

Protective effect of resveratrol and vitamin E against ethanol-induced oxidative damage in mice: Biochemical and immunological basis

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Received 06 June 2009; revised 09 October 2009

The metabolism of ethanol gives rise to the generation of excess amounts of reactive oxygen species and is also associated with immune dysfunction. We examined the efficacy of resveratrol and vitamin E on the immunomodulatory activity and vascular function in mice with liver abnormalities induced by chronic ethanol consumption by measuring the protein, liver-specific transaminase enzymes, antioxidant enzymes and non-enzymes such as reduced glutathione (GSH) content, thiobarbituric acid reactive substance (TBARS) level, nitrite level, and activities of superoxide dismutase (SOD), catalase (CAT), glutathione reductase (GR) and glutathione peroxidase (GPx) and glutathione-S-transferase (GST), and cytokines such as interleukin (IL)-2, IL-4, IL-10, tumor necrosis factor (TNF)- α , gamma interferon (IFN- γ), vascular endothelial growth factor (VEGF)-A and transforming growth factor (TGF)- β 1 in mice blood. Ethanol (1.6 g/kg body wt/day) exposure for 12 wks significantly increased TBARS and nitrite levels and GST activity, and significantly decreased GSH content and the activities of SOD, CAT, GR and GPx in whole blood hemolyzate of 8-10 wks-old male BALB/c mice (weighing 20-30 g). Ethanol exposure also elevated the activities of transaminase enzymes (AST and ALT), IL-10, TNF- α , IFN- γ , VEGF-A and TGF- β 1, while decreasing the albumin concentration and IL-4 activity in the serum. Both resveratrol (5 mg kg⁻¹ day⁻¹) and vitamin E (80 mg kg⁻¹ day⁻¹) treatment significantly reduced AST, ALT, GST, IL-10, TNF- α , IFN- γ , VEGF-A and TGF- β 1 activities and levels of TBARS and nitrite, and elevated albumin content, GSH level and activities of SOD, CAT, GR and GPx, compared to ethanol-treated group. Thus, results from the study demonstrated that both resveratrol (5 mg kg⁻¹ day⁻¹) and vitamin E (80 mg kg⁻¹ day⁻¹) can effectively ameliorate ethanol (1.6 g kg⁻¹ day⁻¹)-induced oxidative challenges, immunomodulatory activity and angiogenesis processes.

Keywords: Cytokines, Ethanol, Oxidative stress, Resveratrol, Vitamin E.

Ethanol is related to more than 60 medical conditions and overall, 3.5% of the global burden of diseases is attributable to alcohol¹. Acute as well as chronic toxic effects of ethanol may result in irreversible organ damage². Following ingestion, alcohol is rapidly absorbed by the gastric and small intestinal mucosa and is metabolized primarily in the liver³. The metabolism of ethanol produces reactive oxygen

species (ROS), detrimental to the cellular antioxidant defense system⁴. The ROS cause oxidation of cellular lipids, proteins or DNA, and finally leading to cell injury⁵. Tissue repair encompassing regeneration of hepatic extracellular matrix and angiogenesis plays a critical role in restoring the structure and function of the liver. This complex process is governed by intricate cellular signaling, involving a number of chemokines, cytokines and growth factors⁶.

Despite understanding in the pathogenesis of alcohol-induced organ damage, current therapies for this are not effective. There is increasing evidence that oxidative stress plays an important role in the development of alcoholic liver disease (ALD)^{4,7}. Numerous interventions have been found to counteract the vulnerability of the oxidative challenges during alcohol consumption⁷. Based on the hypothesis that oxidative stress occurs only when the

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Abbreviations: ALD, alcoholic liver disease; ALT, alanine transaminase; AST, aspartate transaminase; CAT, catalase; GPx, glutathione peroxidase; GR: glutathione reductase, GSH, reduced glutathione; GST, glutathione-S-transferase; IFN- γ , γ -interferon; IL, interleukin; ROS, reactive oxygen species; SOD, superoxide dismutase; TBARS, thiobarbituric acid reactive substance; TGF, transforming growth factor; TNF, tumor necrosis factor; VEGF, vascular endothelial growth factor.

antioxidant capacity is insufficient to cope with the generation of pro-oxidants⁸, many studies have focused on the ethanol-induced changes in the liver antioxidants. Fruits and vegetables play an important role in maintaining the physiological redox equilibrium by supplying several antioxidants.

