ORIGINAL

Experimental Studies on Protective Effects of FK506 Against Hepatic Ischemia-Reperfusion Injury

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Abstract : Purposes ; FK506 (strong immunosuppressive agent) was investigated experimentally whether to protect the hepatic IRI. Methods ; Warm ischemic experiment using pigs and rats were performed and examined whether FK506 is effective. Results ; The results obtained are as follows. 1. Warm ischemia allowed time of the pigs without FK506 was 150 minutes, but as for that of FK506 group, the extension of 30 minutes was got in 180 minutes. 2. Biliary excretion rate of BSP after reperfusion were better in the group of 180 minutes ischemia with FK506 than in without FK506 group. 3. Chemiluminescence intensity in the peripheral neutrophils and adhered and infiltrated leukocytes in the liver were suppressed markedly by FK506. 4. The vascular endothelium with the scanning electron microscope was relatively preserved in the FK506 group comparing to the placebo group on 30 minutes after reperfusion. 5. Stress gastric ulcer was controlled and myeloperoxidase activity in the gastric mucosa was suppressed by FK506. Conclusion ; Based on the results of theses experiments, it was suggested that FK506 has a protective effect on IRI by suppressing : the impairment of sinusoidal endothelial cells ; the activation of KCs ; the disturbance of micro-circulation ; oxidative stress ; inflammation ; and the accumulation of leukocytes. J. Med. Invest. 63 : 262-269, August, 2016

Keywords : *hepatic warm ischemia, ischemia-reperfusion injury (IRI), FK506 (tacrolimus), protective effect for IRI.*

INTRODUCTION

In recent years, liver resection and liver transplantation have been widely adopted in clinical practice for treatment of liver diseases. Hepatic ischemia-reperfusion injury (IRI) occurs substantially during liver resection with resection of the portal vein and/or the hepatic artery, or liver transplantation and remains a major cause of liver non-function or functional failure following liver surgery. This IRI has become an obstacle which has restricted the development of extensive liver resection and liver transplantation using marginal liver donors.

The mechanism of hepatic IRI have been widely investigated, but nevertheless remains largely unclear. More importantly, an effective prevention or treatment method is still lacking. Therefore in Experiment 1, FK506 (tacrolimus), which is a powerful immune-suppressive agent and also a hepato-trophic agent for hepatic IRI (1), was used to perform the hepatic warm ischemic experiment on pigs. This was in order to identify the impact on liver damage of ischemic time and the effects of FK506 on IRI ; serum liver function tests ; bile flow ; biliary excretion rate of bromsulfophtalein (BSP) ; and animal survival rates. Furthermore, in Experiment 2 an immuno-staining by the anti-CD18 antibody was performed and the adhesive state of neutrophils in the liver after reperfusion was examined in order to explore the mechanism of the protective effects of FK506. As well, the vascular endothelium was examined with the scanning electron microscope after reperfusion. Finally, in Experiment 3 we examined the effect of FK506 on stress gastric mucosal damage where the participation of superoxides had been shown (2-4).

MATERIALS AND METHODS

Experiment 1

Twenty-three (23) female pigs weighing 18 to 24 kg were fasted for twenty-four (24) hours prior to their participation in the experiment. After the making of a porto-caval shunt and cannulation with a tube into the common bile duct, clamping of the hepatic artery and the portal vein at the portal triad allowed hepatic warm ischemia to be carried out for various lengths of time.

The twenty-two (22) pigs were divided into four (4) groups.
Group 1 : 120 minutes clamping (n=5) without FK506 treatment.
Group 2 : 150 minutes clamping (n=5) without FK506 treatment.
Group 3 : 180 minutes clamping (n=7) without FK506 treatment.
Group 4 : 180 minutes clamping (n=5) with FK506 treatment.

For Group 4, FK506 1 mg/kg was injected intra-muscularly for four (4) days pre-operatively.

All the animals were examined for survival rate ; serum liver function test such as total bilirubin, aspartate aminotransferase (AST), alanine aminotransferase (ALT), and lactate dehydrogenase (LDH) ; bile flow ; the biliary excretion rate of BSP ; chemiluminesence of the neutrophils in the peripheral blood ; and the histology of the liver.

Initially, as a normal control, one (1) pig was used to ascertain the bile flow, and the biliary excretion rate of BSP after 5 μg/kg of BSP was injected intravenously. And BSP in the bile was measured for five (5) minutes at one (1) hour after reperfusion and the biliary excretion rate was calculated.

A monitor tube for portal vein pressure was inserted from the branch of the superior mesenteric vein to the portal trunk, and a

Abbreviations
BSP : bromsulfophtalein ; AST : Aspartate aminotransferase ; ALT : Alanine aminotransferase ; LDH : Lactate Dehydrogenase ; ROS : Reactive Oxygen Species ; KCs : Kupffer Cells ; TNF-α : tumor necrosis factorα ; ICAM-1 : intracellular adhesion molecule 1 ; VCAM-1 : vascular cell adhesion molecule 1 ; (IL)-1β : interleukin-1β ; H2O2 : hydrogen peroxide ; HOCl : hypochlorous acid ; ENA-78 : chemokine epithelial neutrophil activating protein-78 ; NF-κB : nuclear factor-κB ; JNK : c-Jun N-terminal kinase ; OKY046 : thromboxane synthetase inhibitor ; TXA2 : Thromboxane A2 ; ONO-4057 : Leukotriene B4 receptor antagonist.

