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ORAL SUPPLEMENTATION WITH RUTIN, A FLAVONOL GLYCOSIDE, INHIBITS ETHANOL INDUCED HEPATIC LIPID ACCUMULATION IN EXPERIMENTAL RATS

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ABSTRACT

Our aim was to determine the effect of rutin, a flavonol glycoside on ethanol induced hepatic lipid accumulation in experimental rats. Male albino Wistar rats were randomized into four groups. Groups 1 and 2 received isocaloric glucose. Hepatic injury was induced in groups 3 and 4 by administering 20% ethanol via intragastric intubation for 60days. In addition, groups 2 and 4 were given rutin (100 mg/kg) daily for the last 30 days of the experiment. Ethanol alone administered rats showed a significant increase in the levels of total cholesterol (TC), triglycerides (TG), free fatty acids (FFA), phospholipids (PL), low density lipoproteins (LDL) and very low density lipoproteins (VLDL) with a concomitant decrease in the levels of high density lipoproteins (HDL). Haematoxylin and eosin staining of the liver of ethanol alone fed rats showed inflammatory cell infiltrates and fatty changes Supplementation with rutin reversed the ethanol induced alterations in lipid profile and also restored the liver histology which appeared similar to the control rats. Together the results obtained clearly reveal the protective effects of rutin against lipid accumulation in the circulation and liver.

INTRODUCTION: Liver is the most important organ, which plays a pivotal role in regulating various physiological processes in the body. It is involved in several vital functions, such as metabolism, secretion and storage. It has great capacity to detoxify toxic substances. Ethanol abuse is a major health problem and a public health issue [1]. Alcoholism is associated with numerous degenerative and inflammatory disorders affecting the liver like steatosis, necrosis, fibrosis, cirrhosis and hepatitis [2]. Hyperlipidemia associated with ethanol consumption is an important prime risk factor for cardiovascular disease (CVD) and is predicted to be the most important cause of mortality in India by the year 2015 [3].

Ethanol is known to have a profound effect on the metabolism of lipids and lipoproteins. The two most conspicuous features of alcoholic fatty liver are the deposition of fat and the enlargement of the organ. Accumulation of lipids in the hepatocytes is the striking initial manifestation of ethanol-induced liver injury [4]. In chronic lipid accumulation, the liver cells become fibrotic leading to impaired liver function. Enhanced lipid peroxidation has also been reported in ethanol-induced hyperlipidemia [5-6].

Flavonoids are ubiquitous in higher plants and have been shown to be beneficial to human health due to its diverse pharmacological properties, including vasodilatory, anti-inflammatory, antiviral, antioxidant, and anticarcinogenic effects [7]. Rutin or quercetin-3-rutinoside, is one of the most commonly found flavonol glycoside together with quercetin and hesperidin, which are widely found in the buckwheat plant [8]. In addition rutin is an important dietary constituent of food and plant-based beverages. It has been reported to have clinically relevant functions, including antioxidant, antihypertensive, anti-inflammatory and antihemorrhagic activities, ability to strengthen the capillaries, regulate capillary permeability, and stabilize the platelets [9-10]. Rutin glycoside can relax smooth muscles and it has been observed to lower the hepatic and plasma cholesterol levels [11]. However, little efforts have been made to determine the impact of rutin against chronic ethanol induced hyperlipidemia in experimental rats. The present study hypothesized the effect of rutin on serum and tissue lipids in the alcohol induced hepatotoxic rats.

MATERIALS AND METHODS:

Materials: Rutin hydrate was purchased from Sigma Chemical Co., St. Louis, MO, USA. Absolute ethanol used in our study was obtained from Nellikuppam, Cuddalore District, India and all other chemicals and solvents used were of analytical grade.

Animals: Healthy male albino Wistar rats (150–180 g) were purchased from the Central Animal House, Department of Experimental Medicine, Rajah Muthiah Medical Collage, Annamalai University. They were housed in polypropylene rat cages with controlled temperature (24±2°C) and light (lights on 06:00 to 18:00). They were fed with standard pellet diet (Pranav Agro Industries, Pune, India) and water *ad libitum*. The rats were cared for according to the principles of the Institutional Animal Ethics Committee (IAEC), Annamalai University (Registration number: 160/1999/CPCSEA/559) and the experiments were conducted in accordance with the “Guide for the care and Use of Laboratory Rats”.

