

• BRIEF REPORTS •

Resveratrol prolongs allograft survival after liver transplantation in rats

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Abstract

AIM: To study the immuno-modulatory effect of resveratrol (RES) on allograft rejection after liver transplantation in rats.

METHODS: Male Sprague-Dawley (SD) rats were selected as donors and male Wistar rats as recipients for a rejection model. The recipients were divided into four groups after orthotopic liver transplantation (OLTx). In the RES A, B, and C groups, RES was given intra-peritoneally once a day (25, 50, and 100 mg/kg, respectively) after OLTx, whereas in the control group, vehicle buffer was given intra-peritoneally once a day. The survival time, serum chemistry, production of cytokines, activation of transcription factor NF- κ B, and histopathologic findings were then compared among these groups.

RESULTS: The mean survival time after OLTx in the RES C group was significantly longer than that in the control group (16.7 ± 1.2 d vs 9.3 ± 0.6 d, $P < 0.01$). On the 7th post-transplant day the serum albumin level significantly improved in the RES C group, the serum total bile acid and alanine aminotransferase (ALT) levels were significantly lower in the RES C group, the serum IL-2 and INF- γ levels were significantly lower in the RES C group, and the activation of transcription factor NF- κ B in peripheral blood T lymphocytes was significantly suppressed in the RES A, B, and C groups in comparison to those in the control group. On the 7th post-transplant day, a histological examination revealed apparent difference in the severity of rejection between the RES C group and control group.

CONCLUSION: RES has an immuno-suppressive property as well as protective effect on hepatocytes under allograft rejection. It might serve as a novel agent for reducing the severity of hepatic allograft rejection in rats.

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Key words: Resveratrol; Liver; Transplantation; Rat

INTRODUCTION

Since the introduction of cyclosporine and tacrolimus for the control of post-operative rejection, liver transplantation has become an established surgical technique and is now performed on patients with various terminal liver diseases. Most instances of mortality after liver transplantation are still attributed to infection, and such infection is apt to occur as a result of over-immunosuppression to severe rejection^[1-3]. Rejection is still a leading cause of morbidity and mortality after liver transplantation. To improve the survival after liver transplantation, novel strategies are still needed for the treatment of the rejection.

Resveratrol (3,5,4'-trihydroxystilbene, RES) is a polyphenol present in a limited number of plants^[4], mainly in grapes with levels up to tens of grams per kg^[5], where it is synthesized in response to stress conditions such as fungal infections and trauma^[4]. Many studies have demonstrated that this molecule exhibits a wide range of biological and pharmacological activities both *in vitro* and *in vivo*^[6]. A series of studies showed that resveratrol has anti-oxidant properties^[7], anti-inflammatory properties^[8,9], and cancer-chemopreventive activity^[10,11]. Many of the biological activities of resveratrol, like the inhibition of cyclooxygenase^[8], induction of CD95 signaling-dependent apoptosis^[12], effects on the cell division cycle^[13] and modulation of NF- κ B activation^[14], indicate a possible effect on immune response and many *in vitro* experiments have proven that resveratrol has immuno-modulatory activity^[15-17].

To our knowledge, there has not been a report on the *in vivo* immuno-modulatory effect of RES on hepatic allograft after transplantation. We therefore investigated whether RES might have any beneficial effect on hepatic allograft after transplantation.

MATERIALS AND METHODS

Materials

Male Sprague-Dawley (SD) rats 9-10 wk old weighing 190-210 g as donors and male Wistar rats 7-8 wk old weighing 190-210 g as recipients were purchased from the Animal Center of Xi'an Jiaotong University (Xi'an, China) and Animal Center of Shanxi Medical College (Shanxi, China), respectively. All rats were allowed free access

to water and standard laboratory chow. Before operation the rats were fasted for 12 h and only allowed free access to water. All animal protocols were approved by the Xi'an Jiaotong University Institutional Animal Care and Use Committee.

Resveratrol, dimethyl sulfoxide (DMSO), and IL-2 and INF- γ ELISA kits were purchased from Sigma Chemical Co. RPMI-1640, HEPES, EDTA, EGTA, DTT, PMSF, and NP-40 were from Gibco BRL. NF- κ B consensus oligonucleotide and single base pair mutant were from Promega. Fetal bovine serum was from SiJiQing Co., Hangzhou, China. 32 P-ATP was from Beijing Isotope Co., China.