The resveratrol (*trans*-3,4',5-trihydroxystilbene), a phytoalexin present especially in grapes and peanuts is known to exert antioxidant property at low concentrations, normally found in foods. Besides nutritional supplement, it also shows beneficial effects on the cardiovascular system^{9,10} and neuroprotection¹¹ by its antioxidant action. In another study, the beneficial effect of resveratrol against alcoholism in rats has been attributed to its antioxidant property¹². Similarly, vitamin E is one of the most important endogenous antioxidants, contributing against oxidative stress in human. Thus, in this study, the possible role of resveratrol and vitamin E in protecting chronic ethanol-induced oxidative stress in mice has been evaluated by measuring the biochemical and immunological parameters. The effectiveness of resveratrol has also been compared with vitamin E. As many processes related to the consumption or breakdown of ethanol are mediated by cytokines¹³, the treatment with the test compounds on cytokine levels has also been investigated.

Materials and Methods

Materials

Resveratrol and vitamin E were obtained from Sigma-Aldrich (St. Louis, MO, USA). The cytokine kits (Becton, Dickinson Biosciences and Co., Franklin Lakes, USA), transforming growth factor (TGF)- β 1 and vascular endothelial growth factor (VEGF)-A ELISA kits (Bender Med Systems, Austria) were used. The other chemicals used were ethanol (Bengal Chemicals Limited, Kolkata, India), trichloroacetic acid (TCA) (Thomas Baker, Mumbai, India), thiobabaturic acid (TBA), 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB) (Himedia Lab. Pvt. Ltd., Mumbai, India), N-(1-naphthyl) ethylenediamine (NEDD) (SRL, Mumbai, India), and 1-chloro-2,4-dinitrobenzene (CDNB) (Aldrich, Milwaukee, WI).

Animals and treatment

The male BALB/c mice (8–10 weeks old, 20–30 g) were housed in plastic cages inside a pathogen-free well ventilated room, maintained under standard husbandry conditions. The mice had free access to standard diet¹⁴ and water *ad libitum*. The animals

were weighed daily and their general conditions including liquid intake were recorded. The experiments were approved by the Animal Ethics Committee of the institution in accordance with the CPCSEA guidelines.

The mice were divided into the following four groups of 6 each: Group I: control mice were fed isocaloric glucose solution instead of ethanol (1.6 g/kg body wt) per day; Group II: mice fed with ethanol (1.6 g/kg body wt) per day; Group III: mice fed with ethanol (1.6 g/kg body wt) and resveratrol (5 mg/kg body wt) per day; and Group IV: mice fed with ethanol (1.6 g/kg body wt) and vitamin E (80 mg/kg body wt) per day.

The feeding of ethanol (as aqueous solution), resveratrol or vitamin E (both in ethanol) was done orally by intragastric infusion technique for 12 weeks.

Biochemical assays

At the end of experimental period, blood samples were collected from retroorbital plexus of mice. The serum was separated, and used for estimation of protein¹⁵, albumin¹⁶, aminotransferases¹⁷ and nitrite¹⁸. Blood ethanol concentration was determined with an ethanol assay kit (Sigma). The whole blood samples collected into vacutainers containing EDTA were centrifuged at $1000 \times g$ for 10 min at 2°C. After removing the plasma and buffy coats, the packed erythrocyte was washed with 4.0 ml cold normal saline, centrifuged at $3000 \times g$ for 15 min at 2°C and hemolyzed by adding 30 ml chilled double-distilled water. The hemolyzate was used for assaying TBARS¹⁹, reduced glutathione (GSH) content²⁰, as well as the activities of catalase (CAT)²¹, superoxide dismutase (SOD)²², glutathione reductase (GR)²³, glutathione peroxidase (GPx)²⁴ and glutathione-S-transferase (GST)²⁵.

Nitrite estimation

Nitrite was measured using Griess reagents¹⁸. Sulfonamide (1%, 50 μ l) in 2.5% ortho-phosphoric acid (Griess reagent 1) was added to the serum/homolysate, followed by 50 μ l of 0.1% N-(1-naphthyl) ethylenediamine (NEDD) in double-distilled water (Griess reagent 2). The mixture was incubated in dark at room temperature for 10 min, and the absorbance at 540 nm was read. The concentration of nitrite was measured from a standard curve of NaNO₂.