Rutin preparation: Rutin was suspended in carboxymethyl cellulose (CMC) at a concentration of 0.5% and was administered orally by intragastric intubation every day at the dose of 100mg/kg body weight.

Study design: The rats were randomly divided into four groups of 8 rats each. Groups 1 and 2 received standard pellet diet and isocaloric glucose from 40% glucose solution. Hepatic injury was induced in rats

of groups 3 and 4 by administering 20% ethanol (2.5 ml in the morning and 2.5 ml in the afternoon), equivalent to 7.9g/kg bodyweight as an aqueous solution by intragastric intubation for 30 days. At the end of this period, the dietary protocol of groups 1 and 3 were unaltered. Group 2 animals received rutin for the next 30 days, while group 4 continued to receive ethanol daily along with rutin as in group 2. The study design was as follows

Group 1: Control rats received standard pellet diet and isocaloric glucose from a 40% glucose solution daily by intragastric intubation.

Group 2: Rats received standard pellet diet and isocaloric glucose from a 40% glucose solution and in addition to rutin (100mg/kg bodyweight) by intragastric intubation daily.

Group 3: Rats received standard pellet diet and 20% ethanol daily.

Group 4: Rats received standard pellet diet and 20% ethanol and in addition to rutin (100mg/kg bodyweight) by intragastric intubation daily.

Rats in all the groups were fasted overnight, anesthetized by intramuscular injection of ketamine hydrochloride (30 mg/kg) and sacrificed by decapitation 24 h after the last treatment. Blood was collected with heparin as anticoagulant. Plasma was separated by centrifugation at 1500×g.

Lipids were extracted by the method of Folch *et al*., (1957) [12] using chloroform- methanol mixture (2:1 v/v). The levels of cholesterol (TC) were measured by the method of Zlatkis et al (1953) [13] and triglycerides (TG) by the method of Foster and Dunn (1973) [14]. Free fatty acids (FFA) and phospholipids (PL) were evaluated according to the methods of Falholt *et al*., (1973) [15] and Zilversmit & Davis (1950) [16] respectively and high-density lipoprotein (HDL) was analyzed in the supernatant obtained after precipitation of plasma with phosphotungstic acid/Mg^{2+}. Low density lipoprotein (LDL) and very low-density lipoprotein (VLDL) levels were calculated by the Friedwald formula [17].

LDL = Triglycerides/5

LDL = Total cholesterol – (HDL +VLDL)

Histopathological analysis: For histopathological studies, rats from the control and experimental groups were perfused with 10% neutral formalin solution. Livers were removed immediately from the rats; paraffin sections of 5μm thickness were made and stained with hematoxylin-eosin (H&E) stain. After staining, the sections were observed under a light microscope.

Statistical analysis: The results were statistically evaluated using one-way analysis of variance (ANOVA) followed by Duncan's multiple range test. All the grouped data were statistically determined using SPSS/11.0 software (SPSS Inc., Chicago, IL, USA). The difference were considered significant at $p<0.05$.

RESULTS: Table 1 shows the liver and body weight ratio of control and experimental rats. The liver and body weight ratio of ethanol fed rats (Group 3) were significantly ($P<0.05$) increased as compared to those of the control rats (Group 1). Ethanol fed rats supplemented with rutin (100 mg kg/b.w.), (Group 4) significantly ($P<0.05$) showed decreased liver and body weight ratio as compared to ethanol alone administered rats (Group 3).

Figures 1, 2, 3 and **4** show the concentrations of plasma TC, TG, FFA and PL of control and experimental rats. The concentrations of TC, TG, FFA and PL were significantly ($P<0.05$) higher in ethanol fed rats (Group 3) as compared to those of the control rats (Group 1). Rutin (100 mg kg/b.w.), supplementation to ethanol fed rats (Group 4) significantly ($P<0.05$) lowered the levels of plasma lipids as compared to those of the ethanol

alone treated rats (Group 3). Rutin alone supplemented rats (Group 2) did not show any significant change in the levels of plasma lipids. **Table 2** shows the concentrations of liver TC, TG, FFA and PL of control and experimental rats. The concentrations of TC, TG, FFA and PL were significantly (P<0.05) higher in ethanol-treated rats (Group 3) as compared to those of the control rats (Group 1). Rutin supplementation (100 mg kg/b.w.,) to ethanol administered rats (Group 4), significantly (P<0.05) lowered the levels of lipids in the liver as compared to those of the ethanol-alone treated rats (Group 3). Rutin alone supplementation (Group 2) did not show any significant change in the concentrations of lipids in the liver.

