Ambrisentan improves the outcome of rats with liver transplantation partially through reducing nephrotoxicity

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Abstract. – OBJECTIVE: Tacrolimus is a potent immunosuppressive agent mainly used for allogeneic solid organ transplantation. Although usage of tacrolimus led to a significant increase in short-term allograft survival, the long-term morbidity remains high. Endothelin-1 (ET-1) is reported to be associated with increased vascular resistance, CNI-induced nephrotoxicity and chronic rejection.

MATERIALS AND METHODS: In the present study, we first detected the serum and renal ET-1 level of rats treated by tacrolimus and found strong positive correlations were existed between the ET-1 level and the tacrolimus dosage and treated time. Furthermore, we studied the protective effect of ambrisentan in liver transplantation rats when co-administrated with tacrolimus. Healthy inbred male Wistar and Sprague-Dawley (SD) rats were used in this study. The post-operative general condition, transplantation survival time, hepatic aminotransferase, serum IFN-γ and level kidney injury biochemical index were recorded and compared to evaluate the immune response and outcomes in the recipient rats after liver transplantation.

RESULTS: Our results indicate that ambrisentan prevents the changes of ET-1 content in rats of non-operative treatment group and reduced the nephrotoxicity in the rats with liver transplantation. Rats from ambrisentan co-administration group exhibited good postoperative condition and prolonged survival.

CONCLUSIONS: Ambrisentan reverted some effects induced by tacrolimus in the kidney and indicated a positive potential for therapeutic benefit.

Keywords:

Tacrolimus, Ambrisentan, Liver transplantation, Endotheline, Nephrotoxicity.

Introduction

Calcineurin inhibitors (CNIs), which are widely used for immunosuppressive therapy in solid organ transplantation patients, lead to a remarkable increase in allograft short-term survival. The two CNIs utilized in transplantation are cyclosporine and tacrolimus. Tacrolimus is one of the most successful CNIs, the occurrence of negative effects of which seems to be lower than others of its kind. Although widespread adoption of tacrolimus has led to a significant increase in short-term allograft survival, the long-term allograft survival remains high, and improvements in late allograft attrition are not as pronounced as the short-term gains in graft survival[1]. The main side effects of tacrolimus are exhibited on nephrotoxicity, hypertension (HTN), neurotoxicity and so on.

Endothelins (ETs) exist as a family of three separate peptides, ET-1, ET-2, and ET-3, with ET-1 being the predominant subtype present *in vivo*[2]. ET-1 exerts its physiologic effects through the endothelin receptor, which has two distinct subtypes – endothelin receptor A (ETA) and endothelin receptor B (ETB) – with a similar affinity to each receptor[3]. As a vasoconstrictor, endothelin (ET)-1, is suggested to be closely related with the development of CNI-induced nephrotoxicity. After released from endothelial cells, about 80% of ET-1 interacts with endothelin receptor A (ETA). This interaction gives rise to release of intracellular calcium, causing vasoconstriction finally. Furthermore, interaction between ET-1 and ETA receptor in mesangial cells and podocytes, causes glomerulosclerosis, proteinuria and renal dysfunction ultimately[2]. ET-1 increases after isograft implantation in preclinical

Table I. Survival time of rats after liver transplantation.

Group	Number	Survival time (days)	Median survival time (days)	95% CI
A	7	24, 26, 35, 43, >50×3	43	29.13-50.30
B	7	8, 11×2, 12, 14, 16, 18	12	9.72-16.00
C	7	11, 15,16, 19×2, 23, 30	19	13.32-24.68
D	7	16, 22, 23, 27, 33×2, 34	27	20.51-33.21

studies, suggesting that ET-1 enhanced expression might be a natural consequence of transplantation[4]. Expression of endogenous ET-1 is also induced by CNIs treatment[5-8]. This accumulative expression is probably responsible for the progressively renal dysfunction.

ET-1 is studied firstly in pulmonary arterial HTN, and the endothelin receptor antagonists (ERAs) are developed to treat it. Nevertheless, according to the potential contribution of ET-1 to the progression of posttransplantation complications, ERAs are supposed to be used in transplant patients. Ambrisentan, a selective ETA receptor blockade, might play a positive role in transplantation of preventing HTN and renal dysfunction.