Orthotopic liver transplantation

Orthotopic rat liver transplantation (OLTx) was performed by the cuff technique as described by Kamada and Calne^[18], with some slight modifications. With the rat under ketamine anesthesia (75 mg/kg), the liver was gently skeletonized and flushed with chilled lactated Ringer's solution through the abdominal aorta. Special care was taken for minimal manipulation of the graft and portal vein and bile duct for reconstruction. The liver was harvested and stored at 4 °C in lactated Ringer's solution until transplantation. OLTx was performed without hepatic artery reconstruction. The suprahepatic vena cava was anastomosed with 7-0 Prolene continuous suture (Ethicon, Somerville, NJ) and portal vein and inferior vena cava reconstruction was performed by the cuff technique. The bile duct connection was made with an intra-luminal epidural catheter stent. In rat liver transplantation, the cold ischemic time and anhepatic phase were 40-50 min and 14-16 min, respectively, and no significant difference was recognized among these groups.

RES administration and graft survival

The RES was dissolved and sterilized in DMSO and then diluted in RPMI-1640 to 5, 10, and 20 mg/mL. The recipients were randomly divided into four groups after OLTx. In the RES A, B, and C groups, 1 mL of these preparations was administered by intra-peritoneal route once a day after OLTx (25, 50, and 100 mg/kg, respectively) and in the control group, vehicle buffer was given by intraperitoneal route once a day after OLTx.

Six rats were left in each group until they died. The rats used to evaluate graft survival were given RES or vehicle buffer until they died.

Liver function test and ELISA

Six animals in each group were killed on the 7th posttransplant day for blood collection. A 6-mL blood sample was obtained from the vena cava. Two milliliters blood was centrifuged immediately at 3 000 r/min at 4 °C for 10 min and stored at -80 °C until analysis. Albumin, total bile acid, and alanine aminotransferase (ALT) were assayed by standard enzymatic methods, while serum IL-2 and INF- γ levels were assayed by ELISA.

EMSA

The remaining 4 mL of blood was used for the detection of NF- κ B activation in peripheral blood T lymphocytes. First, the peripheral blood mononuclear cells were separated

by standard Ficoll-Hypaque gradient centrifugation, and then incubated in RPMI-1640 culture medium containing 10% calf serum. The cell suspension was separated from the adherent cells the next day and T-lymphocyte subpopulation was obtained by magnetic separation column according to the instructions of the manufacturer (Miltenyi Biotec, Germany). The purity of the cell population was confirmed by FACScan analysis and cell viability was determined by trypan blue dye exclusion.

Then T-lymphocyte nuclear extracts were prepared by the modified procedure of Dignam *et al.*^[19]. Following treatment, cells were washed thrice with PBS, resuspended, and incubated on ice for 15 min in hypotonic buffer A (10 mmol/L HEPES, pH 7.9, 10 mmol/L KCl, 0.1 mmol/L EDTA, 0.1 mmol/L EGTA, 1 mmol/L DTT, 0.5 mmol/L PMSF, and 0.6% NP-40). Cells were vortexed gently for lysis, and nuclei were separated from cytosolic components by centrifugation at 12 000 *g* for 1 min at 25 °C. Nuclei were resuspended in buffer C (20 mmol/L HEPES, pH 7.9, 25% glycerol, 0.4 mol/L NaCl, 1 mmol/L EDTA, 1 mmol/L EGTA, 1 mmol/L DTT, 0.5 mmol/L PMSF) and shaken for 30 min at 4 °C. Nuclear extracts were obtained by centrifugation at 12 000 *g* for 10 min at 25 °C. Protein concentration was measured by the Bradford assay (Bio-Rad).

For binding reactions, nuclear extracts (10 μ g of protein) were incubated in a 25- μ L total reaction volume containing 20 μ mol/L HEPES, pH 7.9, 60 mmol/L NaCl, 0.1 mmol/L EDTA, 1 mmol/L DDT, 8% glycerol, and 2.55 μ g/mL of poly (dl-dC, Pharmacia). Double-stranded radiolabeled NF- κ B oligonucleotide probe (5'-CGCTT-GATGAGTCAGC-CGGAA-3') was added to the mixture after pre-incubation for 15 min at 4 °C, and the reaction mixture was then incubated for 20 min at room temperature. Samples were loaded on 6% polyacrylamide gels in low-ionic-strength 0.25 \times TBE buffer (22.3 mmol/L Tris, 22.2 mmol/L borate, 0.5 mmol/L EDTA) and run at 150 V/cm with cooling. The gels were dried and analyzed by autoradiography.