Table 3 depicts the lipoprotein levels of control and experimental rats. Ethanol fed rats showed significant (P<0.05) decrease in HDL and increased LDL, VLDL levels (Group 3) as compared to those of the control rats (Group 1). Supplementation with rutin (100 mg kg/b.w.,) to ethanol-fed rats (Group 4) showed significant (P<0.05) increase in the HDL and a decrease in the LDL, VLDL levels as compared to those of the ethanol alone treated rats (Group 3).

TABLE 1: EFFECT OF RUTIN ON LIVER WEIGHT TO BODY WEIGHT RATIO OF CONTROL AND EXPERIMENTAL RATS

Groups	Cholesterol (mg g^{-1} tissue)	Triglycerides (mg g^{-1} tissue)	Free fatty acids (mg g^{-1} tissue)	Phospholipids (mg g^{-1} tissue)
Control	3.23±0.24^{a}	3.89±0.29^{a}	6.53±0.48^{a}	13.04±0.96^{a}
Control + rutin (100mg/kg b.w.)	3.15±0.233^{a}	3.62±0.27^{a}	6.68±0.49^{a}	13.11±0.96^{a}
Ethanol	6.10±0.45^{c}	6.68±0.49^{c}	14.09±1.04^{c}	20.84±1.53^{c}
Ethanol + rutin (100mg/kg b.w.)	3.91±0.29^{b}	4.41±0.32^{ab}	8.15±0.60^{b}	17.17±1.26^{b}

Values are means ± SD of six rats from each group. Values not sharing a common superscript letter differ significantly at p<0.05 one-way ANOVA followed by DMRT

TABLE 2: EFFECT OF RUTIN ON LIVER CHOLESTEROL, TRIGLYCERIDES, FREE FATTY ACIDS AND PHOSPHOLIPIDS, OF CONTROL AND EXPERIMENTAL RATS

Groups	Cholesterol (mg g^{-1} tissue)	Triglycerides (mg g^{-1} tissue)	Free fatty acids (mg g^{-1} tissue)	Phospholipids (mg g^{-1} tissue)
Control	3.23±0.24^{a}	3.89±0.29^{a}	6.53±0.48^{a}	13.04±0.96^{a}
Control + rutin (100mg/kg b.w.)	3.15±0.233^{a}	3.62±0.27^{a}	6.68±0.49^{a}	13.11±0.96^{a}
Ethanol	6.10±0.45^{c}	6.68±0.49^{c}	14.09±1.04^{c}	20.84±1.53^{c}
Ethanol + rutin (100mg/kg b.w.)	3.91±0.29^{b}	4.41±0.32^{ab}	8.15±0.60^{b}	17.17±1.26^{b}

Values are means ± SD of six rats from each group. Values not sharing a common superscript letter differ significantly at p<0.05 one-way ANOVA followed by DMRT

TABLE 3: EFFECT OF RUTIN ON PLASMA LIPID PROFILE OF CONTROL AND EXPERIMENTAL RATS

Groups	HDL-C (mg dl^{-1})	LDL-C (mg dl^{-1})	VLDL-C (mg dl^{-1})
Control	49.27±3.62^{a}	27.65±2.03^{a}	14.17±1.04^{a}
Control + rutin (100mg/kg b.w.)	48.82±3.59^{a}	26.98±1.98^{a}	13.77±1.01^{a}
Ethanol	37.08±2.73^{c}	49.00±3.60^{c}	32.12±2.36^{c}
Ethanol + rutin (100mg/kg b.w.)	43.20±3.17^{b}	32.60±2.40^{b}	20.42±1.50^{b}

Values are means ± SD of six rats from each group. Values not sharing a common superscript letter differ significantly at p<0.05 one-way ANOVA followed by DMRT