Our group of researchers has been focused on liver transplantation for years, including optimizing the therapeutic strategy of posttransplantational patients, especially the renal damages. The present study is aimed to find out that, in a rat model of liver transplantation, whether ET-1 and ETA levels in kidney are affected by Tacrolimus. Furthermore, our study demonstrates that whether Tacrolimus co-administration with Ambrisentan could decrease or prevent the CNI-induced nephrotoxicity without interfering the immunosuppression of graft rejection, to provide new evidences for the treatment of transplant patients.

Materials and Methods

Animals

Male inbred Wistar rats and a closed colony of Sprague Dawley (SD) rats (body weight range from 200 to 250 g) were purchased from the animal facility of the Institute of Radiation Medicine, Chinese Academy of Medical Sciences.

For studying the effect of Tacrolimus dosage on ET-1 and ETA expression

Experiments were completed in 20 male SD rats with age range between 12 and 16 weeks.

Rats were maintained in temperature controlled room under 12-hour dark/light cycles and free access to food (standard laboratory chow) and water. Rats were divided into four groups: a) control (n=5); b) low tacrolimus (Fujisawa Pharmaceutical Co., Ltd., Osaka, Japan) (0.1 mg/kg bw/day for 28 days, n=5) which was daily intramuscularly injected; c) medium dose tacrolimus (0.5 mg/kg bw/day for 28 days, n=5) was daily intramuscularly injected and d) high dose tacrolimus (2.5 mg/kg bw/day for 28 days, n=5) was daily intramuscularly injected. Tacrolimus was dissolved in isotonic saline and was injected in a different leg each day; moreover, the injection site on leg was also changed to avoid muscle damages. Injection volumes were limited to 20-25 μl. The control group only received isotonic saline following the same schedule of tacrolimus-treated groups. Twenty eight days after tacrolimus administration, rats were sacrificed. Kidney, liver tissue and sera were collected for the subsequent assays.

For studying the effect of time course of Tacrolimus treatment and the expression of ET-1 and ETA

These experiments were completed in 25 male SD rats with age range between 12 and 16 weeks. Rats were divided into five groups: a) control (n=5); b) tacrolimus (0.5 mg/kg bw/day for 7 days, n=5) was daily intramuscularly injected for the first week; c) tacrolimus (0.5 mg/kg bw/day for 14 days, n=5) was daily intramuscularly injected for the first two weeks; d) tacrolimus (0.5 mg/kg bw/day for 21 days, n=5) was daily intramuscularly injected for the first 21 days; e) tacrolimus (0.5 mg/kg bw/day for 28 days, n=5) was daily intramuscularly injected for 28 days. Twenty eight days after tacrolimus administration, rats were sacrificed. Kidney, liver tissue and sera were collected for the subsequent assays.

For studying the effect of tacrolimus and ambrisentan co-treatment on the expression of ET-1 and ETA

Fifteen male SD rats with age range between 12 and 16 weeks were divided into three groups: a) control (n=5); b) tacrolimus (0.5 mg/kg bw/day for 28 days, n=5) was daily intramuscularly injected; c) tacrolimus-administered rats which they were co-treated with ambrisentan (0.5 mg/kg bw/day for 28 days, n=5). Twenty eight days after administration, rats were sacrificed. Kidney, liver tissue and sera were collected for the subsequent assays.

For studying the effect of different treatments on the survival time of liver recipient rats

Forty six male SD rats with age range between 7 and 9 weeks were divided into three groups: a) the intragraft transplantation control (n=16), in which the donor liver tissue was from SD rats; b) no-treatment transplantation control in which the donor liver tissue was from Wistar rats; (n=16); c) after transplantation, tacrolimus (0.5 mg/kg bw/day, n=16) was daily intramuscularly injected; d) after transplantation, tacrolimus-administered rats which they were co-treated with ambrisentan (0.5 mg/kg bw/day, n=16). They underwent orthotopic liver transplantation according to a protocol previously described[9]. Three recipient rats from each group were sacrificed on days 1, 3 and 9 post-surgery. Liver tissue and sera were collected for the subsequent assays, and additional fresh liver tissue from each rat was stored in liquid nitrogen. All animals were cared for according to the international guidelines on Animal Care, and ethical approval was obtained from the Ethical Committee at Changzhen Hospital, Second Military Medical University.

