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THE PROTECTIVE EFFICACY OF VITAMINS (C AND E), SELENIUM AND SILYMARIN SUPPLEMENTS AGAINST ALCOHOL TOXICITY

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Abstract: This study aimed at investigating the efficacy of vitamins (C and E), selenium and silymarin (an antioxidant complex from *Silybum marianum*) supplementation in reducing toxic effects of ethanol on liver weight and some blood parameters. Sixty male rabbits, individually housed in steel cages, were randomly divided into three groups. The first was a control group, the second received balanced diet and daily 20% (v/v) ethyl alcohol in their drinking water, the third received the same diet and 20% (v/v) ethanol in their drinking water and treated with vitamin C (1 mg/100 g body weight, BW), vitamin E (1 mg/100 g BW), selenium (0.01 mg/100 g BW) and silymarin (1 mg/100 g body weight) by gastric tube daily. Five animals per group were slaughtered every two weeks and liver and blood samples were taken after 2, 4, 6 and 8 weeks of treatment. Ethanol decreased body weight of rabbits and induced hepatomegally and apoptotic DNA fragmentation in hepatocytes. Chronic alcohol consumption induced significant increases in serum glucose, triglycerides and cholesterol levels whereas serum total protein content decreased. Significant increases in serum ALT, AST, ALP and LDH activities were observed in ethanol-treated rabbits. The treatment of alcohol-abused animals with vitamins (C and E), selenium and silymarin enhanced significant improvement in the biochemical, physiological and molecular aspects indicating their protective effects against alcohol toxicity.

Key words: Rabbits; alcohol toxicity; vitamin C; vitamin E; selenium, silymarin.

INTRODUCTION

Alcohol consumption represents a large problem all over the world (Kumar and Clark, 2002). Alcohol cannot be stored and obligatory oxidation must take place predominantly in the liver via alcohol dehydrogenase. The production of potentially toxic acetaldehyde is enhanced and conversion to acetate reduced (Sherlock and Dooley, 2002). The hydrogen produced replaces fatty acid as a fuel so that fatty acids accumulate with consequent ketosis, triglyceridaemia, fatty liver and hyperlipidaemia (Lieber, 1990). The conversion of alcohol to acetaldehyde also leads to inhibition of protein synthesis (Bernal *et al*., 1992) and alter its metabolism (Apte *et al*., 2004). Ethanol was also reported to cause many alterations in the activities of several enzymes under different nutritional conditions which appeared to play a role in modulating this effect (Shalan, 1996). Ethanol was recorded to alter also carbohydrate (Martin *et al*., 2004) and lipid (Ruf, 2004) metabolism.

Koyuturk *et al*. (2004) showed the protective effect of combination therapy with vitamins C and E, and selenium on ethanol-induced duodenal mucosal injury. Marino *et al*. (2004) showed that vitamin E protects against alcohol induced oxidative stress. The presence of selenium in combination with vitamin E enhanced its activity in removing free radicals and prevented their formation (Saito *et al*., 2003).

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Silymarin is an antioxidant flavonoid complex derived from the herb milk thistle (*Silybum marianum*). It was proved to have a protective effect against experimental hepatotoxicity by regulating the actions of the ultrastructures of the liver cells and improving the activities of hepato-cellular enzymes and bile production (Hagymasi *et al*., 2002).

Thus the main purpose of our study was to investigate the protective effect of combined supplementation with vitamins (C and E), selenium, and silymarin against alcohol toxicity.

MATERIAL AND METHODS

Animals and experimental treatments

Sixty male laboratory New Zealand White rabbits 8 weeks old (*Oryctolagus cuniculus*) weighing (1000 ± 100 g) were housed individually in steel cages where laboratory balanced diet and water were initially provided under standard controlled conditions (25 ± 2°C and relative humidity of 25 ± 5%).

Animals were randomly divided into three groups. The first group was normal controls (20 animals). The second was ethanol group (20 animals). Each animal received 30 ml 20% (v/v) ethanol/day as drinking water (absolute ethanol purchased from Al-Gomhoria Chemical Co., Egypt) and fed with balanced diet. The third one was alcohol + antioxidant group (20 animals). Each animal received 30 ml 20% (v/v) ethanol/day as drinking water, fed with balanced diet and supplemented with 1 mg vitamin C/100g body weight (BW), 1 mg vitamin E/100 g BW, 1 mg silymarin/100 g BW and 0.01 mg selenium/ 100g BW by gastric tube daily.