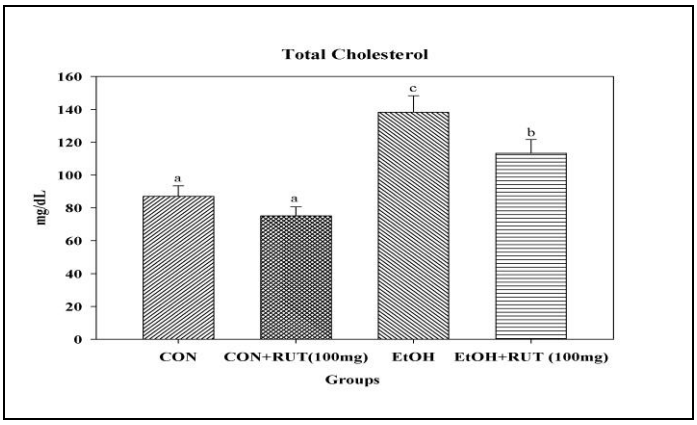

FIG. 1: CHANGES IN THE LEVELS OF PLASMA TOTAL CHOLESTEROL OF CONTROL AND EXPERIMENTAL RATS. VALUES ARE MEAN±SD OF SIX RATS FROM EACH GROUP. VALUES NOT SHARING A COMMON SUPERSCRIPT LETTER DIFFER SIGNIFICANTLY AT *P*<0.05 ONE-WAY ANOVA FOLLOWED BY DMRT

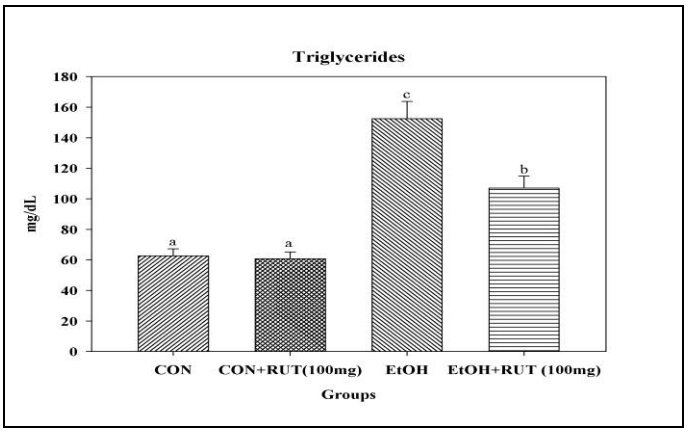

FIG. 2: CHANGES IN THE LEVELS OF PLASMA TRIGLYCERIDES OF CONTROL AND EXPERIMENTAL RATS. VALUES ARE MEAN±SD OF SIX RATS FROM EACH GROUP. VALUES NOT SHARING A COMMON SUPERSCRIPT LETTER DIFFER SIGNIFICANTLY AT *P*<0.05 ONE-WAY ANOVA FOLLOWED BY DMRT

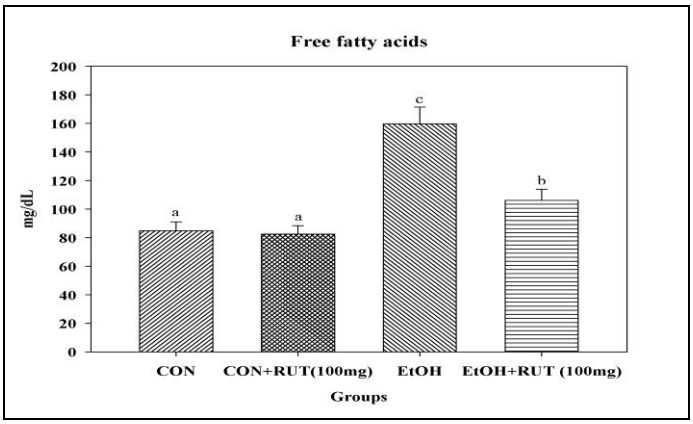

FIG. 3: CHANGES IN THE LEVELS OF PLASMA TOTAL FREE FATTY ACIDS OF CONTROL AND EXPERIMENTAL RATS. VALUES ARE MEAN±SD OF SIX RATS FROM EACH GROUP. VALUES NOT SHARING A COMMON SUPERSCRIPT LETTER DIFFER SIGNIFICANTLY AT *P*<0.05 ONE-WAY ANOVA FOLLOWED BY DMRT

