Mechanism research on the effects of fasudil to postoperative acute hepatic failure induced by hepatic ischemia & hepatectomy on rats with obstructive jaundice

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ABSTRACT

Objective: To establish a kind of animal model of postoperative acute hepatic failure induced by hepatic ischemia & hepatectomy on rats with obstructive jaundice, which could show similar clinical pathophysiological changes in human beings. To investigate the influence of fasudil to this model. Method: Selected 96 Wistar big rats as animal model of obstructive jaundice, which were treated with ligation and cutting off common bile duct. Rats in low-dose group were immediately injected fasudil of 10 mg/kg through portal vein after hepatectomy, while rats in high-dose group were immediately injected fasudil of 30 mg/kg through portal vein after hepatectomy, rats in control group were immediately injected equivalent normal saline through portal vein after hepatectomy. To determine the serum ALT, AST, TBIL (tumor necrosis factor-α, TNF-α) and (interferon-γ, INF-γ) levels in postoperative rats with hepatic failure within 6 h; to determine the (superoxide dismutase, SOD) activity and (malondialdehyde, MDA) content in hepatic tissue; hepatic tissue HE staining to observe the pathological injury; to observe animal model 96 h of survival rate.

Results: That Proceeding internal biliary drainage operation to rats after obstruction for 14 h, and blocking 70% of hepatic blood supply, excising remnant liver after 30 min was in accordance with criteria of hepatic failure animal, and was deserved to further research. Compared with control group, serum AST, ALT, TBIL, TNF-α levels decreased in fasudil treatment group, SOD activity increased in hepatic tissue, MDA content decreased, pathological injury in hepatic tissue reduced, rats 96 h of survival rate increased, and the effects of high-dose group were more obvious than that in low-dose group.

Conclusion: A surgical hepatic failure model in rat was established, which showed similar clinical pathophysiological changes in human beings. In addition, we have found that fasudil possibly played a role of protection to hepatic failure through regulating the inflammatory response; Reducing hepatic lipid peroxidation and strengthening the removal of the free radicals.

1. Introduction

The clinic treatment of acute hepatic failure usually used etiological treatment & supportive treatment, including application of non-biologic artificial liver and bioartificial liver. Liver transplantation was still the only confirmed effective therapeutic method to patients who have underwent supportive treatment but not effective[1-2]. These therapeutic methods have achieved a certain effect, but the overall effect was still not satisfied. In addition, the therapeutic method was more complicated, expensive, which was still needed to solve. Drug treatment had the characteristics of simple operation and was easy for clinical acceptance, could be used as one of the ideal methods of hepatic failure treatment. But at present therapeutic drugs to hepatic failure were not ideal, except that toxic liver damage resulting in hepatic failure was clear, not yet found special therapeutic drugs, other liver protectant still existed deficiency in the clinical curative effect and use safety, so looking for a safe,
effective and low cost of liver protectant was currently one of the focuses in research on treatment of hepatic failure[3-4]. Fasudil was a kind of Rho kinase inhibitors, and its clinical application security has been confirmed. Through inhibiting ROCK mediated oxidative stress and inflammatory response to give play to the ability to resist oxidative damage, also fasudil could improve the microcirculation of organization, promote cells metabolism, thus played a role of protecting organs. Researches of fasudil to organ protection were mainly found in brain,cardiac ischemia reperfusion, pulmonary hypertension, arteriosclerosis etc., but was uncommon in the research of liver diseases, mainly involved liver ischemia-reperfusion and chronic liver fibrosis, but there was little intensive study in hepatic failure[5]. Therefore, Through this study to establish a kind of animal model of postoperative acute hepatic failure induced by hepatic ischemia & hepatectomy on rats with obstructive jaundice, which chould show similar clinical pathophysiological changes in human beings. To investigate the influence of fasudil to this model.