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monitor tube for arterial pressure was inserted in the femoral artery. Arterial pressure and portal pressure were measured in real time before declamping of the hepatic artery (HA) and the portal vein (PV) for about seven minutes. After having observed it about ten minutes until arterial pressure was stable after declamping of HA and PV, portal vein pressure was measured after clamping in Porto-Caval shunt in each three pigs of Group 3 and Group 4.

The intensity of chemiluminescence of the peripheral neutrophils was measured during ischemia and reperfusion, and was compared between Group 3 and Group 4.

Experiment 2

Experiment 2 was carried out in order to explore the mechanism of the protective effects of FK506 on the anti-CD18 antibody ; the adhesive state of neutrophils in the sinusoid and the vascular endothelium after reperfusion.

Twenty-two (22) male Wister rats, weighing 200 to 250 g were fasted for sixteen (16) hours prior to the experiment. The rats were divided into two (2) groups, that is, the group receiving FK506 and the placebo group. Under pentobarbital anesthesia (50 mg/kg), laparotomy was carried out on each rat. Clamping of the inflow in the hepatic hilus was done for fifteen (15) minutes as warm ischemia. At thirty (30) minutes, three (3) hours, and twenty-four (24) hours after reperfusion, the liver was excised following the removal of blood from the whole body via the left ventricle with 140 cm H_2O pressure. An immuno-staining was performed using ant-CD18 antibody on a fresh frozen specimen of the liver. The number of white blood cells in the liver stained with ant-CD18 antibody that was found in one field of view of 200 magnification, was then measured. As well, the hepatic sinusoid was observed with the scanning electron microscope on 30 minutes after reperfusion.

Experiment 3

Because it has been suggested that superoxides cause the origin of stress gastric mucosa damage (2-4), the effect of FK506 on the development of stress ulcers was examined in Experiment 3.

Thirty-nine (39) male Wister rats, weighing between 200 and 250 g, were fasted for sixteen (16) hours prior to the experiment. The rats were placed in restraint gauge, and were dipped into a bath with a constant temperature of 22°C up to the xiphoid process. The stress load time was six (6) hours. After that, the stomach of each rat was excised, and fixated with a 1% formalin solution overnight. The lengths of the bleeding plaques were then measured, and their lengths expressed as an ulcer index.

To ascertain the time dependent effect and dose dependent effect, the ulcer index was examined and comparison was made between the placebo group and the FK506 group of rats. The ulcer index was compared between three groups (Group A : one (1) time of FK506 1 mg/kg on the day of the examination ; Group B : two (2) times of FK506 1 mg/kg, one (1) on the day before the examination and one (1) on the day of the examination ; Group C : three (3) times of FK506 1 mg/kg, one (1) on the two (2) days prior to the examination and one (1) on the day of the examination).

Next the ulcer index was compared between three (3) groups of the dosage of FK506 0.1 mg/kg, 0.5 mg/kg, 1.0 mg/kg by twice administration on the day before and the day of the examination.

Myeloperoxidase activity in the gastric mucosa was examined and comparison made between the placebo group and the FK506 group of rats.

All animal procedures complied with the animal care guidelines of the Institute of Animal Experimentation in the Medical School of Kumamoto University.

STATISTICS

Statistical analyses were performed using the Student's t-test with a p value of less than 0.05 considered to be significant. Data were shown as mean±SD. Differences in survival were determined using the Kaplan-Meier Survival analysis.

RESULTS

Experiment 1

One (1) week survival (rate) was 5/5 (100%) in Group1 (120 minutes ischemia without FK506), 5/5 (100%) in Group 2 (150 minutes ischemia without FK506), 1/7 (14.3%) in Group 3 (180 minutes ischemia without FK506), and 5/5 (100%) in Group 4 (180 minutes ischemia with FK506).

The results showed that all the pigs included in the experiment survived until 150 minutes ischemia in the groups without FK506 (Groups 1 and 2). However, although six (6) of seven (7) cases without FK506 died under 180 minutes ischemia (Group 3), all five (5) cases with FK506 survived under 180 minutes ischemia (Group4) [Figure 1]

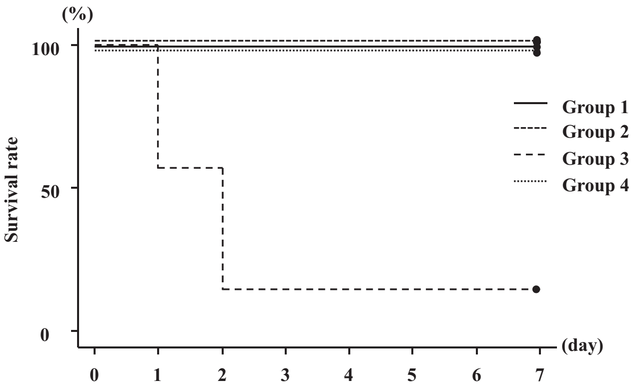

Figure 1. The 7-day survival rate is shown. All pigs survived for 7 days in Group 1, 2, 4. However, 6 of 7 pigs died within 3 days by circulatory disturbance or liver failure. Survival rate was a significant difference between Group 1, 2, 4, and Group 3 ($p < 0.02$).