Vitamin E (DL-α-tocopherol) and selenium (sodium selenite) were obtained from Merk (Darmstadt, Germany). Silymarin was commercially available from Sedico Pharmaceutical Co. (Cairo, Egypt).

Sample collection and biochemical analyses

Animals were weighed at the beginning of the experiment and before each sample collection. Every 2 weeks 5 animals per group were anaesthetized and rapidly dissected. Livers were weighed immediately after dissection.

Samples were collected after 2, 4, 6 and 8 weeks of alcohol consumption. Blood samples were collected from the inferior vena cava in glass centrifuge tubes, then centrifuged for 15 min at 1000 g in a cooled centrifuge (4°C). Sera were separated and stored at –30°C in deep freezer till further biochemical measurements. Serum total protein, glucose, triglycerides, cholesterol, ALT (alanine aminotransferase), AST (aspartate aminotransferase), ALP (alkaline phosphatase) and LDH (lactate dehydrogenase) concentrations were determined automatically using Integra 800 auto-analyzer (Liver Institute, Menoufiya University, Egypt).

Preparation of tissues, gel preparation and electrophoresis of lysate tissue

After dissection, liver was removed, blotted on filter paper and weighed. Portions of 10 mg were taken immediately for gel examinations and the remaining portions were stored at –30°C.

Gels were prepared with 1.8 % electophoretic grade agarose (BRL). The agarose was boiled in Tris-borate EDTA buffer (1 × TBE buffer; 89 mM tris, 89 mM boric acid, 2 mM EDTA, pH 8.3). The 0.5 µg/ ml ethidium bromide was added to gel at 40°C. Gels were poured and allowed to solidify at room temperature for 1h before samples were loaded. The 10 mg hepatic tissue was squeezed and lysed in 200 µl lysing buffer (50 mM NaCl, 1mM Na_2 EDTA, 0.5% SDS, pH 8.3) for at least 30 min. For electophoretic pattern of nucleic acids of tissue lysate, 20 µl of lysate hepatic cells was loaded in well, 5 µl 6 × loading buffer was added on the lysing tissue.

Electrophoresis was performed for 2 hours at 50 V in gel buffer (1 × TBE buffer). Gel was photographed using a Polaroid camera while the DNA and RNA was visualized using a 312 nm UV transilluminator.

Nucleic acids extraction and molecular assessment for apoptosis

Nucleic acids extraction was based on salting out extraction method (Aljanabi and Martinez, 1997). For apoptosis, the extracted DNA was gently resuspended with TE buffer supplemented with 5% glycerol, gently pipetting, then the samples were mixed with 6 × loading buffer and loaded directly on the gel (Hassab El-Nabi, 2004). The remained DNA was kept at –20°C for another loading. Apoptotic bands appeared and located at 180, 360 and 540 bp.

Statistical analysis:

Data are presented as means ± s.d. in tables. Data were statistically analyzed by one-way analysis of variance (Anova-Tukey test) using SPSS 10.1 software pakage. The *P* values < 0.05 were considered significant.

RESULTS AND DISCUSSION

Effect of alcohol intake

The impact of prolonged alcohol consumption on the growth of experimental animals has been used as a means to determine the overall toxicity of this compound (Wartburg and Popenberg, 1970). The results of present study demonstrated significant decreases in body weight which reached its maximum value (–20%) by the end of the 2nd week of alcohol intake (Table 1). The magnitude of depression in body weight was attenuated thereafter by prolonged ethanol consumption (–15% after 8 weeks of treatment). It is relevant in this respect to mention that chronic treatment of pregnant rats with ethanol depressed feed and water consumption and weight gain (Abel, 1978). This depression in weight gain of the alcohol-treated animals probably reflects the complication of the depressant effects of ethanol on feed intake and impaired feed efficiency (Abel and Dintcheff, 1978).

