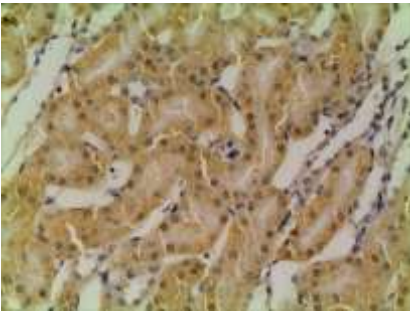


Figure 33: Treated group-28d Expression (+++) NF-KB×200

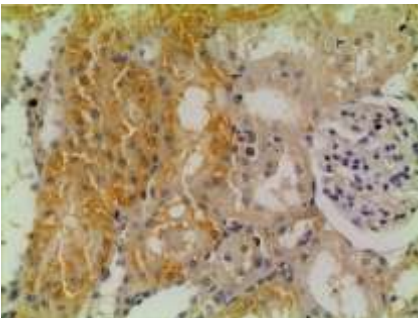


Figure 34: Model control group-21d Expression (+++) NF-KB×200

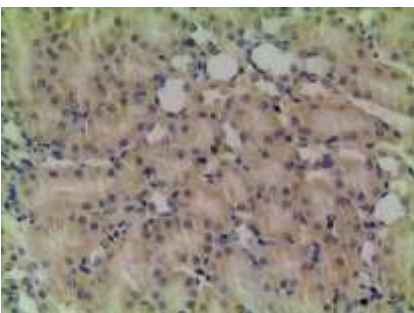


Figure 35: Treated group-21d Expression (++) NF-KB×200

Comparison of the product of the staining intensity and the positive staining rate of NF-κB p65 protein of kidney: At all-time points after operation, no significant difference in the product of the staining intensity and the positive staining rate of NF-κB p65 protein of kidney was noted between sham-operated group and model control group, between sham-operated group and *Salvia miltiorrhizae* treated group as well as between model control group and *Salvia miltiorrhizae* treated group ($P>0.05$). The results were shown in Table 7.

Discussion

With the gradual deepening of clinical and experimental studies, more and more researchers began to realize that one of the crucial measures for shortening the course and reducing the mortality rates of SAP was to prevent the damage to multiple organs, of which the kidney was very important. Among all factors that are involved in SAP-induced renal dysfunction, besides the main factors such as inflammatory mediators, oxygen free radicals, endotoxin, etc. (Bonegio et al., 2002; Kimmings et al., 2008; Kylänpää et al., 2000; Zhang et al., 2008), excessive apoptosis of renal cells also has some effects (Ortiz et al., 2005; Takase et al., 1999; Wen et al., 2011).

Apoptosis is a form of programmed cell death that belongs to an active and spontaneous process. Different from cell necrosis, apoptosis does not induce dramatic inflammatory reaction. However, as a mode of cell loss, apoptosis can cause renal tubular dysfunction, such as exposure of basement membrane, loss of transport capacity, tubular back-leak, etc., thereby playing an important role in inducing the injury of renal function (Zhang et al., 2009; Zhang et al., 2009; Zhang et al., 2007). Bax gene, a member of the Bcl-2 family, encodes a key protein that controls apoptosis (Gu et al., 2005; Joza et al., 2002; Ortiz, et al., 2005; Wen, et al., 2011). Upon apoptotic stimulation, the configuration of Bax protein is modified. The modified Bax protein can bind to the outer membrane of the mitochondria, reduce its stability and induce cell injury. In the kidney of SAP rats, no significant difference in the apoptosis index was noted among each group ($P>0.05$), suggesting that apoptosis does not play a crucial role in SAP-induced renal injury. The staining intensity of Bax protein in the kidney of SAP rats in model control group was significantly greater than that in sham-operated group while the staining intensity in *Salvia miltiorrhizae* treated group at 3 and 6 hours after operation was significantly lower than that in model control group ($P<0.01$), indicating that Bax protein was significantly upregulated in SAP but obviously downregulated after treatment with *Salvia miltiorrhizae*. Thus, *Salvia miltiorrhizae* injection can not only improve the pathological changes in kidney and reduce the occurrence of necrosis but also significantly inhibit the expression of Bax protein. Some studies have shown that, instead of causing an increase in apoptosis, excessive Bax protein can induce cell necrosis (Chia, et al., 2000; Thomas, et al., 2000). Therefore, we believe that, through directly suppressing the excessive expression of Bax protein in SAP, *Salvia miltiorrhizae* can reduce the occurrence of cell necrosis, prevent the presence of excessive inflammatory reaction and thereby exert its protective effects.

Nuclear transcription factor- κ B (NF- κ B) is a member of Rel family. Its expression levels can directly affect the transcription of pro-inflammatory factors. The excessive activation of pro-inflammatory factors can induce the strong expression of TNF- α , IL-6 and ICAM-1, thus promoting the toxicity to cells in multiple organs (Algül et al., 2002; Kan et al., 2010; Wang et al., 2004). The results of this study showed that the expression levels of NF- κ B protein, which were well correlated with the severity of pathological changes in renal tubules and glomeruluses, were significantly enhanced in the kidney of SAP rats in model control group. However, *Salvia miltiorrhizae* had no obvious effect on the expression of this protein though it could significantly improve the pathological changes in kidney. Therefore, we think that the protective effect of *Salvia miltiorrhizae* on the kidney of SAP rats may be unrelated with the expression levels of NF- κ B protein. Nevertheless, NF- κ B protein, as an important inflammatory factor in SAP, is indirectly involved in apoptosis and necrosis in multiple organs (Liu, et al., 2001; Rakonczay, et al., 2008; Zhang, et al., 2007; Zhang, et al., 2007; Zhao, et al., 2007). In general, NF- κ B refers to the p50-Rel-A (p65) complex. At present, we can only buy the reagent kits to detect NF- κ B is usually detected NF- κ B p65. In this study, it is feasible to reflect the changes of the content of NF- κ B by detecting the expression of NF- κ B p65.

Therefore, its effect should not be neglected. *Salvia miltiorrhizae* is able to significantly improve the pathological changes in the kidney of SAP and OJ rats, which is mainly manifested as reducing inflammatory cell infiltration, protecting glomerular capillary, decreasing the shedding and necrosis of renal tubular epithelial cells, and stabilizing the structure of cell organelles. Additionally, *Salvia miltiorrhizae* also has some protective effects on

excessive apoptosis-induced renal injury in SAP and OJ through downregulating the expression of Bax protein. This protective effect is beneficial to the recovery of renal function. *Salvia miltiorrhizae* has features of diverse pharmacological actions, low cost and few side-effects, it has better application prospect and economic values, and deserves to be popularized.

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