The biochemical data such as total bilirubin, AST, ALT, and LDH values at one (1) hour after reperfusion were not different between the four (4) groups.

The bile flow was 1,200 μl/5 minutes in the normal pig used as control. By contrast, the bile flow at one (1) hour after reperfusion was 890 μl/5 minutes in Group 1 ; 400 μl/5 minutes in Group 2 ; 240 μl/5 minutes in Group 3 ; and 490 μl/5 minutes in Group 4 (180 minutes ischemia with FK506 treatment). It was noted that the bile flow in Group 4 was almost equal to that in Group 2 [Figure 2].

The biliary excretion rate of BSP was 70% in the normal control pig, but it was 25% in Group 1 ; 13% in Group 2 ; 5% in Group 3 ; and 15% in Group 4, respectively. The biliary excretion rate in Group 4 (180 minutes ischemia with FK506 treatment) was almost equal to that in Group 2 (150 minutes ischemia without FK506) [Figure 3].

The color of the liver was dark red into a map form, and the hardness was also increased at fifteen (15) minutes after reperfusion in Group 3 (180 minutes ischemia without FK506), on the other hand, the liver was a bright red color in the whole uniform, enhancement of hardness was also mild in Group 4 (180 minutes ischemia with FK506) [Figure 4].

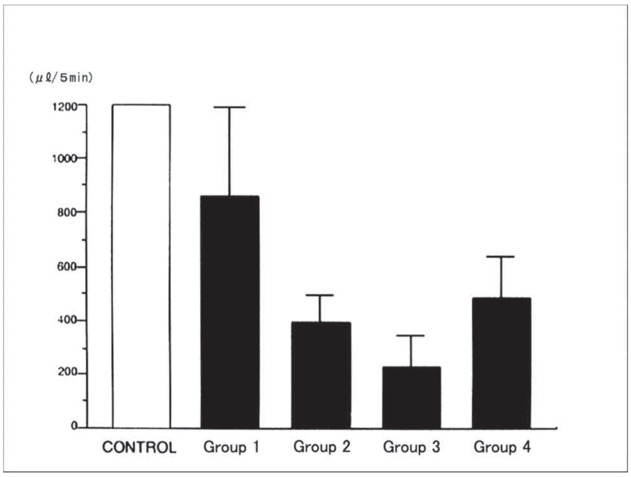

Figure 2. Bile flow was 1,200 μl/5 minutes in normal pig as control, but, bile flow on one hour after reperfusion was 890 μl/5 minutes in Group 1, 400 μl/5 minutes in Group 2, 240 μl/5 minutes in group 3 and 490 μl/5 minutes in Group 4 (180 minutes ischemia with FK506 treatment), respectively. And bile flow in Group 4 was almost equal to it in Group2.

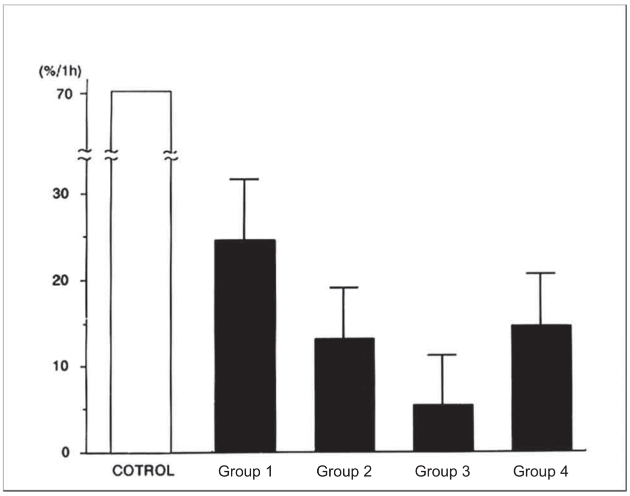

Figure 3. Biliary excretion rate of BSP was 70% in normal pig, but it was 25% in Group 1, 13% in Group 2, 5% in Group 3, and 15% in Group 4, respectively. Biliary excretion rate in Group 4 (180 minutes ischemia with FK506 treatment) was almost equal to in Group 2 (150 minutes ischemia without FK506).

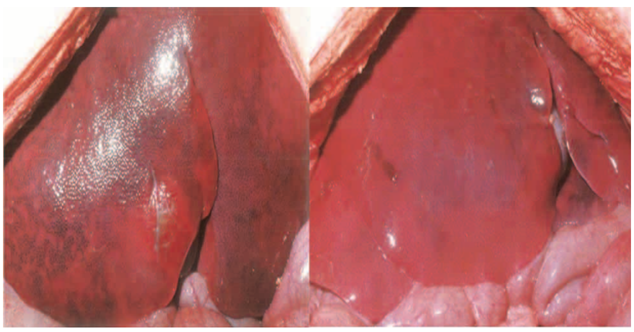

Figure 4. The color of the liver surface at fifteen (15) minutes after reperfusion was dark red in Group 3 (180 minutes ischemia without FK506), and clear red in Group 4 (180 minutes ischemia with FK506).