The present data demonstrate that alcohol toxicity leads to not significant increase in the liver weight and hepatosomatic index (Table 1). This alteration is accompanied with significant decreases in total serum protein (Table 2). Such results lead to the suggestion that proteins are accumulated in

Table 1: Body weight, liver weight and hepatosomatic index during the trial

		Control group	Alcohol group	Alcohol+antioxidant group
Rabbits, no.		5	5	5
Body weight (g):	2 week	1110±102[b]	890±143[a]	1010±82[ab]
	4 week	1150±206	1046±177	1125±95
	6 week	1270±186	1050±100	1150±98
	8 week	1300±216[b]	1100±99[a]	1200±141[ab]
Liver weight (g):	2 week	59±14	60±12	60±14
	4 week	60±15	64±14	61±10
	6 week	61±15	68±16	61±11
	8 week	61±16	72±16	62±14
Hepatosomatic index[1] (%)	2 week	5.0±0.5	6.3±1.0	5.2±0.6
	4 week	4.9±0.7	6.1±1.2	5.0±0.8
	6 week	4.7±0.5	6.0±0.9	4.8±0.9
	8 week	4.6±0.6	5.9±0.9	4.6±1.7

[1] Hepatosomatic index: liver weight (g) / body weight (g) x 100). [a, b]: $P<0.05$

Table 2: Serum total protein and glucose concentration.

		Control group	Alcohol group	Alcohol+antioxidant group
Rabbits, no.		5	5	5
Total protein (g/l):	2 week	59.2±5.2	53.8±5.1	54.6±5.7
	4 week	62.2±8.9^{b}	50.9±5.2^{a}	57.2±5.3^{ab}
	6 week	60.0±7.7^{b}	44.8±4.7^{a}	53.4±5.8^{b}
	8 week	63.6±5.4^{b}	43.0±4.5^{a}	56.4±6.1^{b}
Glucose (mmol/l):	2 week	4.92±0.51	5.28±0.54	5.02±0.52
	4 week	4.67±0.48	5.50±0.60	4.87±0.54
	6 week	4.66±0.47^{b}	5.61±0.64^{a}	4.78±0.49^{b}
	8 week	5.02±0.52^{b}	6.77±1.04^{a}	5.89±0.69^{ab}

$^{a, b}$: $P<0.05$

the liver instead of being transported to blood, in response to toxic effects of alcohol. It is of significant importance, in this context, to mention the results of Shalan (1995) showing that hepatic protein content markedly increased after alcohol intoxication.

Moreover, ethanol was reported to decrease the number of hepatic macrotubules which are the key promoting secretion and intracellular transport of proteins and accordingly protein retention occurs accompanied by accumulation of lipids due to the increase in fatty acid binding protein leading to fatty liver and hepatomegally (Pignon *et al*., 1987), whereas other authors attributed hepatomegally, produced in response to chronic alcohol consumption, to the increase in the size of the hepatic cells and not to the increase of hepatocyte number (Israel *et al*., 1979). Cunnane *et al*., (1985) showed that increased liver weights resulted from hepatic triglyceride accumulation after chronic alcohol abuse. The decreased serum protein content might be interpreted in the light of the fact that alcohol inhibits secretion of the newly synthesized glycoprotein and albumin by hepatocytes (Lakshman *et al*., 1989).

Serum glucose was increased significantly at 6 and 8 weeks of alcohol consumption compared with normal controls (Table 2). It was shown that alcohol induced hyperglycemia (Forsander *et al*., 1958) that resulted from release of glucose from hepatic glycogen stores (Ammon and Estler, 1968). At the same time decreased peripheral utilization of glucose with alcohol intake helps in rising blood glucose level (Lochner *et al*., 1967); this may be associated with alcohol effect that decreases blood insulin and rises glucagon level (Bucher and Weir, 1976).

Table 3: Serum triglycerides and cholesterol concentration

		Control group	Alcohol group	Alcohol+antioxidant group
Rabbits, no.		5	5	5
Triglycerides (mmol/l):	2 week	1.07±0.16^{b}	1.58±0.17^{a}	1.44±0.15^{a}
	4 week	1.11±0.18^{b}	1.61±0.18^{a}	1.45±0.14^{a}
	6 week	1.16±0.14^{b}	1.66±0.23^{a}	1.54±0.16^{a}
	8 week	1.21±0.19^{b}	1.64±0.20^{a}	1.43±0.16^{ab}
Triglycerides (mmol/l):	2 week	2.58±0.30	3.03±0.42	2.70±0.29
	4 week	2.48±0.32^{b}	3.07±0.30^{a}	2.62±0.36^{ab}
	6 week	2.41±0.44	2.77±0.39	2.59±0.31
	8 week	2.45±0.73	2.70±0.46	2.53±0.55

$^{a, b}$: $P<0.05$

Table 4: Serum ALT, AST, ALP and LDH activities (U/L)