2. Materials and methods

2.1. Clinical information

Experimental animal: Selected 96 healthy wister rats, randomly divided into survival rate observation group and specimen determination group, then the groups were randomly divided into control group, low dosage of fasudil treatment group(low-dose group),high dosage of fasudil treatment group(high-dose group),13 rats in survival rate observation group and 19 rats in specimen determination group, to ensure that after the success of the model-making, each group had 10 rats for observation in survival rate observation group, each group had at least 10 rats for specimen collection in specimen determination group. Given conventional feed, 5 rats/cage, separate cages to feed, feeding environment was clean and had constant temperature.

Experimental reagents and consumables: prepared the following reagents and consumables before the experiment: normal saline, 4% of paraformaldehyde, 10% of chloral hydrate, fasudil (10 mg/mL, Tianjin Red Sun pharmaceutical co., LTD, Lot 091012), surgical operating instrument, glass culture dish, disposable blood taking needle, tube, 1 mL + syringe, 5 mL EP + tube, pipettor; SOD, MDA kit(Bioengineering Research Institute, Nanjing).

2.2. Treatment method

Hepatic failure model-making: experiment preparation:one day before molding in the afternoon, fasting but no water deprivation, surgical instruments disinfection and drying,set aside; after weighting, using10% of chloral hydrate for ntraperitoneal anesthesia(dosage of 2 mL/kg), after satisfied anesthetic effect, took supine position fixed on the operating table; skin around surgical incision using polyvinylpyrrollidone for disinfection, spreading aseptic towel; along the original incision into the abdomen, cautiously separating adhesion intestinal canal and omentum, exposing the inflated common bile duct, carefully separating adhesion part of duodenum, lobes of liver and common bile duct; avoiding damaging intestinal wall and bleeding; at the antetheca of inflated common bile duct, using 70 suture with needle for purse string suture, did not fasten the suture for the standby application; presetting cotton ball around common bile ductin the pouch, using 1 mL of syringe to suck out part of the bile for decompression;using ophthalmic forceps to lift gently the pouch intrahepatic bile duct walls,using eye scissors to cut a small opening, inserting the prepared silicone tube (length 3 cm,inner diameter 1 mm, outer diameter 2 mm) and fastening the pouch, as the circumstances may require, suturing and fixing silicone tube, the other end of silicone tube indwelled as duodenal bile internal drain-age, dissociating in the left lobe and middle lobe of the liver(account for about 70% of the whole liver), except for 0 min + group, each experimental group using small size of zero damage vascular clamps to block the left lobe and middle lobe vasculars of liver, only leaving about 30% of the right lobe, nipple lobe as the backflow channel for the portal vein and vena cava, in order to prevent portal vein from blood clot. After occlusion, color of the left lobe and right lobe of liver changed from red to dark purple, which indicated that Vascular blocking was successful. Removing the vascular clamps to recover the blood-supply in the block area after blocking blood-supply for 0 min, 15 min, 30 min, 45 min + respectively, meanwhile, excising parts of liver without ischemia treatment,to establish the hepatic ischemia & hepatectomy damage model.

Fasudil intravenous injection: (First operation:obstructive jaundice 14 d. Second operation: biliary tract duodenum crossover main internal drainage, 70% of hepatic ischemia, excising the remnant liver after 30 min). Using 1ml of injection syringe to inject slowly 600 μL 0.9% of normal saline through main portal vein immediately in control group after hepatectomy; while in low-dose group, to inject slowly 200 mL + of fasudil (10 mg/kg) which was diluted to 600 μL + by normal saline, through main portal vein immediately; in high-dose group, to inject slowly 600 mL + of fasudil (30 mg/kg) through main portal vein immediately, light pressure to stop the bleeding after injection. All rats in specimen collection group were killed after operation 6 h+ to collect specimen; survival rate observation group to observe 96 h+ survival rate.