Cytokines assay

The cytokines (TNF- α , IL-2, IL-4, IL-10, IFN- γ , VEGF-A and TGF- β 1) levels in serum were estimated

using representative Sandwich ELISA kits according to manufacturer's instruction.

Statistical analysis

All data were analyzed using the statistical package SPSS (version 11.0, SPSS Inc., Chicago, IL) and the results were expressed as mean \pm SD (standard deviation). The sources of variation for multiple comparisons were assessed by the analysis of variance (ANOVA), followed by *post-hoc* test. The difference were considered significant at $P < 0.05$. In the present study, group I served as normal control, while group II served as experimental control.

Results

During the experimental period, no observable change was noted in physical appearance in the animals which had normal food and water as well as stool. Although we adopted a forced feeding of ethanol, the experimental mice showed only a marginally less weight gain, compared to any other group. Even the blood ethanol levels in the ethanol-treated and ethanol with food derived supplementation-fed mice were not significantly different (Table 1).

No significant change was observed in serum protein and globulin levels, but albumin level decreased significantly ($P < 0.01$) and AST and ALT activities elevated significantly ($P < 0.001$) in the ethanol-fed group, compared to the control group (Table 2). Except protein content, albumin level, globulin content, AST activity and ALT activity were significantly ($P < 0.05$) reversed by resveratrol and vitamin E treatment.

Chronic ethanol administration significantly ($P < 0.001$) increased TBARS (89.6%) and nitrite (52.9%) levels and GST activity (47%), but significantly ($P < 0.05$) decreased GSH content (42.7%) and activities of SOD (13.2%), CAT (19.3%), GR (39.3%) and GPx (30%) compared to the normal group (Table 3). Supplementation with resveratrol or vitamin E to the ethanol-fed mice significantly ($P < 0.05$) lowered TBARS level by 26.1% and 30.4% respectively, GST activity by 8% and 12% respectively, and nitrite levels by 19.2% for both test compounds; while significantly ($P < 0.05$) elevated GSH content by 44.6% and 53.6% respectively, GR activity by 41.1% and 47% respectively, and CAT activity by 12% in both cases, compared to the experimental group. Though supplementation with resveratrol did not show any significant effect on SOD (11.8%) or GPx (12%) activity, but vitamin E supplementation significantly ($P < 0.05$) increased SOD (13.8%) and GPx (20.2%) activities (Table 3).

Ethanol administration increased the levels of TNF- α (4.2-fold, $P < 0.001$), IFN- γ (2.9-fold, $P < 0.001$), IL-10 (5.2-fold, $P < 0.001$), VEGF-A (2.6-fold, $P < 0.001$) and TGF- β 1 (1.6-fold, $P < 0.001$), but reduced IL-4 (45.3%, $P < 0.01$), compared to the normal mice (Table 4). Treatment with resveratrol increased IL-4 (51.17%, $P < 0.01$), but reduced IL-10 (57.9%, $P < 0.001$), TNF- α (65.4%, $P < 0.001$), IFN- γ (52.13%, $P < 0.001$), VEGF-A (32.91%, $P < 0.01$), and TGF- β 1 (27.58%, $P < 0.01$), compared to the

Table 1—Body weight gain and plasma alcohol profile of normal, ethanol-fed, and resveratrol and vitamin E-treated mice

[Values represent mean \pm SD of three independent experiments with 6 mice in each group]

Parameters	Normal	Ethanol-treated	Ethanol + Resveratrol-treated	Ethanol + vitamin E-treated	F-variance	Significance
Weight gain (%) after 12 weeks	14.35 \pm 1.94	12.51 \pm 1.6	13.25 \pm 1.78	13.51 \pm 1.44	0.651	0.592
Ethanol consumption/ day (g/kg)	Nil	1.6	1.6	1.6	-	-
Plasma alcohol level (mM)	--	55.5 \pm 5.13	53.17 \pm 4.21	52.33 \pm 5	250.58	<0.001

Table 2—Effect of resveratrol and vitamin E on protein and liver-specific enzyme activities in serum of chronic ethanol-fed mice

[Values represent mean \pm SD of 6 mice in each group]