Since the results of portal pressure in each three pigs in Group 3 and Group 4 on portal pressure were the same, each one example of Group 3 (left) and Group 4 (right) was shown in Figure 5 as representative example. The portal pressure was increased soon in Group 3 after clamping of the porto-caval shunt. However, it did not increased in Group 4, showing clearly from these results that FK506 improved micro- circulation of the liver after reperfusion [Figure 5].

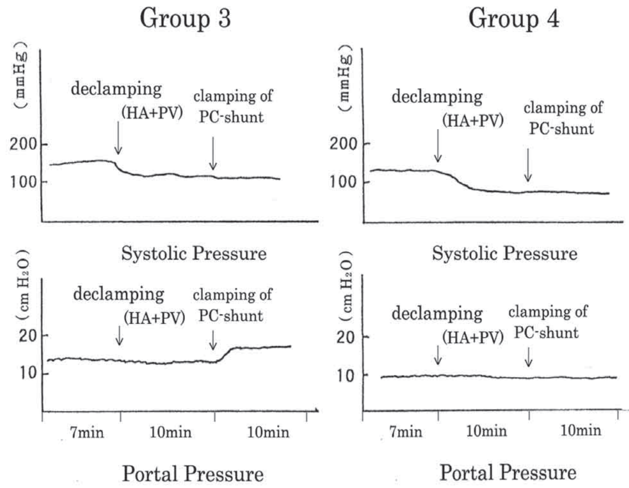

Figure 5. Portal pressure was increased in Group 3 after clamping of portocaval shunt. But it did not increased in Group 4. It was clear that FK506 improved microcirculation of the liver after reperfusion from these results.

The intensity of chemiluminescence of neutrophils began to rise from two (2) hours after hepatic warm ischemia, and continued to rise until two (2) hours after reperfusion in Group 3. In contrast, the intensity of chemiluminescence of neutrophils remained slightly increased after reperfusion in Group 4 [Figure 6]. It is suggested from these results that FK506 inhibits the activity of superoxides as well as immunological metabolism.

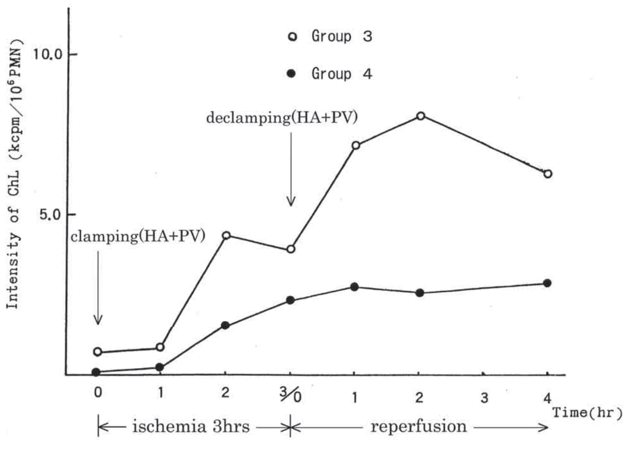

Figure 6. The intensity of chemiluminescence of neutrophils began to rise from 2 hours after hepatic ischemia, and continued to rise until 2 hours after reperfusion. In contrast, the intensity of chemiluminescence of neutrophils remained slightly increased after reperfusion in Group 4.

Microscopic findings of the liver in Group 3 at one (1) hour after reperfusion showed balloon degeneration, sinusoidal dilatation and inflammatory cell infiltration. In contrast, these findings were minor in Group 4.

Experiment 2

Adhered and infiltrated leukocytes that were stained with anti-CD18 antibody, were observed approximately four (4) times before ischemia in the placebo group of rats and in the FK506 group at thirty (30) minutes following reperfusion. As well, a large number of adhered and infiltrated leukocytes were observed in the placebo group between three (3) hours and twenty-four (24) hours following reperfusion, while in the FK506 group, adhered and infiltrated leukocytes decreased between three (3) hours and twenty-four (24) hours, significantly less in comparison with the placebo group [Figure 7].

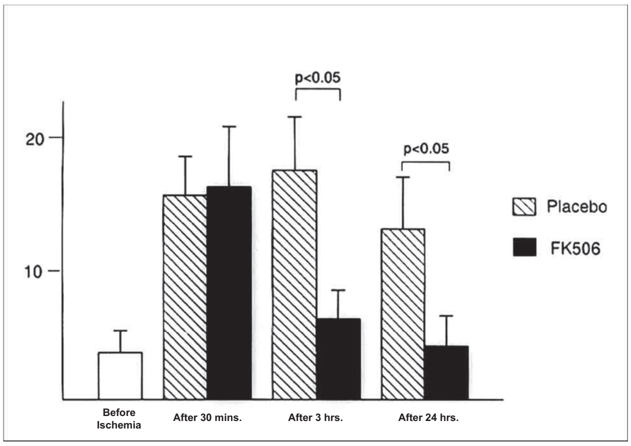

Figure 7. In the placebo group, a large number of adhered and infiltrated leukocytes were observed until 3 hours and 24 hours. But in the FK506 group, adhered and infiltrated leukocytes decreased on 3 hours and 24 hours, and it was significantly less in comparison with the placebo group.