Serum specimen collection: anesthetizing survival rats in each group after operation 6 h+,picking up vena cava blood through laparotomy, 3 000 rpm,10 min centrifuging to get the upper layer of serum, putting into 5 mL ep tube, -80 °C refrigerator for storage use. Hepatic tissue specimen collection: Immediately clipping liver tissue of the same position with the left lobe of liver after picking up blood, a portion of which was put into 4% of paraformaldehyde for fixation, while the remaining liver tissue was put into -80 °C refrigerator for
rats became worse after 1-2 h, that meant they were entering into get up, then they could stand wiggly. General conditions of the about 60 min after operation, at first, they could not turn over and each group. Rats in control group underwent anesthesia recovery After successful model-making, to observe general conditions in 10 min, the second time for 10 min, neutral resins sealing piece.

time for 10 min; transparency, sealing piece: xylene the first time for 5 min, the second time for 1 min, 95% of ethyl alcohol: the first time for 1 min, the second time under the tap water; water deprivation: 80% of ethyl alcohol for 1 short rinsing; weak ammonium hydroxide turned blue, microscopic separation for 30s; tap water soaking for 15 min; distilled water for the tap water; 1% of hydrochloric acid & ethyl alcohol colour concentration to high concentration of ethanol soaking: xylene for the second time 30 min, xylene for the second time 5 minutes, 90% of ethyl alcohol: the first time for 30 min, xylene for the second time 30 min, 100% of ethyl alcohol for the second 10 min 5 min, 90% of ethanol 5 min, finally distilled water rinsing for 5 min; dropwise adding hematoxylin staining cell nucleus for 5 min, rinsing it under the tap water; 1% of hydrochloric acid & ethyl alcohol colour separation for 30s; tap water soaking for 15 min; distilled water for short rinsing; weak ammonium hydroxide turned blue, microscopic observation; putting into eosin liquid staining for 15 min, rinsing it under the tap water; water deprivation: 80% of ethyl alcohol for 1 min, 95% of ethyl alcohol: the first time for 1 min, the second time for 1 min; 100% of ethyl alcohol: the first time for 5 min, the second time for 10 min; transparency, sealing piece: xylene the first time for 10 min, the second time for 10 min, neutral resins sealing piece.

2.3. Statistical analysis

Measurement data using Mean ± standard deviation to show, using SPSS18.0 statistical software for statistical analysis, measurement data underwent normality test first, and then homogeneity test of variance, comparison among groups using complete randomized design of single factor variance analysis; comparisons between the two of multiple sample mean using Student-Newman-Keuls q for examination; survival rate analysis using Kaplan-Meier method, P<0.05 was considered as statistical significance.

3. Results

3.1. General conditions

After successful model-making, to observe general conditions in each group. Rats in control group underwent anesthesia recovery about 60 min after operation, at first, they could not turn over and get up, then they could stand wiggly. General conditions of the rats became worse after 1-2 h, that meant they were entering into condition of hepatic failure, there appeared a coma one after another after 6-8 h, mainly showed depression, lethargy, unresponsive, denied food, yellow color of urine, activity decreased significantly. About 80% of rats died within 24 h, after postoperative injection of fasudil through portal vein, general conditions of rats improved obviously, recovery time became shorter, hepatic failure symptom decreased etc., and high-dose group was better than low-dose group.

3.2. Survival rate analysis

To observe 96 h survival rate of these 3 groups, rats in control group began to die one after the other the 3-6 h after molding, death mainly concentrated within 18 h, 24 h death rate was 80%, 96 h death rate was 90%, while the survival rate of rats in drug treatment group was relatively higher than that in control group, death rate decreased, among them between the high-dose group and control group, difference was statistically significant (P<0.05). 96 h death rate in low-dose group was 70%, 96 h death rate in high-dose group was 50% (Figure 1).

Table 1
Liver function determination after given fasudil in each group.