Parameters	Control	Ethanol-treated	Ethanol + resveratrol-treated	Ethanol + vitamin E-treated	F-variance	Significance
Protein (g%)	4.48 \pm 0.05	4.25 \pm 0.19	4.30 \pm 0.23 (+1.1)	4.38 \pm 0.13 (+3.0)	2.146	0.126
Albumin (g%)	3.27 \pm 0.17	2.91 \pm 0.15 ^b	3.19 \pm 0.15 ^c (+9.6)	3.27 \pm 0.18 ^d (+12.3)	6.617	0.003
Globulin (g%)	1.21 \pm 0.13	1.34 \pm 0.06	1.12 \pm 0.13 ^d (-16.4)	1.11 \pm 0.09 ^d (-17.1)	6.299	0.003
AST (IU/L)	18.5 \pm 4.59	187.17 \pm 17.24 ^a	113.3 \pm 5.46 ^{ac} (-39.4)	107.17 \pm 6.04 ^{ac} (-42.7)	297.184	<0.001
ALT (IU/L)	19.67 \pm 2.58	162.5 \pm 6.41 ^a	97.67 \pm 4.5 ^{ac} (-39.8)	94 \pm 5.06 ^{ac} (-42.1)	874.045	<0.001

Values in the parentheses are % increase (+) or decrease (-), compared to the experimental control group.

P values: ^a<0.001, ^b<0.01 compared to control; ^c<0.001, ^d<0.01, ^e<0.05 compared to ethanol-fed group.

Table 3—Effect of resveratrol and vitamin E on non-enzymatic and enzymic antioxidant parameters in serum of chronic ethanol-fed mice

Parameters	[Values represent mean \pm SD of 6 mice in each group]				F-variance	Significance
	Control	Ethanol-treated	Ethanol + resveratrol treated	Ethanol + vitamin E treated		
GSH ($\mu\text{g}/\text{mg}$ protein)	3.16 \pm 0.26	1.77 \pm 0.2 ^a	2.56 \pm 0.17 ^{ad} (+44.6)	2.72 \pm 0.16 ^{bd} (+53.6)	47.967	<0.001
TBARS (nmol/ml)	3.19 \pm 0.24	6.05 \pm 0.42 ^a	4.47 \pm 0.32 ^{ac} (-26.1)	4.21 \pm 0.32 ^{ac} (-30.4)	75.924	<0.001
Nitrite (nM)	0.17 \pm 0.02	0.26 \pm 0.02 ^a	0.21 \pm 0.02 ^e (-19.2)	0.21 \pm 0.03 ^e (-19.2)	17.098	<0.001
SOD (U/mg hemoglobin)	3.4 \pm 0.31	2.95 \pm 0.16 ^c	3.3 \pm 0.16 (+11.8)	3.36 \pm 0.22 ^f (+13.8)	5.221	0.008
CAT (mmol H ₂ O ₂ decomposed/mg protein /min)	0.31 \pm 0.02	0.25 \pm 0.01 ^a	0.28 \pm 0.01 ^{cf} (+12)	0.28 \pm 0.01 ^{bf} (+12)	16.64	<0.001
GR (nmol NADPH oxidized/min/mg protein)	0.28 \pm 0.03	0.17 \pm 0.01 ^a	0.24 \pm 0.01 ^{bd} (+41.1)	0.25 \pm 0.01 ^{bd} (+47)	56.07	<0.001
GPx (U/g hemoglobin)	3.03 \pm 0.18	2.12 \pm 0.14 ^a	2.41 \pm 0.17 ^a (+12)	2.55 \pm 0.18 ^{bf} (+20.2)	25.065	<0.001
GST (nmol CDNB conjugate formed/mg protein/min)	0.17 \pm 0.01	0.25 \pm 0.02 ^a	0.23 \pm 0.01 ^{ae} (-8)	0.22 \pm 0.01 ^{ad} (-12)	59.671	<0.001

Values in the parentheses are % increase (+) or decrease (-), compared to the experimental control group.

P values: ^a<0.001, ^b<0.01, ^c<0.05, compared to control; ^d<0.001, ^e<0.01, ^f<0.05 compared to ethanol-fed group.