The hepatic sinusoid was observed with the scanning electron microscope on 30 minutes after reperfusion. The dilatation of the vascular endothelial pore, ruptures of the sieve plate, and adhered leukocytes were observed much more in the placebo group (left photo). In contrast, the dilatation of the vascular endothelial pore, ruptures of the sieve plate, and adhered leukocytes were hardly observed (right photo) [Figure 8].

Experiment 3

After six (6) hours restriction a clear hemorrhagic ulcer was seen in the placebo group of rats but not in the FK506 group [Figure 9].

As the administrated times increased (Groups A to C), the ulcer index decreased, and its effect was improved more by FK506 in Time dependent [Figure 10].

The ulcer index decreased as the dose of FK506 increased to 0.1 mg/kg, 0.5 mg/kg, 1 mg/kg, and its effect was improved more in Dose dependent [Figure 11].

The Myeloperoxidase activity rose to four (4) times that of the control in the placebo group, but it was hardly the same as the control in the FK506 group where the activity did not rise [Figure 12]. Myeloperoxidase activity in a gastric mucosa was controlled by FK506 pre-treatment [Figure 12].

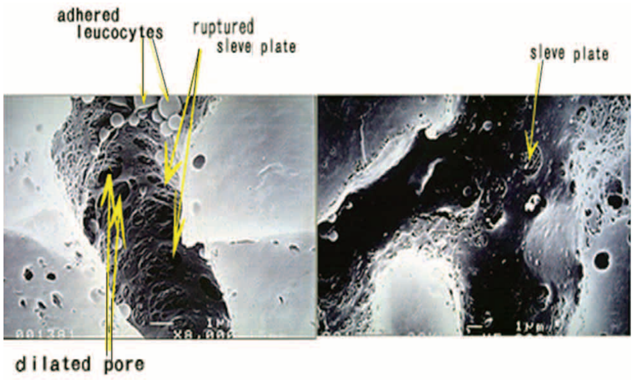

Figure 8. The hepatic sinusoid was observed with the scanning electron microscope on 30 minutes after reperfusion. The dilatation of the vascular endothelial pore, ruptures of the sieve plate, and adhered leukocytes were observed much more in the placebo group (left photo). In contrast, the dilatation of the vascular endothelial pore, ruptures of the sieve plate, and adhered leukocytes were hardly observed (right photo)

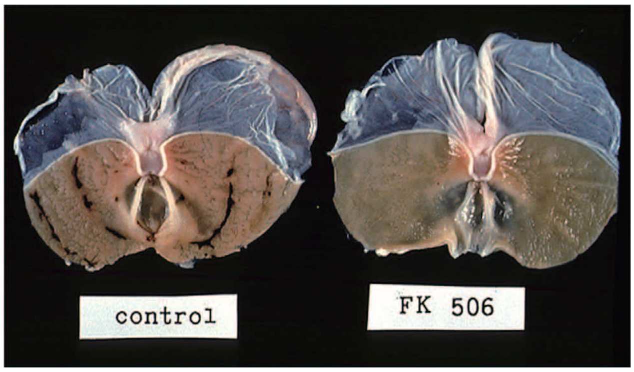

Figure 9. A clear hemorrhagic ulcer was seen by 6-hour restriction in the placebo group (left photo), but the ulcer was not seen in the FK506 group (right photo).

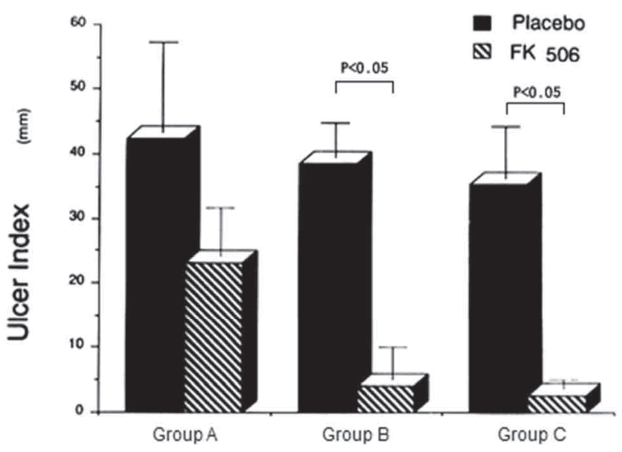

Figure 10. As the administrated times of FK506 increased (Group A to C), the ulcer index decreased, and its effect was improved more by FK506 in Time dependent.