Western blotting

Protein extracts were boiled in SDS/β-mercaptoethanol sample buffer, and 30 μg samples were loaded into each lane of 15% polyacrylamide gels. The proteins were separated by electrophoresis, and the proteins in the gels were blotted onto polyvinylidene fluoride (PVDF) membranes (Amersham Pharmacia Biotech, St. Albans, Herts, UK) by electrophoretic transfer. The membrane was incubated with rabbit anti-ET-1 and ET_A type receptor monoclonal antibody (Abcam, Cambridge, MA, USA), mouse anti-β-actin monoclonal antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) for 1 h at 37°C. The specific protein antibody complex was detected by using horseradish peroxidase conjugated goat anti-rabbit or rabbit anti-mouse IgG. Detection by the chemiluminescence reaction was carried using the ECL kit (Pierce, Appleton, WI, USA). The β-actin signal was used as a loading control.

Determination of serum ET-1, ETA and IFN-γ level by ELISA

Serum ET-1, ETA and IFN-γ were determined by enzyme-linked immunosorbent assay (ELISA) kits (Abcam, Cambridge, MA, USA) following the manufacturers' instructions.

Assessment of kidney and liver dysfunction

Blood samples were centrifuged at 3000 g for 10 min at 4°C. To detect signs of liver rejection, serum liver function tests, including aspartate aminotransferase (AST), alanine aminotransferase (ALT) and bilirubin were assessed. For kidney function assessment, serum creatinine (Cr) and urea nitrogen (BUN) were analyzed. Samples from three animals in each group were analyzed.

Statistical analysis

SPSS17.0 software was used for statistical analysis (SPSS Inc., Chicago, IL, USA). The Kaplan-Meier method was utilized for survival analysis. Normally distributed data were analyzed by the least significant difference (LSD) t test for comparison of two samples. $p < 0.05$ was considered to indicate statistical significance.

Results

The correlation between tacrolimus treatment and the expression of ET-1 and ETA

Tacrolimus is a macrolide with potent immunosuppressive properties and is a good therapeutic alternative to cyclosporine-A (CsA) with low graft rejection rates. The drug inhibits the mixed lymphocyte reaction and the formation of interleukin-2 by T lymphocytes. It is highly efficacious, safe, and well tolerated but is associated with significant dose related toxicity such as neurotoxicity, nephrotoxicity, glucose intolerance, gastrointestinal toxicity, posttransplant lymphoproliferative disease, and infections[10,11]. In the present study, our work focused on studying the
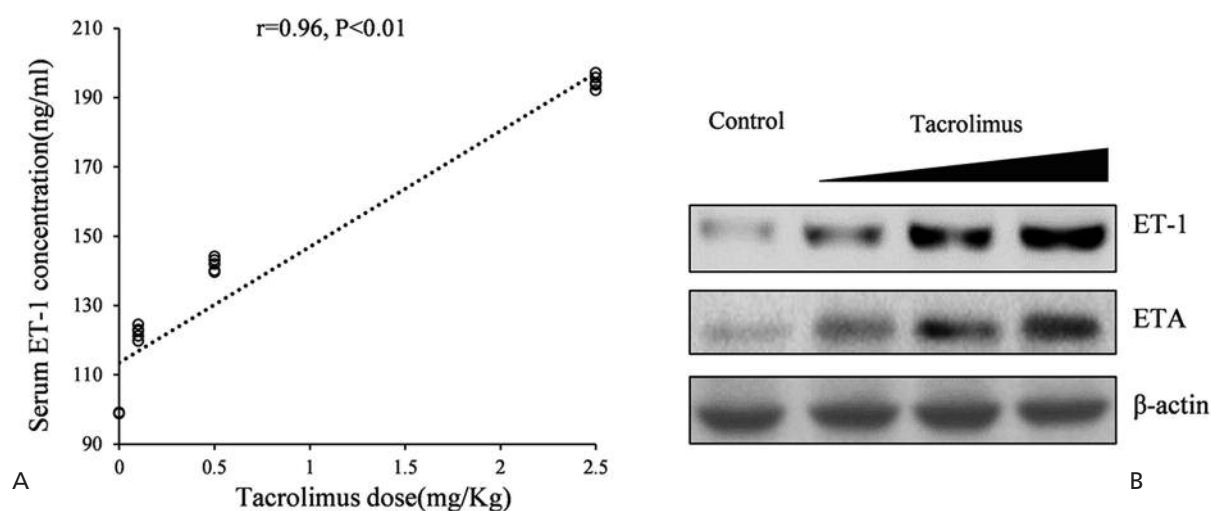
Figure 1. The correlation between ET-1 expression and tacrolimus dosage. Rats were divided into four groups: control (n=5); low tacrolimus (0.1 mg/kg bw/day for 28 days, n=5) which was daily intramuscularly injected; medium dose tacrolimus (0.5 mg/kg bw/day for 28 days, n=5) was daily intramuscularly injected and d) high dose tacrolimus (2.5 mg/kg bw/day for 28 days, n=5) was daily intramuscularly injected. The serum ET-1 level was detected by ELISA (**A**), and renal ET-1, ETA expression was detected using western blot (**B**).

