Using Biochemical Findings to Study the Effect of Silymarin on the Liver of Pregnant Rat that Consumed Ethanol

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Received: 23 Aug. 2010; Received in revised form: 10 Dec. 2010; Accepted: 4 Jan. 2011

Abstract- In pregnancy period, there is high risk of hepatic diseases and alcohol consumption increases such risk. Some pregnant mothers are not able to quit the habit of drinking alcohol or they are unaware of its dangers. Finding a drug which is effective and efficient in reducing ethanol misuse consequences during pregnancy can assist the decrease of harmful effects of this habit. The purpose of the current research is to investigate the effects of oral administration of silymarin in preventing consequences of ethanol consumption on the liver during pregnancy, using the rat animal model as well as biochemical findings and clinical symptoms. 45 female rats were randomly divided into 3 groups, each composed of 15 rats. After the first day of pregnancy, the study was performed as follows. The first group received distilled water. The second group was given ethanol equivalent to 35% of their total required calorie. Furthermore, the third group received the same amount of ethanol plus 200 mg/kg silymarin. In order to evaluate liver's activity, biochemical analysis was performed at days 1, 7, 14, and 21, to measure the amount of the enzymes ALT, AST, ALP, and bilirubin. The nutrition and clinical status of animal in the groups was studied and recorded 2 times daily. This study showed that silymarin's protective effects are expressed from the first day of treatment.

Acta Medica Iranica, 2011; 49 (7): 407-413.

Keywords: Silymarin; Biochemical findings; Pregnancy; Ethanol; Liver; Rat

Introduction

Alcohol dependence is the tenth prevalent disease in Europe (1). It cannot also be overemphasized that pregnancy period is one of the most significant and critical periods in a mother's life and the infant to be born. Many mothers who drink alcohol are unaware of the consequences of this habit, including hepatic fibrosis and cirrhosis of alcoholic origin, Fetal Alcohol Syndrome (FAS), and intensification of the cholestasis of pregnancy (2,3), on themselves and their fetus during pregnancy. Besides, risks caused by drinking alcohol during pregnancy are increased as a result of not giving information concerning this habit to specialist or hygienic centers prior to and during pregnancy, due to cultural and social issues or lack of access to facilities. Hence, finding an effective drug for decreasing ethanol misuse effects which can reliably be used during pregnancy even without specialist's prescription and be simultaneously inexpensive and available, helps to decrease consequences of this habit in the society.

Liver is one of the organs with most vulnerability against ethanol misuse. It is vulnerable during pregnancy period even in normal conditions due to biochemical and physiological changes (4); thus any damage to this organ during pregnancy period will endanger the health of both mother and fetus. Acute or chronic hepatic toxicity caused by alcohol is mainly resulted from production of free radicals (5,6).

Silymarin is the mixture of flavonolignans extracted from milk thistle (*Silybum marianum*) (6). It appears that silymarin is hepatoprotective and the its use during pregnancy period results in no risk for mother and the fetus (7,8). Furthermore, silymarin is capable of passing

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through placenta and can prevent weight loss of fetus caused by alcohol consumption during pregnancy (9). Therefore, this study was designed to investigate the effect of silymarin in reducing hepatic damage caused by ethanol consumption during pregnancy period in laboratory animals.

Materials and Methods

In this study, 45 female rats were purchased from Pasteur Institute, Iran, with almost the same age and weight of 140-160 g (10). They were then randomly divided into 3 groups each including 15 rats, and each 3 animals were put in a cage. To be adapted to the new environment, cages were put for 14 days in the same environmental (22-26^{o}C, relative humidity of 40-60%, and 12/ 12 h light-cycle) and nutritional conditions (10-16). After this period, sibling male rat was kept in the same cage with female rats. After observation of vaginal plug in female rats, the male rat was taken out from the cage and it was recorded as embryonic day 0 (E0). This day was the beginning of the study on groups as follows. Every day at the same time, the first group as control was given distilled water by gavage. The second group daily received ethanol (Merck) by gavage equivalent to 35% of their total required calorie. Also, the third group received daily ethanol (Merck) equivalent to 35% of their total required calorie as well as 200 mg/kg silymarin. To assure that they have received the total prescribed silymarin dose, initially the pallets containing silymarin were made available to rats and after its consumption, their normal ration was given to them (17). To evaluate and compare the degree of hepatic injury in each group, venesection of tail vein was performed at days 1, 7, 14, and 21 of pregnancy (18). For the purpose of serum isolation, samples were put into incubator at 37^{o}C for 1 h. using automatic device with photometric base (Clima Co.), the biochemical analysis was subsequently performed to study the level of the enzymes alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkalen phosphatase (ALP), and bilirubin (Bill) as biochemical indicators of hepatic activity. During the study, the amount of received water and food as well as the clinical status of animals in the groups were recorded two times daily. Water and standard diet were given to animals *ad libitum* during preparation and the experiment (19,20). To prevent probable damage to liver, their food ration was controlled concerning the absence of fungal contamination and toxins such as aphlatoxin. Also no other chemical or drug was used to condition the animals. Finally, for description of obtained results, mean and standard error of dependent variables were calculated and one-way ANOVA was used to compare the acquired mean values in each group.