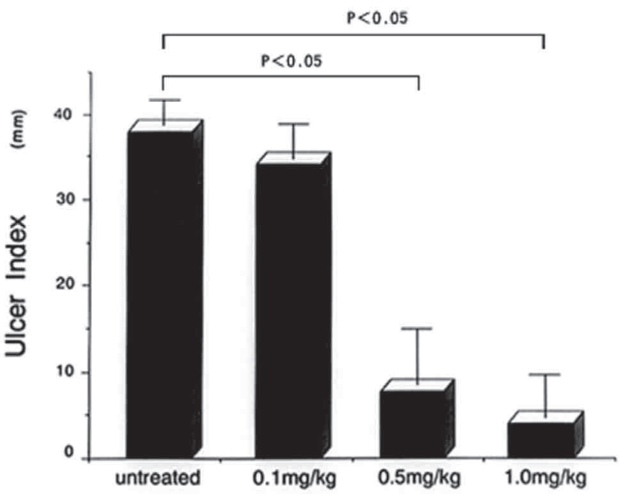

Figure 11. The ulcer index decreased as the dose of FK506 increased to 0.1 mg/kg, 0.5 mg/kg, 1 mg/kg, and its effect was improved more in Dose dependent.

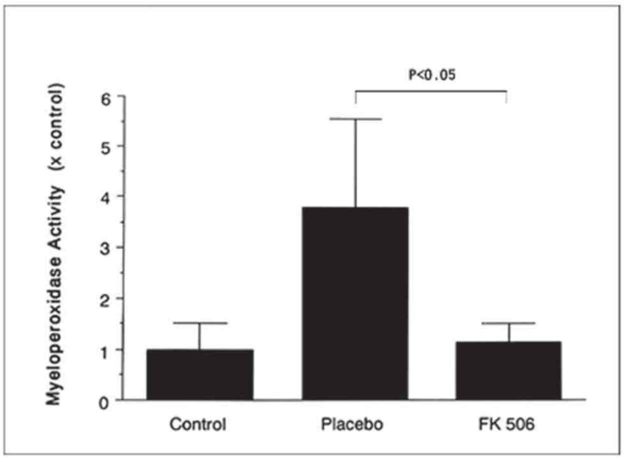

Figure 12. The Myeloperoxidase activity in the gastric mucosa rose to 4 times of control in the placebo group, but it was the hardly same as control in FK506 group, and the activity did notrise.

DISCUSSION

Hepatic IRI includes both warm and cold IRI-two types that share similar pathophysiological processes. The mechanism of hepatic IRI have been widely investigated, but nevertheless remains largely unclear. The factors/pathways that have been implicated in the hepatic IRI process include anaerobic metabolism, mitochondria, oxidative stress, intracellular calcium overlord, liver Kupffer cells (KCs) and neutrophils, and cytokines and chemokines.

During the state of hepatic ischemia, the metabolic pattern is shifted from aerobic to anaerobic, the redox process of the hepatocytes is blocked, ATP-dependent cellular metabolic activities are gradually stopped, and intracellular ATP is rapidly depleted. Conversely, there is an accumulation of acidic metabolites, such as lactic acid and ketone bodies, which is caused by enhanced anaerobic glycolysis. This is accompanied by hypo function of mitochondrial oxidative phosphorylation, resulting in the decrease of pH values between tissues and cells, known as metabolic acidosis.

When the blood flow is reopened to the ischemic organ, the pH values restore to normal after reperfusion, and further enhance pH-dependent enzyme activation, such as activation of proteases and phospholipases, further worsening the damage of tissues and organ. This is called the pH paradox (5).

IRI has biochemical ramification. The oxidative stress plays a key role in reperfusion injury. Many highly reactive molecules, such as Reactive Oxygen Species (ROS), are induced during the period of hepatic IRI. ROS can also damage endothelial cells and destroy the integrity of the microvasculature.

Among the biochemical factors affected by IRI, calcium has an especially important role. When the calcium level is elevated by ischemia or hypoxia, oxidative stress, toxic substance release or other harmful event occur, this is call Ca^{2+} overload. Intracellular Ca^{2+} overload can activate Ca^{2+}-dependent enzymes such as calpains, protein kinase C, and phospholipase C, and ultimately leads to cell death or apoptosis.

The liver KCs and neutrophils are involved in the hepatic IRI process. The KCs mainly mediate liver ischemic injury in the earlier stage of reperfusion (within two (2) hours) by synthesizing and releasing ROS and the pro-inflammatory cytokines tumor necrosis factor-alpha (TNF-α) and interleukin (IL)-1β to further activate liver sinusoidal endothelial cells, enhance the expression of the adhesion molecules inter cellular adhesion molecule 1 (ICAM-1)/vascular cell adhesion molecule 1 (VCAM-1), further promote the adhesion, migration, and chemotaxis of neutrophils and endothelial cells and accumulate and activate neutrophils, resulting in subsequent liver cell damage (6). Activation of neutrophils can directly damage liver cells by the release of oxidants and proteases after reperfusion. Ultimately, myeloperoxidase (halide form, such as Cl-) released from neutrophils changes hydrogen peroxide (H2O2) into hypochlorous acid (HOCl), which is a potent oxidant. These oxidants can directly cause liver cell damage and/or induce protease-mediated injury through inactivation of the endogenous anti-protease system (7, 8), suggesting that anti-oxidant or anti-protease therapy would be helpful for preventing IRI. In our experiment of stress gastric ulcer in rats, it is very interesting that FK506 controlled the activity of myeloperoxidase of the gastric mucosa significantly. It is considered that there is enough possibility to suppress the activity of myeloperoxidase which is released upon hepatic IRI.