		Control group	Alcohol group	Alcohol+antioxidant group
Rabbits, no.		5	5	5
ALT activity (U/l):	2 week	62±9[b]	165±9[a]	62±7[b]
	4 week	61±9[b]	170±10[a]	64±9[b]
	6 week	61±6[b]	172±8[a]	65±8[b]
	8 week	61±10[b]	170±6[a]	67±8[b]
AST activity (U/l):	2 week	183±20[b]	235±23[a]	187±16[b]
	4 week	182±16[b]	233±20[a]	183±19[b]
	6 week	181±20[b]	249±22[a]	182±16[b]
	8 week	185±10[b]	243±17[a]	186±13[b]
ALP activity (U/l):	2 week	162±17[b]	200±24[a]	166±19[b]
	4 week	163±11[b]	200±22[a]	164±13[b]
	6 week	150±16[b]	202±19[a]	162±24[b]
	8 week	162±13[b]	207±19[a]	165±13[b]
LDH activity (U/l):	2 week	5.12±0.63	6.76±0.95	6.11±0.58
	4 week	5.18±0.56[b]	7.98±0.84[a]	6.05±0.64[b]
	6 week	5.21±0.50[b]	9.17±0.94[a]	5.36±0.63[b]
	8 week	5.24±0.[b]	10.96±0.94[a]	5.26±0.59[b]

[a, b]: $P<0.05$

Ethanol induced significant increase in triglycerides concentrations at 2, 4, 6 and 8 weeks of treatment compared with normal controls (Table 3). A significant increase in serum cholesterol content was reported only at 4 weeks of treatment of rabbits with ethanol compared with normal controls (Table 3). Fatty liver is an important feature of alcohol abuse (Bernal *et al*., 1992). Alcohol decreased fatty acids oxidation levels in the liver (Lieber, 1991) and that resulted in hepatic triglycerides accumulation (Lamb *et al*., 1994). Baraona *et al*. (1973) indicated that rising serum triglycerides level related to increased triglycerides synthesis resulted from increased fatty acids and alpha-glycerophosphate availability during alcohol metabolism, and seemed that alcohol enhanced lipogenesis through microsomes enhancement (Jenkins, 1984).

Results showed increased AST, ALT, ALP and LDH activities in response to alcohol administration (Table 4). It was documented that alcohol causes modifications in the fluidity of membranes (Freund, 1979), permeability of these membranes (Ross, 1977), and their lipid composition (Hoek *et al*., 1988). Therefore, alcohol may exert its effect through alteration of synthesis in the endoplasmic reticulum, intracellular translocation and/or possibility of solubilization at the site of plasma membrane, hence increasing the level of serum enzymes especially membrane-bound enzymes, like ALP, and cytosolic enzymes, such as LDH and transaminases (ALT and AST). Elevated serum levels of ALP and LDH were also observed by Yokoyame *et al*. (1993). Meanwhile, it was showed in previous studies that chronic alcohol intoxication depresses hepatic enzyme activities, suggesting that elevated serum enzyme activities might be induced as a result of enhanced release of hepatic enzymes into blood stream due to liver cell injury (Singer and Kaplan, 1978).

Ethanol induced apoptotic DNA fragmentation of hepatocytes (Figure 1). Studies in mice and rats revealed that both acute and chronic alcohol administration resulted in significant increases in hepatocyte apoptosis (Goldin *et al*., 1993, Yacoub *et al*., 1995). Potential mechanisms of acute ethanol-induced liver apoptosis include increased cytokine activity, Fas ligand (FasL) expression, and/or

oxidative stress (Kurose *et al*., 1997, Neuman *et al*., 2001). Ethanol-induced liver apoptosis involves the activation of cysteine proteases or caspases (Deaciuc *et al*., 1999, Zhou *et al*., 2001). Endonucleases and DNA fragmentation factors are activated during apoptosis, resulting in degradation of chromatin DNA into internucleosomal units (Cohen and Duke, 1984, Liu *et al*., 1997).

Effect of antioxidant treatment

Supplementations with vitamins (C, E), selenium and silymarin reduced the effect of alcohol intake on body weight (Table 1). There were significant differences in serum total protein and glucose concentrations between alcohol and alcohol + antioxidants groups at 6 weeks of treatment only (Table 2). Ethanol + antioxidants group showed increased triglycerides content significantly at 2, 4 and 6 weeks of treatment compared with normal controls, however not significant differences was reported at 8 weeks (Table 3). Supplementations with antioxidants significantly reduced the effect of alcohol intake on serum ALT, AST, ALP and LDH activities (Table 4).