<table>
<thead>
<tr>
<th>Group</th>
<th>ALT (U/L)</th>
<th>AST (U/L)</th>
<th>TBIL (U/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (n=10)</td>
<td>1436.5±258.1</td>
<td>1519.1±274.6</td>
<td>120.3±30.7</td>
</tr>
<tr>
<td>Low-dose (n=12)</td>
<td>1211.5±213.4*</td>
<td>1272.5±221.6*</td>
<td>90.4±26.3*</td>
</tr>
<tr>
<td>High-dose (n=13)</td>
<td>908.2±174.2#</td>
<td>952.4±118.9#</td>
<td>67.3±21.2#</td>
</tr>
</tbody>
</table>

Notes: compared with control group *P<0.05; Compared with other groups #P<0.05.

Table 2
TNF-α, IFN-γ concentration after given fasudil in each group.

<table>
<thead>
<tr>
<th>Group</th>
<th>TNF-α (pg/mL)</th>
<th>IFN-γ (pg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control group</td>
<td>427.5±104.3</td>
<td>581.3±128.1</td>
</tr>
<tr>
<td>Low-dose fasudil group</td>
<td>272.1±82.4*</td>
<td>398.3±81.0*</td>
</tr>
<tr>
<td>High-dose fasudil group</td>
<td>182.8±56.9*#</td>
<td>217.3±69.4*#</td>
</tr>
</tbody>
</table>

Notes: compared with control group *P<0.05; Compared with other groups #P<0.05.

Figure 1. Comparison of 96 h survival rate in each group.
3.3. Serum ALT, AST, TBIL level

ALT, AST, TBIL level in control group was obviously higher than that in fasudil treatment group, and the difference had statistical significance \((P<0.05)\), after given fasudil treatment, AST, ALT, TBIL values declined obviously, and high-dose group was higher than low-dose group, difference had statistical significance \((P<0.05)\) (Table 1).

<table>
<thead>
<tr>
<th>Table 3</th>
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<tbody>
<tr>
<td>SOD activity, MDA content changes after given fasudil in each group.</td>
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<tr>
<td></td>
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<tr>
<td>Control group</td>
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<tr>
<td>Low-dose fasudil group</td>
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<tr>
<td>High-dose fasudil group</td>
</tr>
</tbody>
</table>

Notes: compared with control group \(*P<0.05\); compared with other groups \(#P<0.05\).

3.4. Serum cytokines TNF-α, IFN-γ level

Results showed that cytokines concentration declined obviously after given fasudil through portal vein, compared with control group, the difference had statistical significance \((P<0.05)\), and degree of declining in high-dose group was higher than that in low-dose group, difference between two groups had statistical significance \((P<0.05)\) (Table 2).

3.5. Liver tissue homogenate SOD, MDA content

SOD activity in control group was lower than that in fasudil treatment group, difference had statistical significance \((P<0.05)\), SOD activity rised after given fasudil treatment, and high-dose group was higher than low-dose group, difference between two groups had statistical significance \((P<0.05)\), while MDA content showed the opposite trend, content in control group was higher than that in drug treatment group, difference had statistical significance \((P<0.05)\) (Chart 3).

3.6. Histopathological observation

Observation from light microscope (Figure 2): the control group showed the typical symptoms of liver failure, after given fasudil through portal vein, the hepatic lobule structure degree of damage alleviated, liver necrocytosis area decreased, the existence of residual liver cells was more than control group, high-dose necrocytosis area was smaller than that in low-dose group, but through pathological observation, not sure it had obvious difference.

4. Discussion

In animal experiment, the common administration methods of therapeutic drugs including tail vein injection, intraperitoneal injection and intragastric administration treatment, and the common administration method of fasudil was intraperitoneal injection, but in the process of the experiment of animal model-making, animals often had ascites, which had a certain influence on drug absorption, but after peripheral intravenous injection, drugs may affect the intrahepatic concentration through the systemic circulation of dilution, this experiment choosed portal vein injection, could make the drug more quickly act on the liver, and increased the drug concentration in liver, operation was relatively simple. In the research of protective effect of fasudil to liver injury, dose of common usage for fasudil was 10 mg/kg and 30 mg/kg, results reported in references [8-10]. In this study, the final decision for the therapeutic dose of fasudil was low-dose (10 mg/kg) and high-dose (30 mg/kg) through trial test.