nephrotoxicity coursed by tacrolimus and how to reduce the nephrotoxicity. We first detected the ET-1 and ETA expression in tacrolimus treated rats. As shown in Figure 1A, there is a positive correlation between serum ET-1 levels and the tacrolimus dose in 28 days treated rats (r = 0.96, $p < 0.01$). Furthermore, we detected the expression of ET-1 and ETA in the renal tissues. As Figure 1B shows, the renal ET-1 and ETA expressions are increased with the increase of tacrolimus dosage. Meanwhile, we detected the serum ET-1 levels of rats that were treated with tacrolimus for different times. As shown in Figure 2A, the serum ET-1 level has a positive correlation with tacrolimus treated times (r = 0.99, $p < 0.01$). Meanwhile, the renal ET-1 and ETA expressions are also increased with the increase of tacrolimus (Figure 2B).

Ambrisentan reduced tacrolimus toxicity in rats

To detect the protection effect of ambrisentan, we treated rats with tacrolimus combined with ambrisentan. As shown in Figure 3A, the expression of ET-1 and ET-A was up-regulated 28 days after tacrolimus administration compared with control rats. Ambrisentan prevented the increased ET-1 and ET-A level induced by tacrolimus. Serum ET-1 and ET-A levels were also increased by tacrolimus with respect to control group ($p < 0.01$) (Figure 3B). Ambrisentan co-administration

can significantly prevent the increased serum ET-1 content induced by tacrolimus ($p < 0.05$).

Postoperative condition and survival time

The recipient rats in Group B began losing body weight on day 3 after liver transplantation, and continued to deteriorate on day 6, when the animals appeared subdued, with disheveled and shedding coats. Subcutaneous hemorrhage was observed around the mouth and eyes in some rats. Successive mortality was observed from day 8. The rats in Groups A, C and D also appeared subdued and lost bodyweight in the first 6 days after liver transplantation, although the general condition and appetite of these animals improved subsequently, and their body weight also increased after 2 weeks. Our findings suggested that the survival rate of the ambrisentan co-administration group following allograft liver transplantation was more similarto that of the intragraft control group compared with only tacrolimus treatment group ($p = 0.036$) (Figure 4).

Hepatic and renal dysfunction after liver tranplantation

Serum liver function tests (AST and ALT) were increased after day three in the allogeneic recipients ($p < 0.05$ vs. syngeneic controls, Figure 5A). Bilirubin level was increased after 9 days in the allogeneic recipients ($p < 0.05$ vs. syngeneic con-
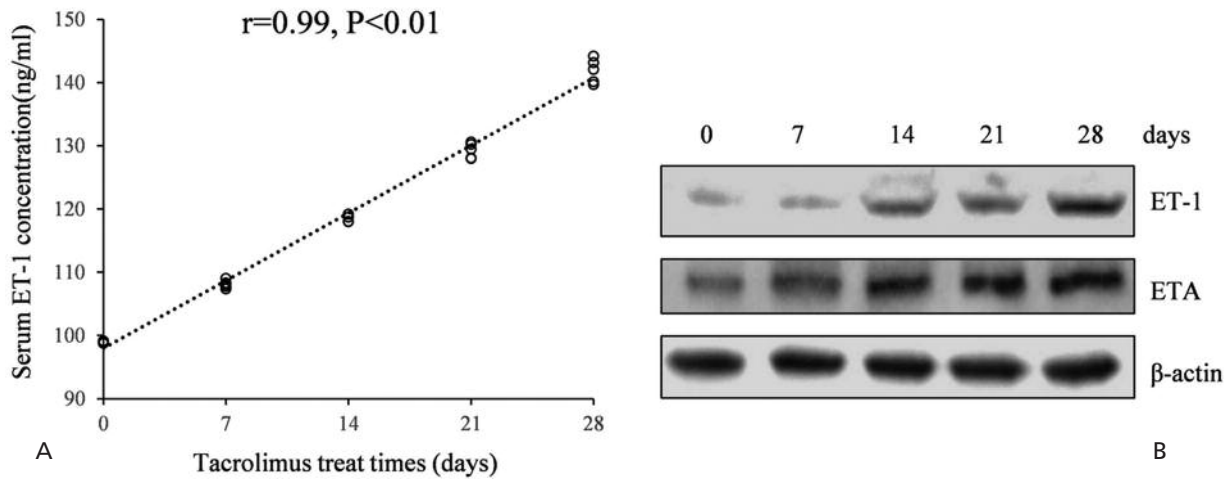
A