Histopathologic examination

Liver specimens were collected from five animals in each group on the 7th post-transplant day and fixed in 10% neutral buffered formalin. Then, the specimens were embedded in paraffin and 3 μ m thick sections were cut and stained with hematoxylin and eosin (H&E). A single blinded pathologist examined the liver graft specimens.

Statistical analysis

All data were expressed as mean \pm SD, and Student's *t*-test was used to evaluate the significance of the difference between experimental groups and control group. The survival time were compared according to the Kaplan-Meier and log rank analysis. *P*<0.05 was considered statistically significant.

RESULTS

Graft survival

The mean survival time was 8.3 \pm 0.6, 11.7 \pm 1.5, and 16.7 \pm 1.2 d in the RES A, B, and C groups, respectively,

and 9.3 ± 0.6 d in the control group. The difference between the RES C group and control group was statistically significant ($P < 0.01$). The survival rates in the four groups are shown in Figure 1.

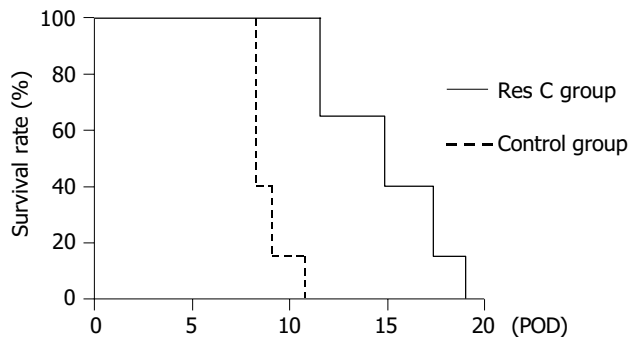


Figure 1 Survival rate of rats after liver transplantation.

Liver function tests

Table 1 shows the serum chemistry data that reflected the liver functions after OLTx. On the 7th posttransplant day the albumin level was 2.0 ± 0.1 , 2.2 ± 0.1 , and 2.7 ± 0.2 g/dL in the RES A, B, and C groups, respectively, whereas it was 2.1 ± 0.3 g/dL in the control group. The difference between the RES C group and control group was statistically significant ($P < 0.05$). The total bile acid was 314.7 ± 97.5 , 270.4 ± 93.7 , and 155.4 ± 34.2 μ mol/L in the RES A, B, and C groups respectively, whereas it was 353.9 ± 84.4 μ mol/L in the control group. The difference between the RES C group and the control group was significant ($P < 0.05$). ALT was 1112.1 ± 159.4 , 806.5 ± 135.2 , and 482.7 ± 101.2 U/L in the RES A, B, and C groups respectively, whereas it was 1137.5 ± 235.4 U/L in the control group. The difference between the RES C group and the control group was significant ($P < 0.05$).

Table 1 Effect of RES on liver function in rats after OLTx (mean \pm SD)

Group	Dose (mg/kg)	n	Albumin level (g/dL)	Total bile acid (μ mol/L)	ALT (U/L)
Control	0.0	6	2.1 ± 0.3	353.9 ± 84.41	1137.5 ± 235.4
RES A	25.0	6	2.0 ± 0.1	314.7 ± 97.5	1112.1 ± 159.4
RES B	50.0	6	2.2 ± 0.1	270.4 ± 93.7	806.5 ± 135.2
RES C	100.0	6	2.7 ± 0.2^a	155.4 ± 34.2^a	482.7 ± 101.2^a

^a $P < 0.05$ vs control.

ELISA

The IL-2 level was 305.1 ± 82.8 , 241.7 ± 38.1 , and 195.7 ± 42.4 ng/L in the RES A, B, and C groups respectively, whereas it was 294.4 ± 38.0 ng/L in the control group. The difference between the RES C group and the control group was significant ($P < 0.05$). The INF- γ level was 96.5 ± 6.5 , 82.8 ± 14.9 , and 66.6 ± 15.7 ng/L in the RES A, B, and C groups respectively, whereas it was 101.3 ± 14.1 ng/L in the control group. The difference between the RES C group and the control group was

significant ($P < 0.05$). The serum cytokine levels on the 7th post-transplant day in the four groups are shown in Table 2.