Results

The results obtained from measurement of biochemical factors in days 1, 7, 14 and 21 are summarized in following tables, categorized according to above mentioned groups. Serum markers of liver during the period of pregnancy were all increased in the second group which received ethanol, compared to the first group which were given only distilled water. This increase was less in the third group which received both ethanol and silymarin.

Table 1. Status of biochemical characteristics of hepatic activity in 1st of pregnancy

Biochemical indicator of Hepatic activity / Group #	Alanine aminotransferase (ALT) U/ml	Aspartate aminotransferase (AST) U/ml	Alkalen phosphatase (ALP) U/ml	Bilirubin (Bill) µM/l
1	^{a}44.13±1.57	^{a}109.53±3.31	^{a}176.60±4.360	^{a}0.81±0.05
2	^{b}55.73±1.99	^{a}116.60±6.37	^{b}198.60±4.36	^{a}0.82±0.04
3	^{ac}45.00±1.28	^{a}105.07±4.00	^{ab}185.60±2.40	^{a}0.69±0.06

Disharmonic subscripts denote a significant difference at level of 0.01.

Table 2. Status of biochemical factors characteristic of hepatic activity in 7th of pregnancy

Biochemical indicator of Hepatic activity / Group #	Alanine aminotransferase (ALT) U/ml	Aspartate aminotransferase (AST) U/ml	Alkalen phosphatase (ALP) U/ml	Bilirubin (Bill) µM/L
1	^{a}44.80±1.43	^{a}114.40±5.15	^{a}182.4±2.78	^{a}0.76±0.07
2	^{b}67.67±1.95	^{b}197.33±6.09	^{b}277.07±7.93	^{a}0.87±0.05
3	^{ac}49.47±1.77	^{ac}128.93±4.15	^{ac}199.73±5.29	^{a}0.71±0.03

Disharmonic subscripts denote a significant difference at level of 0.01.

Table 3. Status of biochemical characteristics of hepatic activity in 14th of pregnancy

Biochemical indicator of Hepatic activity Group #	Alanine aminotransferase (ALT) U/ml	Aspartate aminotransferase (AST) U/ml	Alkalen phosphatase (ALP) U/ml	Bilirubin (Bill) µM/L
1	[a]44.27±1.50	[a]112.13±4.82	[a]186.20±8.89	[a]0.70±0.07
2	[b]86.67±1.49	[b]302.87±4.21	[b]373.87±6.94	[b]1.113±0.06
3	[c]54.27±1.26	[c]208.84±1.66	[c]294.87±2.19	[ac]0.77±0.06

Disharmonic subscripts denote a significant difference at level of 0.01.

Table 4. Status of biochemical factors characteristics of hepatic activity in 21st of pregnancy

Biochemical indicator of Hepatic activity Group #	Alanine aminotransferase (ALT) U/ml	Aspartate aminotransferase (AST) U/ml	Alkalen phosphatase (ALP) U/ml	bilirubin (Bill) µM/L
1	[a]44.47±1.65	[a]113.87±4.03	[a]190.40±7.55	[a]0.65±0.06
2	[b]91.47±2.69	[b]312.20±4.03	[b]390.07±3.20	[b]1.16±0.06
3	[c]55.93±2.22	[c]219.20±6.03	[c]295.93±4.79	[ac]0.87±0.06

Disharmonic subscripts denote a significant difference at level of 0.01.