B

Figure 2. The correlation between ET-1 expression and tacrolimus treated time. Rats were divided into five groups: control (n=5); tacrolimus (0.5 mg/kg bw/day for 7 days, n=5) was daily intramuscularly injected for the first week; tacrolimus (0.5 mg/kg bw/day for 14 days, n=5) was daily intramuscularly injected for the first two weeks; tacrolimus (0.5 mg/kg bw/day for 21 days, n=5) was daily intramuscularly injected for the first 21 days; tacrolimus (0.5 mg/kg bw/day for 28 days, n=5) was daily intramuscularly injected for 28 days. Rats were sacrificed in day 28. Serum ET-1 level was detected by ELISA (**A**) and renal ET-1, ETA expression were detected by western blot (**B**).

trols, Figure 1B). However, there are no significant difference between tacrolimus group and ambrisentan co-administration group in the serum AST, ALT and bilirubin concentration.

Serum IFN-γ level was determined to evaluate the overall immune status of rats after liver trans-

plantation. Serum IFN-γ levels were higher in the allogeneic group and gradually increased with the time extended. However, there are no significant difference between syngeneic, tacrolimus treated allogeneic and ambrisentan co-administration group in the serum IFN-γ levels (Figure 5C).
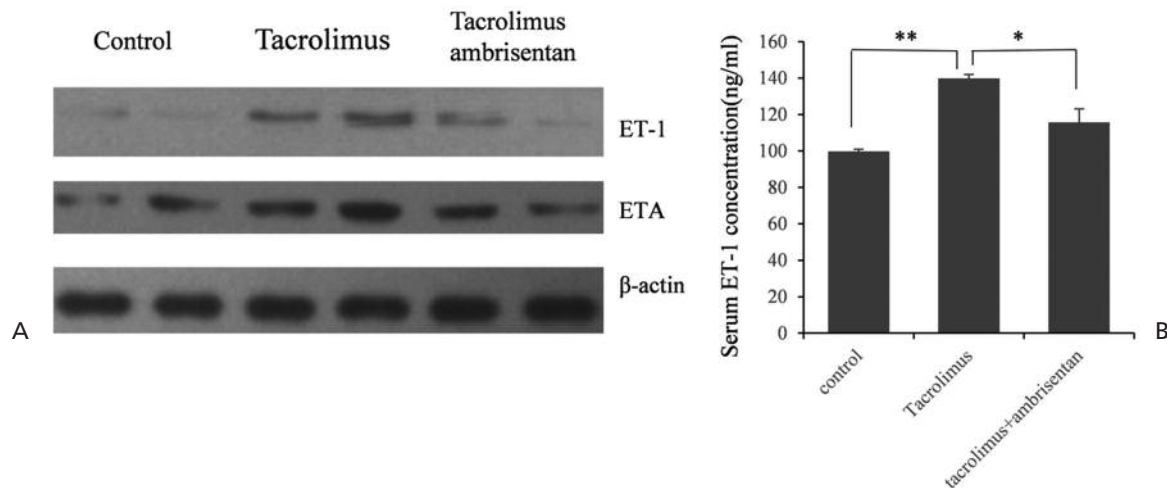