Table 4—Effect of resveratrol and vitamin E on immunological parameters in serum of chronic ethanol-fed mice

Parameters	[Values represent mean \pm SD of 6 mice in each group]				F-variance	Significance
	Control	Ethanol-treated	Ethanol + resveratrol-treated	Ethanol + vitamin E-treated		
IL-2 (pg/ml)	20.26 \pm 3.23	20.27 \pm 2.94	20.93 \pm 2.67 (+3.2)	21.7 \pm 3.18 (+7)	0.307	0.82
IL-4 (pg/ml)	132 \pm 22.35	78.5 \pm 15.04 ^a	118.67 \pm 17.1 ^e (+51.17)	116 \pm 7.48 ^e (+47.7)	11.768	<0.001
IL-10 (pg/ml)	94.17 \pm 15.37	490.5 \pm 97.27 ^a	206.3 \pm 26.39 ^{bd} (-57.9)	143 \pm 21.24 ^d (-70.8)	69.626	<0.001
TNF- α (pg/ml)	161.3 \pm 57.33	678.67 \pm 112.69 ^a	234.33 \pm 49.61 ^d (-65.4)	287 \pm 50.29 ^{cd} (-57.7)	61.239	<0.001
IFN- γ (pg/ml)	172.33 \pm 39.14	508.33 \pm 53.74 ^a	243.3 \pm 22.96 ^{cd} (-52.13)	237.37 \pm 28.87 ^{cd} (-53.3)	91.965	<0.001
VEGF-A (pg/ml)	70.17 \pm 17.81	185.83 \pm 35.66 ^a	124.67 \pm 26.11 ^{be} (-32.91)	122.17 \pm 16.32 ^{ce} (-34.25)	21.162	<0.001
TGF- β 1 (ng/ml)	0.36 \pm 0.04	0.58 \pm 0.07 ^a	0.42 \pm 0.06 ^e (-27.58)	0.39 \pm 0.05 ^d (-32.7)	18.386	<0.001

Values in the parentheses are % increase (+) or decrease (-) compared to experimental control (Group II).

P values: ^a<0.001, ^b<0.01, ^c<0.05 compared to normal control; ^d<0.001, ^e<0.01, compared to ethanol-fed group

experimental group (Table 4). Vitamin E also showed a similar trend by increasing IL-4 (47.7%, P <0.01) and by reducing IL-10 (70.8%, P <0.001), TNF- α (57.7%, P <0.001), IFN- γ (53.3%, P <0.001), VEGF-A (34.25%, P <0.01) and TGF- β 1 (32.7%, P <0.01), compared to the experimental group animals. However, the IL-2 level remained unaffected in different treatments (Table 4).

Discussion

Alcohol consumption and health outcomes are complex and multi-dimensional. The ethanol-fed mice maintained normal functioning as reflected from their food intake and lack of any behavioral abnormality. As all the four groups of mice were kept in the same room without showing any infection or mortality, the observed changes in the biochemical and immunological parameters were due to ethanol and/or drug administration. Since the control mice were fed with isocaloric glucose solution, their nutritional status was practically the same, which was also evident from the weight gain of the respective groups (Table 1).

The low doses of resveratrol have been found to improve cell survival in cardio- and neuroprotection and its high doses increase cell death such as in cancer treatment²⁶. Although the limited literature makes comparison of doses difficult, resveratrol at lower doses (5 mg/kg) activate survival signals by up-regulating the antiapoptotic and redox proteins in the heart, while higher doses (25 mg/kg) potentiate a death signal by downregulating redox proteins and up-regulating pro-apoptotic proteins²⁷. We had also chosen lower dose of resveratrol in this study, while the dose of vitamin E was earlier established by us²⁸.

Chronic consumption of ethanol causes hepatic injury which can be diagnosed from the serum globulin level and transaminase activities (AST and ALT)²⁹. Hypoalbuminemia is a common feature of chronic alcoholic liver disease³⁰. Significantly decreased albumin level and increased liver marker transaminase enzymes activities in response to the chronic ethanol exposure (Table 2) suggested that these animals suffered from liver damage. Although administration of resveratrol or vitamin E to the

ethanol-fed mice decreased the levels of serum AST and ALT, as well as globulin (Table 2), these parameters were not restored to normal level.