Overall results indicated the highly protective effects of vitamins (C, E), selenium and silymarin supplements against alcohol intoxication. Tawfik (1998) reported that vitamin E protects polyunsaturated fatty acids from oxygen effects, and inhibits lipid peroxidation enhanced by ethanol (Situnayake *et al*., 1990) by acting as a free radical scavenger. Vitamin E stabilizes biomembranes and prevents lysis of phospholipids (Koning and Drijver, 1979). It was shown that vitamin E prevents alterations in ionic permeability of cellular membrane occurred after alcohol intake (Aono *et al*., 1978, Littleton, 1980).

Vitamin C acts as a free radical scavenger and reduces alcohol capacity for interacting with critical molecules (Davidson, 1998). Blankenship *et al*. (1997) indicated that vitamin C protects cells from undergoing apoptosis. Upasani *et al*. (2001) showed that the preventive activity of vitamins C and E may related to their antioxidant efficacy that inhibits lipid peroxidation.

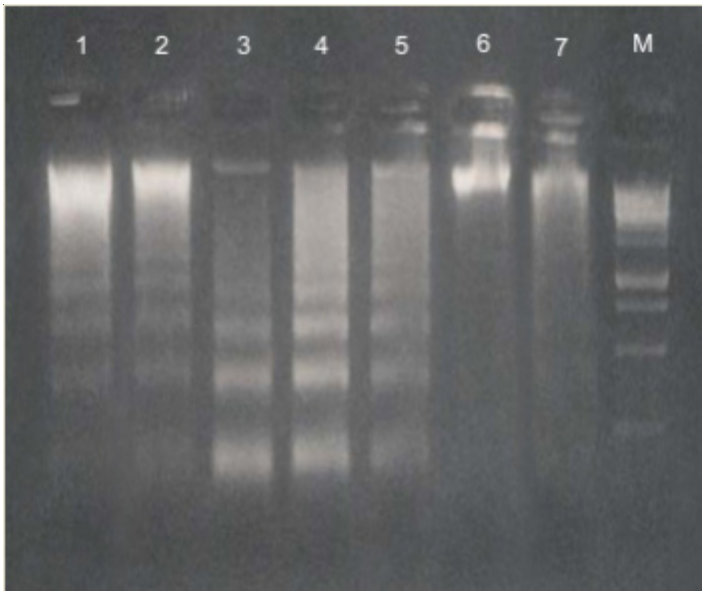

Figure 1: Apoptotic DNA fragmentation in liver of rabbits treated with ethanol and the protective role of vitamins (C, E), selenium and silymarin supplements. Lanes 1 and 2: ethanol treated with antioxidant supplements for 8 weeks; Lanes 3, 4 and 5: ethanol only intake for 8 weeks; Lanes 6 and 7: controls; M: 1 kp ladder.

Silymarin is a natural mixture of antioxidants acting as free radical scavenger and preventing lipid peroxidation (Soto *et al*., 1998). It was reported that silymarin improves liver function tests related to hepatocellular necrosis and/or increases membrane permeability (Buzzelli *et al*., 1993). Ramadan *et al*. (2002) reported that the protective effect of silymarin was attributed to its antioxidant and free radical scavenging properties. It was suggested that silymarin modulates the cellular immunoresponse and restores impaired liver function through its antioxidant capacity (Horvath *et al*., 2001). Feeding of animals on silymarin-phospholipid complex normalized lipid metabolism and inhibited atherosclerosis (Bialecka, 1997). The protective effect of silymarin may be attributed to its ability to scavenge oxygen free radicals and to inhibite liver microsome lipid peroxidation (Mira *et al*., 1994).

It was recorded that alcohol intake may result in a decreased intake of other nutrients, maldigestion and malnutrition (Lieber, 1988). Supplementation with antioxidants may repair the nutritional factors that may be affected by ethanol toxicity. The combined antioxidant supplementation may alter ethanol metabolism through their antioxidant capacities and thereby decreasing its toxic effects. Supplements may exert their effects through rapid elimination of ethanol via bile or decreasing ethanol intestinal absorption. Such suggestions need further studies to ensure the beneficial role of combined treatment, through measuring blood ethanol concentrations and serum alcohol dehydrogenase concentrations.

In conclusion, the present study showed that treatment of alcoholic abused animals with vitamins (C and E), selenium and silymarin supplements reduced toxic effects of ethanol.

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