Cytokines play a dual role of anti-inflammatory and pro-inflammatory responses in the process of liver IRI. TNF-α is a key member of the group of endogenous pro-inflammatory and anti-inflammatory molecules, and is a critical factor in triggering the inflammatory cascade. It is secreted by activated KCs and impacts on liver tissue and distant organs through paracrine signaling and the endocrine system (9). TNF-α can bind to the receptors on the surface of liver cells to induce overproduction of the chemokine epithelial neutrophil activating protein-78 (ENA-78) and ROS, activate nuclear factor (NF)-κB, mitogen-activated protein kinase, and c-Jun N-terminal kinase (JNK), and cause liver injury directly (10). In addition, TNF-α also can up regulate expression of the chemokines ICAM-1, VCAM-1 and P-selectin (11). Moreover, JNK and ROS can directly act on liver cells to cause liver damage.

FK506 was discovered in 1984, it was among the first macrolide immuno-suppressants discovered. It is produced by a type of soil bacterium, Streptomyces Tsukubaensis. FK506 was named from Fujisawa (Pharmaceutical Co) Kaihatsu (Development) Numbers (506). Tacrolimus is named as general name after development of FK506, and it is derived from Tsukuba macrolide immuno-suppressant (12).

FK506 controls the immuno-reaction that a T cell participates in strongly. The reaction includes a cytotoxic T cell, production restraint of IL2, IL3, INF-γ, expression control of IL2 receptor (13).

It has been reported that FK506, in addition to being a powerful immuno-suppressive agent, is also a hepatotrophic agent (1). As well, in view of the mechanisms of IRI mentioned above, this study was undertaken to verify whether FK506 was effective in preventing IRI.

The three experiments in this study were carried out using pigs and rats to know whether FK506 is effective for hepatic IRI. Based on the results of these three (3) experiments, it is suggested that FK506 has a protective effect on IRI by suppressing : the impairment of sinusoidal endothelial cells ; the activation of KCs ; the disturbance of micro-circulation ; oxidative stress ; inflammation ; and the accumulation of leukocytes.

The calcineurin inhibitor FK506 (tacrolimus) acts through a blockade of the intracellular calcineurin-calmodulin complex. This blockade inhibits the calcium-dependent phosphorylation of the nuclear factor of activated T cells (NFAT). As a consequence, IL-2, which is normally involved in the activation of CD4+ and CD8+ T cells, and the IL-2 receptor are downregulated. Thus, the inactivation of T cells is regarded as the central mechanism in the immunosuppressant properties of FK506 (tacrolimus) (14, 15).

In addition, FK506 (tacrolimus) might attenuate allogen-independent hepatic IRI, which is characterized by the release of a complex cascade of cytokines including IL-6 and TNF-α, the generation of ROS, the accumulation and transmigration of different cell types (that is, lymphocytes, neutrophils, platelets), as well as alterations of the microcirculation potentially causing graft dysfunction or even non-function (16). In this respect, T cells have been shown to be critically involved in the induction of IRI of the liver (17-20). A rapid recruitment of CD4+ T cells in hepatic sinusoids as early as 30 minutes after reperfusion is followed by their migration through the endothelial barrier to injured hepatic tissue (18). Although CD4+ T cells themselves are not cytotoxic, they release a panel of cytokines, chemokines and adhesion molecules which are potentially harmful to the organ. Moreover, CD4+ T cells interact with platelets and KCs which further aggravate IRI (21).

Neutrophils are also actively involved in hepatic IRI. The accumulation of neutrophils congests hepatic sinusoids and leads to the release of proinflammatory cytokines (that is, TNF-α and IL-6), as well as ROS (22). Adhesion molecules such as P-selectin and ICAM-1 are involved in the process of neutrophil recruitment (23). The application of FK506 (tacrolimus) decreases the expression of these adhesion molecules, thereby attenuating neutrophil recruitment (24, 25). In addition, direct suppressive effects of FK506 (tacrolimus) on the activation of KCs, which also release proinflammatory cytokines have been demonstrated *in vitro* (26).

Several experimental studies have demonstrated prospective effects of FK506 (tacrolimus) on IRI following liver transplantation (27-29). Despite their promising results, these models were based on systemic pre-conditioning in the same way of our experiments. If we would like to put into clinical practice upon the procurement of donor organs, It is necessary to verify experimentally whether there is protective effects for IRI with pre-conditioning of FK506 during donor's liver extraction for a short time (2~3 hours). And also an ex vivo FK506 (tacrolimus) treatment clinically may represent a solution to this problem. Recently experimental data indicated a protective effect of an ex vivo FK506 (tacrolimus) rinse in a model of experimental liver transplantation in rats (30). In this study preservation of intracellular glutathione levels was suggested as a potential mechanism, and FK506 increases glutathione metabolism, which in turn may protect organ function by reducing ROS toxicity.