A

B

Figure 3. The effect of ambrisentan co-treatment with tacrolimus on the expression of ET-1 and ETA. Fifteen male SD rats with age range between 12 and 16 weeks were divided into three groups: control (n=5); tacrolimus (0.5 mg/kg bw/day for 28 days, n=5) was daily intramuscularly injected; tacrolimus-administered rats which they were co-treated with ambrisentan (0.5 mg/kg bw/day for 28 days, n=5). Twenty eight days after administration, rats were sacrificed. Kidney, liver tissue and sera were collected for the subsequent assays.
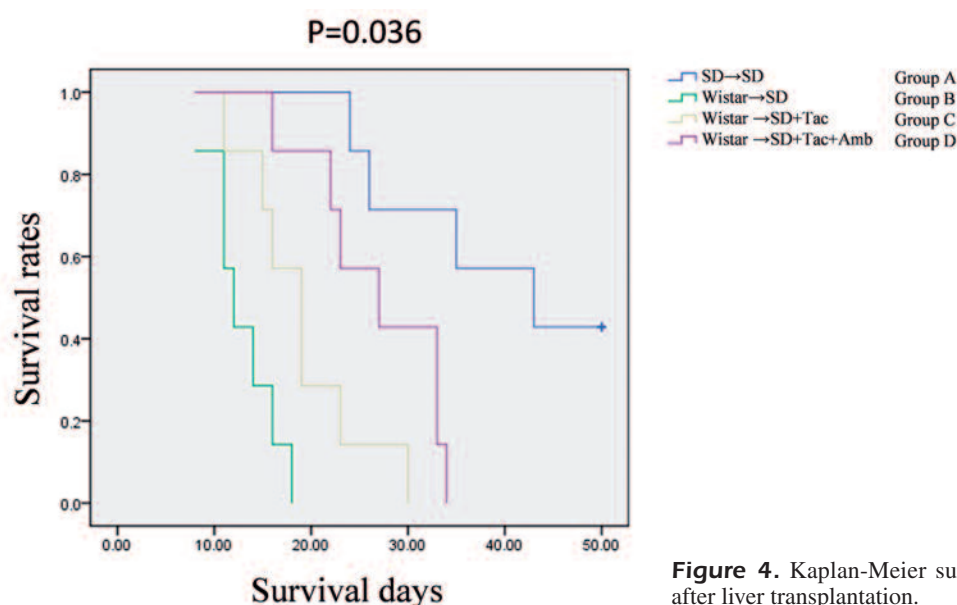
Figure 4. Kaplan-Meier survival curves of each group after liver transplantation.

Serum creatinine and blood urea nitrogen (BUN) levels were detected for presenting the kidney function. As shown in Figure 6A, the serum creatinine level of tacrolimus group was significantly higher in than the ambrisentan co-administration group in the 6 and 9 days post-transplantation. Meanwhile, the BUN level in the ambrisentan co-administration group is lower compared with tacrolimus groups in the ninth day.

As expected, serum ET-1 concentration also presented a higher level in the single tacrolimus treated group in the ninth day post transplantation (Figure 6C).

Discussion

The present work shows that the serum and renal ET-1 level has a positive correlation with the dosage and time of tacrolimus treatment in rats. The ET-A expression in renal was also increased with the increase of tacrolimus dosage or the time extension of tacrolimus treatment. Ambrisentan, an ETA specific antagonist, co-administered with tacrolimus prevented the changes in the serum and renal ET-1 levels. Furthermore, ambrisentan co-administration reduced the nephrotoxicity of tacrolimus accompanied by protected hepatic function.

Tacrolimus is a widespread used calcineurininhibitors (CNIs) in the clinical immunosuppressant therapy. Although usage of tacrolimus led to a significant increase in short-term allograft sur-vival, the long-term morbidity remains high. In this study, ambrisentan co-administration group rats have an increased survival rate, indicates a very fine application prospect for ambrisentan. There reports indicated that ambrisentan is an attractive ERA option in transplant patients because it's minimal effect on hepatic aminotransferase[12]. In this study, AST and ALT levels were nearly the same in the tacrolimus group and ambrisentan co-administrated group rats. These results proved the fact that ambrisentan is minimal hepatotoxicity ERA.