After successful model-making, the portal vein was given different doses of fasudil, while the control group was given normal saline as a comparison, and then began to observe the general conditions of postoperative recovery of rats. Recovery time of rats in control group was generally longer than that in drug therapy group, recovery physical strength was lower than that in drug therapy group, fasudil significantly improved the postoperative general conditions of rats. Picking up liver tissue to proceed HE dyeing observations, found that in the control group rats occurred large necrosis of liver cells, while in fasudil treatment group, hepatocyte necrosis in rats was significantly lighter than that in the control group, around the necrotic area existed more survival liver cells, these showed that fasudil had certain protective effects on liver failure through protecting liver cells. Through the determination of serum ALT, AST, TBIL values in rats, we found that liver biochemical index of rats improved significantly after the treatment \((P<0.05)\), and presented a certain dose-dependent, these reflected the biochemical indicators of liver function, and further confirmed the protective effect of fasudil to liver cells.

After liver failure, except for the primary injuries, meanwhile existed secondary injuries, that was "two hit hypothesis": due to the intestinal barrier damage, increase of endotoxin and mononuclear macrophage function abates, which caused that endotoxin blood...
could not effectively be cleared away in time, endotoxin, in turn, stimulated the liver inside and outside mononuclear macrophage to cause inflammatory reactions, influencing microcirculation, further aggravating liver cell damage[11-13]. Cytokines were involved in this process, and it has now confirmed that cytokines related with liver failure had TNF-α, IFN-γ, IL-1, IL-6 etc. Researchers showed that TNF alpha played an important role in the process of liver failure caused by a variety of reasons, characterized by liver apoptosis and inflammation mediated liver cell necrosis, blocking TNF-α could block the occurrence of liver necrosis. In addition[14,15], IFN-γ was also cytokines closely related with hepatitis or liver failure, Kim WH etc. Through detection of IFN-γ to stimulate signal transduction molecules-STAT1 when target cell receptor gave play to effects, we have proved that IFN-γ was closely related with acute hepatic failure, apoptosis and injuries. Therefore determining serum TNF-α, IFN-γ level could reflect the damage degree of the liver, according to the results of this experiment, serum TNF-α, IFN-γ level in high-dose fasudil group was obviously lower than that in low-dose fasudil group and control group (P<0.05), these showed that fasudil could play a role of liver protection through lowering TNF-α, IFN-γ etc. Cytokine levels, and present dose dependent within a certain range. We also found that low-dose drug treatment group compared with control group, SOD activity increased, MDA content decreased (P<0.05), ability of scavenging free radicals of liver tissue improved, reduced lipid peroxidation reactions, reduced the livercell injuries by free radical ion, thus played a role of liver protection.

Through observing rats 96 h survival conditions and survival analysis,we found that difference of high-dose fasudil group and control group had statistical significance (P<0.05). In Control group, within 6 hours, appeared more animals were killed one after the other, death time of high-dose group was delayed, compared with control group, did not appear massive death in short time, prolonged the survival time of the experimental rats, and the mortality rate decreased from 90% to 50%,which suggested that high-dose fasudil group could effectively improve animals with liver failure survival rate. Death rate in low-dose group was lower than that in control group, survival rate analysis difference had no statistical significance. According to the above test results, we found that the protective effect of fasudil to liver was dose-dependent, therefore speculating the protection effects of low-dose group to liver was not enough to reflect the obvious improvements to survival rate under the condition of the specimen cases (n=10) in this experiment, increasing the sample size may get meaningful results.

In conclusion, we found that fasudil had a certain protection effect on liver failure model induced by liver ischemia and hepatectomy on rats with obstructive jaundice, which could come into effects through reducing inflammatory cytokines levels and relieving lipid peroxidation.

References