The anti-inflammatory effect of FK506 (tacrolimus) was also evident in human liver biopsies after the transplantation of organs rinsed with FK506 (tacrolimus) (31).

With respect to the microcirculation, direct effects of FK506 (tacrolimus) on the expression of vasoconstrictive substances (endothelin-1) in endothelial cells have been shown, which might further improve hepatic microcirculation (32). Increased levels of ROS are known to be involved in the pathogenesis of IRI. The application of FK506 (tacrolimus) *in vivo* is associated with a reduction of ROS (29). FK506 (tacrolimus) has also been found to exert anti-apoptotic effects by preventing Fas-induced apoptosis in human hepatocytes *in vitro* (33), as well as in an *in vivo* model of IRI in rats (34). A decrease in liver apoptosis may contribute to persisting protection of cellular integrity.

Preliminary clinical data have shown beneficial results of FK506 (tacrolimus) preconditioning in human liver transplantation. In a previous trial, Peter *et al.* demonstrated a significant reduction of aminotransferase levels following the transplantation of normal livers rinsed with 20 ng/ml FK506 (tacrolimus) (35). Although the results of this trial were promising, the clinical impact was limited by the small number of patients included (n=20). However, the study population was relatively small, and, as most patients received healthy organs, the results cannot be directly compared to a study of marginal grafts. Now a prospective randomized multicenter trial to reduce IRI in transplantation of marginal liver grafts with an ex vivo FK506 (tacrolimus) perfusion has been performed in a Germany group (36), but a finalized report of the study is not yet reported. We look to accessing the results of this study.

However, because the mechanism of the IRI is very complex, it is difficult to do prevention and treatment of IRI with only the one agent. It is necessary to use various synthetic agents with different action mechanism for prevention and treatment of IRI.

OKY046 is a specific thromboxane synthetase inhibitor which suppresses the production of Thromboxane A2 (TXA), works as a powerful platelet aggregating agent. The action mechanism of FK506 and OKY046 are completely different. Sasaki K, *et al.* (our colleague) reported that all six (6) rat recipients receiving liver transplantation from non-heart beating donor with sixty (60) minutes warm ischemia survived for fourteen (14) days by pretreatment of FK506 and OKY046 (37). It was clear that these two (2) drugs ameliorated graft viability. And Takeichi T, *et al.* reported that pretreatment with ONO-4057 (Leukotriene B4 receptor antagonist) in combination with tacrolimus produced additive effects in a rat model of liver IRI (38).

Nitric oxide (NO)-based therapy has been applied for many years to patients with pulmonary hypertension cardiopulmonary disorders. The therapeutic application of NO in protecting the liver from IRI has been emerging. A prospective randomized trial with liver transplantation patients has demonstrated that NO inhalation in liver recipients during the perioperative period of liver transplantation significantly protects hepatocytes from apoptotic death, accelerates the restoration of liver graft function, and reduces hospital length of stay (39). However, large amounts of NO may in turn paradoxically damage liver tissue by forming nitrogen peroxide (40). Husser N. reported that low-dose FK506 in combination with aminoguanidine, which leads to a reduction of NO levels, reduced IRI of the graft after liver transplantation in a rat model (41).

There have been various experiments performed regarding whether the drugs are something to suppress hepatic IRI, and SM-SOD (superoxide dismutase by linking styrene co-maleic acid butyl ester) and HSP, that derived from geranylgeranylacetone and there have been reports that these are the effects of suppressing the IRI (42-45).

Because it has been found that various drugs can control IRI in this way, it is important to find the integrated method that can easily have clinical applications.

Recently, Sheu GE *et al.* reported that N2 treatment significantly reduced intestinal injury severity scores after ischemia-reperfusion (I/R) injury in humanized mice, generated by transplanting human lymphocytes into immunodeficient mice. Protection from I/R injury correlated with blockade of human antibody deposition on small intestine (46).

This is a study from the new phase taking to I/R injury of the organ, by all means, also verify that there is an effect of N2 against hepatic I/R injury. We want you to have led to the clinical application as an effective treatment of hepatic I/R injury early.

SUPPORTED

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CONFLICT-OF-INTEREST STATEMENT

The authors report no relevant conflicts of interest.

AUTHOR CONTRIBUTIONS

Toshihiko Sawada practiced these three experiments as substantial person, and brought up a paper the results of these three experiments ; Katsuhiko Inoue and Dairo Tanabe participated these three experiments, and contributed to data collections ; Shunji Kawamoto participated and guided the experiment using rats ; Tatsuya Tsuji participated the warm ischemic experiment using pigs and rats, and guided the data analysis ; Seiki Tashiro participated in the experimental design, the method, the data analysis, and writing the manuscript as general manager. All members agree to accept equal responsibility for accuracy of the contents of this paper.

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