Although tacrolimus co-administration with ambrisentan can reduce nephrotoxicity and increase survival rate of liver transplanted rats, the condition might be more complex in the front of diverse genetic background patients. So this potential for therapeutic benefit must be carefully weighed against the small but significant possibility of toxicity related to ERA therapy in these complex patients.

Conclusions

This study showed that tacrolimus treatment increased the ET-1 level in serum and kidney which associated with nephrotoxicity, organ rejection and poor outcome of solid organ transplantation. Ambrisentan co-administration reduced nephrotoxicity and increase survival rate of liver transplanted rats indicates a positive potential for therapeutic benefit.
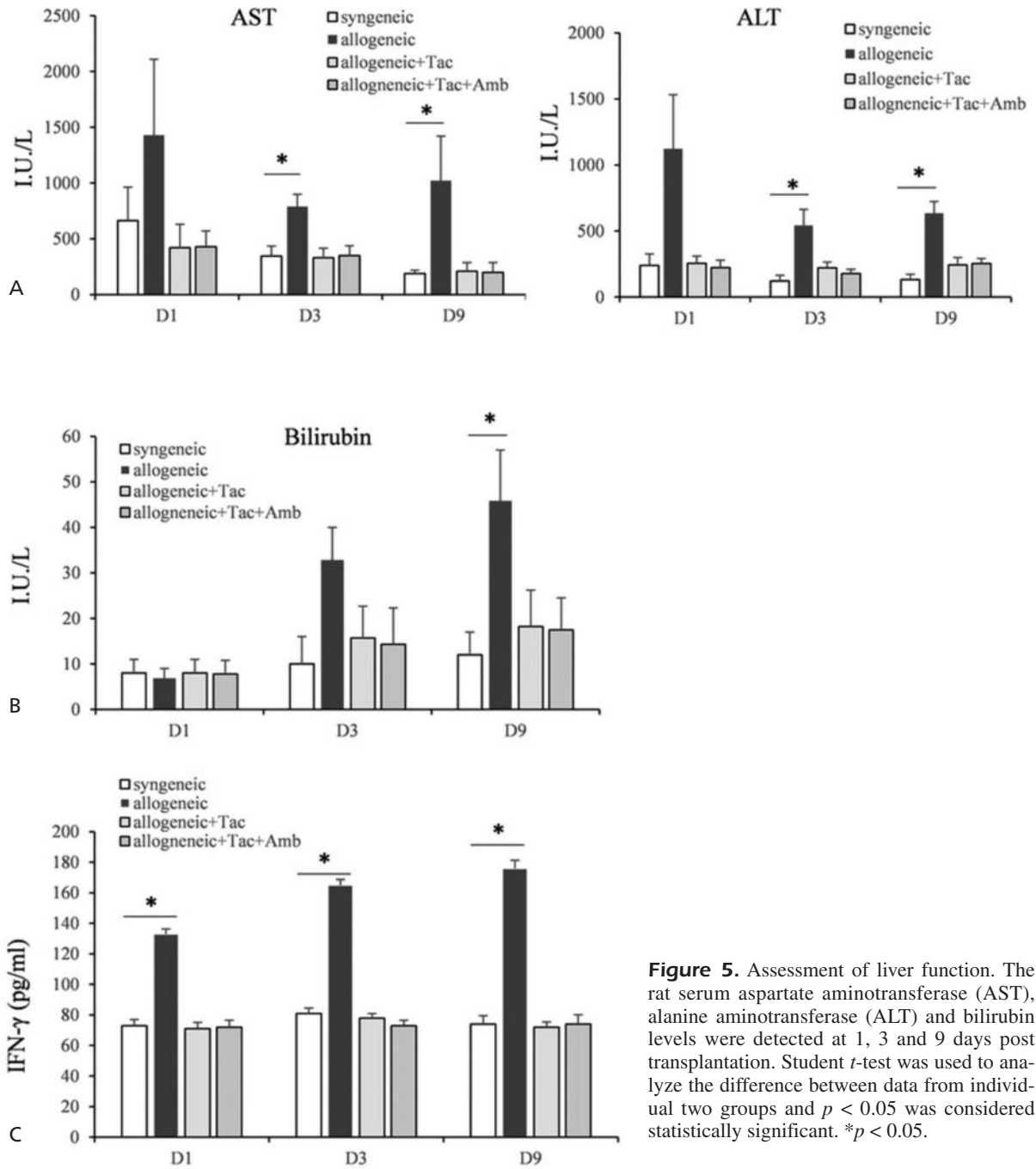
Figure 5. Assessment of liver function. The rat serum aspartate aminotransferase (AST), alanine aminotransferase (ALT) and bilirubin levels were detected at 1, 3 and 9 days post transplantation. Student *t*-test was used to analyze the difference between data from individual two groups and $p < 0.05$ was considered statistically significant. *$p < 0.05$.