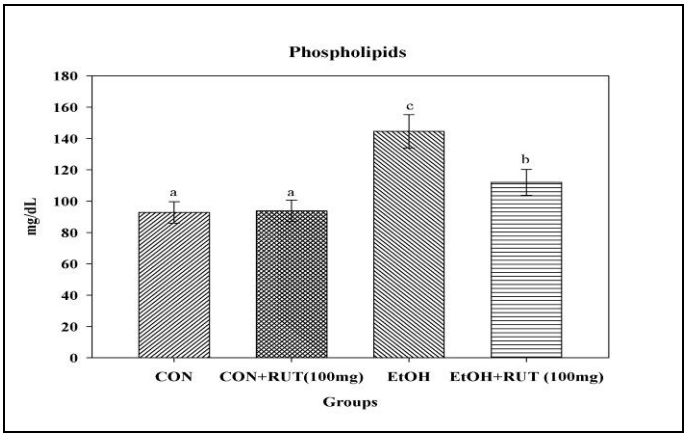

FIG. 5: CHANGES IN THE LEVELS OF PLASMA PHOSPHOLIPIDS OF CONTROL AND EXPERIMENTAL RATS. VALUES ARE MEANS ± SD OF SIX RATS FROM EACH GROUP. VALUES NOT SHARING A COMMON SUPERSCRIPT LETTER DIFFER SIGNIFICANTLY AT *P*<0.05 ONE-WAY ANOVA FOLLOWED BY DMRT

Histopathological findings: Figure 5 shows the histopathalogical changes in the liver of control and experimental rats. The liver of control rats showed normal liver morphology (**Figure 5a**). Control rat liver treated with rutin showed dilated sinusoids with some areas showing degenerated hepatocytes but the hepatocytes closest to the central vein appeared normal (**Figure 5b**).

Ethanol administered rat liver showed feathery degeneration, micro and macrovesicular fatty changes, periportal fibrosis and vascular congestion (**Figure 5c**). The liver of alcohol fed rat which received rutin showed loss of individual hepatocytes by degeneration and the space where the cell had originally been appeared empty, but there was no evidence of fatty change (**Figure 5d**). These findings clearly indicate that exogenous rutin administration shows a protective effect against ethanol induced hepatic injury.

DISCUSSION: Alcoholism is the biggest health problem of the world and most deaths attributed to alcoholism are caused by cirrhosis of the liver. Three common pathologic changes resulting from ethanol abuse are fatty liver, hepatitis and cirrhosis. About 50% of alcoholics have fatty liver, 20-30% acute or chronic hepatitis and 10-20% liver cirrhosis [18]. Ethanol is rich in calories and devoid of nutrients, thus contributing to accumulation of fat in the liver. The accumulation of lipid particles in hepatocytes alters the ultra structure of cellular membranes. Ethanol administration could also increase peripheral fat mobilization, enhance hepatic triacylglycerides synthesis and decrease hepatic lipoprotein release. In addition interaction of ethanol with biological membranes including lipid and protein components is complex and can cause significant changes in membrane function [19]. Our present data shows that rutin significantly protects against alcohol induced liver injury.

Ethanol is known to reduce the absorption of foodstuff and nutrients from the intestines[20]. Ethanol fed rats showed a reduction in the body weight which may be because of decreased food intake and also due to energy wastage when ethanol is metabolized by the microsomal ethanol oxidizing system or the mitochondrial inefficiency in fatty acid oxidation [21]. A better weight gain after rutin administration may be due to the enhancement of detoxification of ethanol directly from the intestines without absorption by up-regulating drug metabolizing enzymes [22]. This shows the beneficial protective effect of rutin against ethanol induced liver injury since body weight is considered to be a putative indicator of health.