Table 2 Effect of RES on IL-2 and INF- γ production in rats after OLTx (mean \pm SD)

Group	Dose (mg/kg)	n	IL-2 (ng/L)	INF- γ (ng/L)
Control	0.0	6	294.4 ± 38.0	101.3 ± 14.1
RES A	25.0	6	305.1 ± 82.8	96.5 ± 6.5
RES B	50.0	6	241.7 ± 38.1	82.8 ± 14.9
RES C	100.0	6	195.7 ± 42.4^a	66.6 ± 15.7^a

^a $P < 0.05$ vs control.

Activation of NF- κ B

The activation of NF- κ B DNA binding activity in peripheral blood T lymphocytes was 57.00 ± 3.00 , 52.33 ± 2.08 , and 41.67 ± 1.53 U in the RES A, B, and C groups respectively, whereas it was 100.33 ± 7.57 U in the control group, being significantly lower in the RES A, B, and C groups than in the control group ($P < 0.05$). Figure 2 demonstrates the measurable NF- κ B DNA binding activity in the four groups on the 7th post-transplant day.

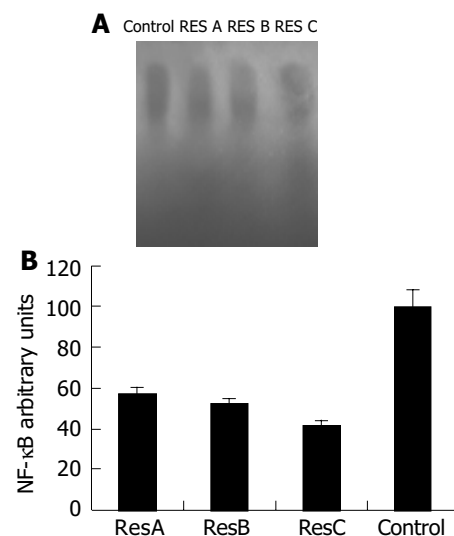


Figure 2 Levels of NF- κ B in peripheral T lymphocytes analyzed by EMSA (A) AND quantitation of NF- κ B activity by PhosphorImager (B).

Histopathologic examination

All the four groups showed the typical signs of severe graft rejection with intense portal infiltrate. There was an apparent difference in the severity of rejection between the RES C group (Banff score 4.3 ± 1.5) and control group (Banff score 7.3 ± 0.6) on the basis of Banff schema by a blinded pathologist ($P < 0.05$). Figure 3 shows the H&E staining of histologic sections in the RES C group and control group on the 7th posttransplant day.

DISCUSSION

The immuno-modulatory effect of resveratrol has been reported

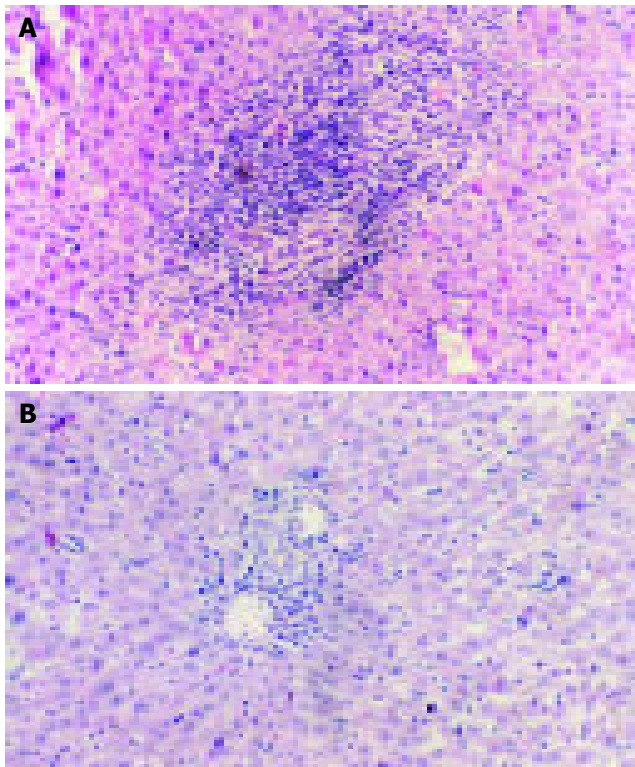


Figure 3 Typical signs of severe graft rejection in control group (A) and RES C group (B).