Conflict of interest

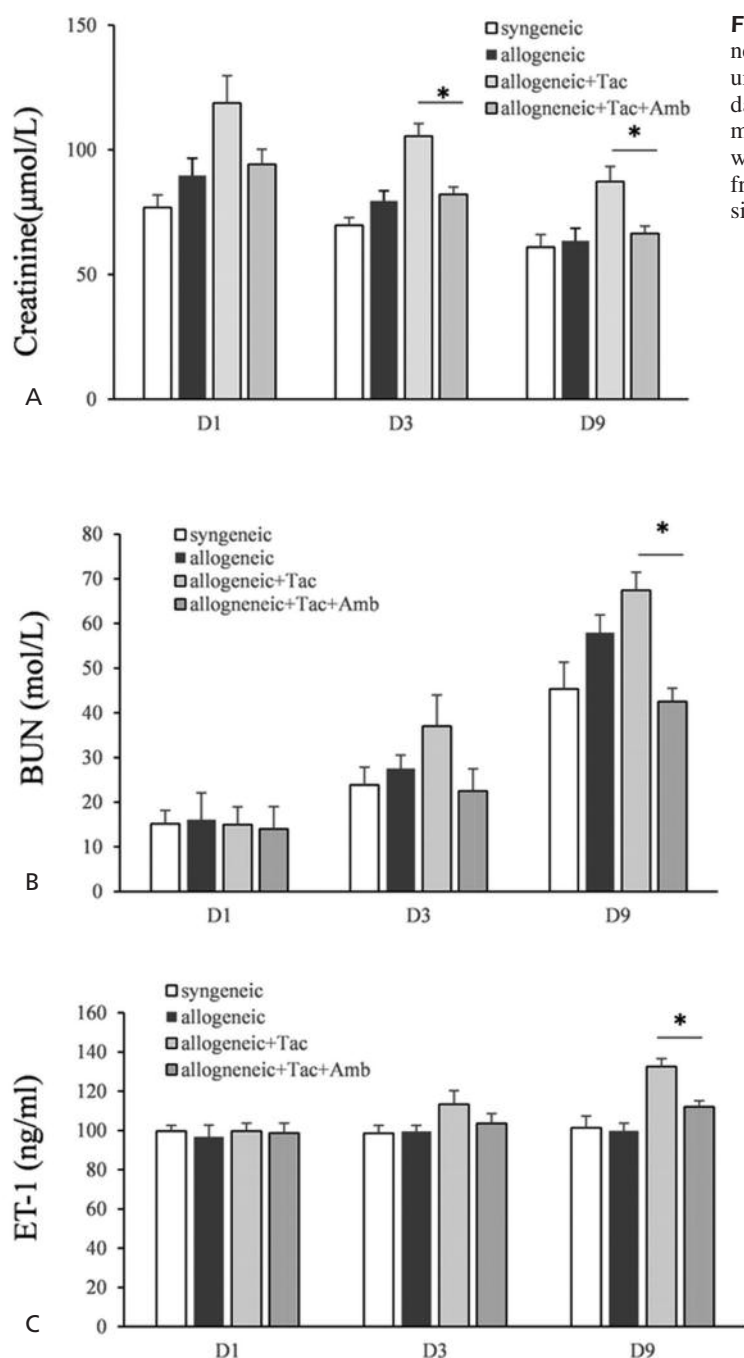The authors have declared no conflict of interest.

Figure 6. Assessment of kidney function. For kidney function assessment, serum creatinine (Cr) and urea nitrogen (BUN) were analyzed at 1, 3 and 9 days post transplantation. Samples from three animals in each group were analyzed. Student t-test was used to analyze the difference between data from individual two groups and $p < 0.05$ was considered statistically significant. *$p < 0.05$.

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