The liver is the chief site of cholesterol synthesis, which competes for available essential fatty acids, can also cause fatty liver. Chronic administration of ethanol results in the accumulation [23] of cholesterol [24] and triglycerides in the liver as well as in the circulation. The interaction of cholesterol with phospholipids may result in an increase in membrane cohesion, leading to a reduction in its passive permeability to small molecules [25]. The elevated serum cholesterol observed in ethanol fed rats may be presumably due to faulty systems of absorption, storage and transportation in this experimental situation. Moreover the serum cholesterol concentrations were lowered on supplementation with rutin to ethanol fed rats. In this context, Park et al (2002) [26] demonstrated that rutin promoted the excretion of faecal sterols, thereby decreasing the absorption of dietary cholesterol as well as lowering plasma and hepatic cholesterol concentrations in rats.

Elevation in TG levels in the liver during ethanol consumption could be due to the increased availability of FFA, decreased fatty acid oxidation and increased secretion of VLDL [27]. Our observation agrees with those of the previous researchers [28]. Increased $NADH/NAD^+$ ratio during ethanol oxidation favours FFA synthesis and increased availability of FFA may elevate TG synthesis. Moreover, the increased FFA levels may cause greater generation of reduced NADPH or NADH, which may result in the activation of NADPH-dependent microsomal peroxidation [29]. Indeed, the elevated plasma TG levels are known to be associated with an increased incidence of coronary artery disease [30].

In addition increased levels of plasma TG in ethanol treated rats may be due to the decreased activity of lipoprotein lipase which is involved in the uptake of TG rich lipoproteins by the extra hepatic tissues. This environment favours the accumulation of TG in tissues [31]. In our study, consistent with previous findings [32], supplementation with rutin to ethanol fed rats showed decreased FFA which in turn decreases the synthesis of TG. Moreover Santos et al. (1999) [33] also demonstrated that rutin is very effective in reducing TG levels in hyperlipidemic rats.

PL are the basic components of cell membranes, mainly acting as regulators of membrane-bound enzymes in the membrane transport processes and also in determining the pathology of alcoholism [34]. It is possible that the effect of ethanol on membranes is mediated through alterations of membrane PL [35]. The high PL content in the liver of ethanol administered rats may be due to increased FFA levels and TC. Chronic administration of ethanol is known to affect the levels of plasma lipoproteins with the development of alcoholic liver disease [36]. The elevated levels of PL can result in the modification of composition, structure and stability of cell membranes, resulting in membrane dysfunction [37].

Reduction of PL levels on rutin supplementation could be due to membrane solubilization, which may lead to the decreased synthesis of TC, FFA and thereby prevent the toxic complications produced by the increased levels of these lipids. Cholesterol is produced in many tissues and transported to the liver for excretion into the bile, either as neutral sterols or as bile salts. To fulfill these transport needs, the liver produces VLDL and HDL. VLDL serves to carry fat from the liver to other tissues. The major function of HDL is to facilitate the mobilization of triglycerides and cholesterol from the extra-hepatic tissues and plasma to liver where it is catabolized and eliminated in the form of bile acids.

HDL concentration decreases and LDL concentration increases with the degree of impairment of liver function in ethanol fed rats [38]. The increased levels of VLDL and LDL in ethanol treated rats observed in our study are known to be cytotoxic to cells and tissues. Moreover Ross and Harker (1976) [39] have reported that during hyperlipidemia, lipoproteins may initiate and maintain atheromatous lesions by endothelial cell injury and lipid accumulation. Supplementation with rutin to ethanol fed rats resulted in a significant lowering of both VLDL and LDL levels and an improvement in the HDL levels. As the flavonoids appeared to act by protecting LDL against oxidation caused by the macrophages, rutin can reduce the cytotoxicity of oxidized LDL cholesterol and lower the risk of heart disease [40]. The decreased LDL levels may occur due to the reduction of VLDL levels [41].

Significant patho-morphological alterations in the liver were observed in ethanol fed rats. These changes can alter the properties of a cell. Hepatic damage observed in the histopathological studies in our present study in ethanol treated rats may be partially attributed to cytochrome-P450 generated metabolic cytochrome-P450 dependant enzyme activities in the liver that tend to be present at their greatest concentration near the central vein, and lowest near the peripheral sites [42]. Supplementation with rutin to ethanol-fed rats reduced the fatty changes and improved the liver histomorphology.

The data presented here suggests that rutin plays a significant role in mitigating abnormal accumulation of lipid during ethanol induced liver damage. Further studies are needed to evaluate the molecular mechanisms of action of rutin during ethanol toxicity.

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