Elevated lipid peroxidation is considered as the primary mechanism of cell membrane damage³. Similarly, despite its beneficial role, such as blood vessel relaxation, proliferation and migration of endothelial cells as well as angiogenesis, nitric oxide (NO[•]) is also cytotoxic at a high concentration, due to the generation of peroxynitrite^{31,32}. Our data indicated that chronic ethanol administration produced hepatic damage in mice, as evident from the significantly elevated TBARS and nitrite levels (Table 3).

GSH, the major non-protein thiol plays a central role in coordinating the body's antioxidant defence processes³³. Besides controlling the glutathione-related enzymes, it acts as a free radical scavenger, assisting in the maintenance of protein sulfhydryl groups. Depletion of GSH due to ethanol exposure (Table 3) renders the cell more susceptible to oxidative stress³⁴. The decrease GPx activity of in ethanol-exposed mice (Table 3) might be due to exhaustion or inactivation of the enzyme by ROS³⁵. While the decreased activity of GR (Table 3) would deplete the GSH content, the increased GST activity in ethanol-fed mice (Table 3) would result in more GSH consumption, and thus reducing its availability for protection against the oxidative damage³⁵. The increased GST activity, decreased GPx and GR activities and GSH depletion are important factors sustaining a pathogenic role for oxidative stress³⁶.

SOD catalytically dismutates superoxide radical anion to H₂O₂, while CAT and GPx render H₂O₂ harmless within cells by converting it into water and oxygen³⁵. The significantly lower activities of SOD and CAT in ethanol-fed mice (Table 3) indicated that chronic ethanol treatment was associated with severe oxidative stress. The decreased CAT activity might be due to loss of NADPH, excessive generation of superoxide radical, increased lipid peroxidation or a combination of all these³⁶. Supplementation with resveratrol or vitamin E to the ethanol-fed mice not only reduced TBARS and nitrite levels, and increased SOD and CAT activities, but also helped to partially reverse the activities of GR, GPx and GST, and thereby significantly restoring the GSH levels (Table 3).

Ethanol-induced damage is associated with oxidative stress and immunological alterations³⁶. It alters cytokines levels in a variety of tissues including plasma, lung, liver and brain. These cytokines play a critical role in cellular communication, activation, inflammation, cell death, cell proliferation and migration as well as healing mechanisms¹³. The acute and chronic alcoholism can increase gut permeability to endotoxins and impair the reticuloendothelial function of the liver^{37,38}, and thereby stimulate the production of cytokines. The low level of GSH and increased level of serum IFN- γ (Table 4) might augment the proinflammatory cytokine TNF- α level³⁵, which is a key factor in various aspects of liver diseases³⁹.

The decreased activities of SOD, CAT, and GPx, as well as increased lipid peroxidation observed in ethanol-fed mice might be due to the augmented TNF- α level⁴⁰. Elevated TGF- β 1 level due to ethanol exposure (Table 4) is implicated as a trigger for collagen deposition and hepatic fibrosis⁴¹. The markedly increased IL-10 level (Table 4) was expected, since it would inhibit monocytes/macrophages, including oxidative burst and production of nitric oxide and proinflammatory cytokines⁴². On the contrary, chronic ethanol exposure reduced the serum IL-4 level (Table 4) and inhibited the IL-4-induced B-cell proliferation and IgG class switching⁴⁰. The chronic ethanol consumption leads to hypoxia^{43,44}, which might indirectly induce production of VEGF-A (Table 4) to stimulate the growth of new blood vessels to meet the increased oxygen demand⁴⁵. However, the cytokines levels were effectively reversed by the test compounds.

In conclusion, both resveratrol and vitamin E appeared to be good candidate in the prevention of ethanol-induced oxidative stress and normalized the cytokine metabolism in mice, which was severely compromised due to chronic ethanol exposure. It is generally believed that vitamin E scavenges free radicals. The effects of resveratrol in biological systems are wide-ranging²⁶. It can maintain the concentrations of intracellular antioxidants found in biological systems⁴⁶. Interestingly, in the present study resveratrol produced similar benefits at a significantly lower concentration, compared to vitamin E. Moreover, the mode of action of resveratrol was primarily by suppression of the TNF- α level, while vitamin E showed stronger effect by downregulating the IL-10 level.

Acknowledgement

Financial assistance from Kerala State Council for Science, Technology and Environment (KSCSTE), Government of Kerala, India and Van Slyke Foundation, American Association for Clinical Chemistry (AACC) is gratefully acknowledged.

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