with use of mouse splenic lymphocytes, lymphokine activated killer (LAK) cells, mouse macrophage-like cell line RAW 264.7, and human peripheral blood T lymphocytes^[15,20-23]. Gao *et al.*^[15], reported that RES inhibits splenic lymphocyte proliferation, induction of cytotoxic T lymphocytes, and cytokine production, at least in part through the inhibition of NF- κ B activation. Yu *et al.*^[20], reported that RES can suppress notably the proliferation and transformation of human lymphocytes and the combination of resveratrol at a given concentration with cyclosporine-A can enhance immune suppression. Although most of these studies focused on the immunosuppressive effects of RES, a few experiments showed that low dose RES could enhance cell-mediated immune response. Feng *et al.*^[23], reported that RES (4 mg/kg, ig) promotes DTH response of mouse. However, the detailed mechanisms of the bi-phasic modulatory effects of RES remain to be studied. Our study revealed that RES could downregulate NF- κ B activation of peripheral T lymphocytes and IL-2 and INF- γ serum levels and decrease portal infiltrate of T lymphocytes in rats with severe rejection. RES has also been shown to promote albumin synthesis and prevent elevation of total bile acid and ALT and prolong the survival time of rats after liver transplantation.

As rejection occurs in the graft, hepatocytes are destroyed by infiltrating T lymphocytes. Hepatic function deteriorates as the number of hepatocytes decreases, and rejection eventually kills the recipient unless immuno-suppressants are given. In this hepatic allograft rejection model, RES decreased the number of accumulated mononuclear cells around the Glissonian triad in the H&E stained section on the 7th posttransplant day. RES may influence lymphocytes in the

hepatic allograft because of its influence on NF- κ B of T lymphocytes^[24].

Transcription factor NF- κ B, and other members of the Rel homology family of transcription factors play a pivotal role in the transcription of genes involved in immune and inflammatory responses^[25], and in cell proliferation and transformation^[26,27]. It is possible that suppression of lymphocyte infiltration and cytokine production by resveratrol may result from suppression of NF- κ B activation. In resting cells, NF- κ B remains sequestered in the cytoplasm in a functionally inactive form, non-covalently bound to an inhibitory protein, I κ B. Upon stimulation of cells with mitogens, antigens, or cytokines, I κ B dissociates from the NF- κ B complex, allowing NF- κ B to translocate to the nucleus where it binds to κ B motifs in the promoter region of the response genes. In our study, peripheral T lymphocytes expressed high levels of activated NF- κ B. However, after administration of RES, the NF- κ B activation due to the stimulation of heterogeneous antigens was blocked partly. These results are consistent with those of other investigators^[14,28].

It has been generally accepted that the immune response leading to graft rejection is accompanied with an increase of cytokine production by the primed T cells. Cytokines are essential for the differentiation, proliferation and amplification of the T cells^[29]. The most important cytokine is IL-2, which is essential for activated T cell proliferation^[30,31], and INF- γ is mainly secreted by activated T cells^[32] and induces MHC class I antigen expression in several kinds of cells such as lymphocytes^[33], myocytes^[34], endothelial cells^[35] and fibroblasts^[36]. Some studies have shown that the gene expression of IL-2 and INF- γ by intra-graft is specific to acute rejection, which precedes histopathologic manifestations in liver transplantation^[37,38]. We also investigated the effect of resveratrol on the production of INF- γ and IL-2, and found that RES could suppress the serum IL-2 and INF- γ levels in rats after liver transplantation when it was administered at a dosage of 100 mg/kg body weight. The results are consistent with other reports^[15,22].

In conclusion, resveratrol downregulates the serum IL-2 and INF- γ levels, decreases the lymphocyte infiltration in allograft liver, prolongs the mean survival time after OLTx. Suppression of the activation of transcription factor NF- κ B in peripheral T lymphocytes appears to be a part of the mechanism by which resveratrol inhibits the *in vivo* development of immunological responses.

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