In all tables, group 1 (control group) received distilled water by gavage. Group 2 received ethanol equivalent to 35% of their total required calorie by daily gavage. Also, group 3 received ethanol equivalent to 35% of their total required calorie as well as 200 mg/kg silymarin daily.

Discussion

Drinking alcohol is the main cause of hepatic failure (21). Alcoholic liver disease which is among the main problems for general health (22) is the consequence of long-term high alcohol consumption and is accompanied by changes in liver enzymes such as AST, ALT, and ALP, as well as bilirubin, protein, albumin, and triglyceride (23). In addition, one of the most significant and critical periods in the lives of both mother and fetus is pregnancy period. Ethanol consumption during pregnancy intensifies hepatic diseases (2). Considering the harmfulness of drinking alcohol and its resultant hepatic diseases, high costs imposed to society by such diseases, and the point that some alcohol drinkers insist on their alcohol consumption even during pregnancy, it is of much significance to find a drug to prevent, treat, or decrease the resulting damages (26). In the absence of efficient chemical drugs, medicinal herbs play an important role (12) since they cause less side-effects especially during pregnancy besides being available and inexpensive. Silymarin is a flavonoid derived from milk thistle (23) which has been used for treatment of different types of hepatic diseases (24) including the one caused by alcohol (17,25). Silymarin has no serious side effects for fetus and mother during pregnancy (7). It is also well tolerated at high doses by pregnant mother. For instance, it has been used by mothers suffering from intrahepatic cholestasis with a dose of 560 mg/day for 16 days and no toxicity has been reported for mother and fetus (27). Although it is able to pass through placenta (9), silymarin has no toxic effect on fetus (8) and can avoid weight loss of fetus caused by alcohol consumption during pregnancy period (9). Considering the significance of liver's health during pregnancy as well as the raised risk for this organ in mothers who drink alcohol, the effect of silymarin was therefore in the current study evaluated on the consequences of alcohol consumption in liver during pregnancy on laboratory animals. In this study, biochemical indicators were used to evaluate the hepatic activities. Compare to other methods, such as liver biopsy, this is a noninvasive method and imposes less stress on pregnant mothers.

This research aimed to study the effects of silymarin on consequences of ethanol consumption during pregnancy period. Therefore, study was initiated immediately after beginning of pregnancy and no pre-treatment period or prescription prior to pregnancy was considered. As the pregnancy period in rats is 21 days, the days for evaluation of liver's status were days 1, 7, 14, and 21 to determine the liver's status in each 1/3 part of pregnancy period.

In the first day (Table 1) all changes in level of indicators were in the normal clinical ranges. Statistically, level of ALT in comparison between

groups 1 and 2, and between groups 2 and 3 differed significantly (P<0.01), but no significant difference was observed between groups 1 and 3 (P=0.93). These findings indicate no remarkable change in level of this enzyme in the group which received silymarin and ethanol compared to control group. Furthermore, ALP changes were significant (P<0.01) in comparison of groups 1 and 2, while no significant difference was observed between groups 2 and 3, nor between groups 1 and 3 (P=0.01 and P=0.13, respectively). In addition, changes in levels of AST enzyme and bilirubin had no significant difference in comparison of different groups. In seventh day (Table 2) significant differences (P<0.01) were observed in all three enzymes compared to control group, but these changes were not significant in case of bilirubin (P=0.08). Also, these changes in group 2 were significantly different (P<0.01) in comparison with group 3, but again bilirubin had no significant difference (P=0.08). Also, no significant differences were observed in level of enzymes and bilirubin in comparison of groups 1 and 3. These imply that silymarin is to a great extent capable of preventing changes in biochemical indicators of liver's damage in ethanol administration until this day and make the status similar to the absence of ethanol. In comparison of samples belonging to the 14^{th} day of pregnancy (Table 3), in addition to the enzymes, bilirubin changes also showed significant difference (P<0.01) when groups 1 and 2 were compared. In comparison of groups 1 and 3, although bilirubin showed no significant difference enzymatic changes were evaluated to be significant (P<0.01). It should be mentioned that differences of all indicators were significant (P<0.01) when comparing groups 2 and 3; so it can be concluded that silymarin has been able to decrease the negative effects of ethanol consumption at this time of pregnancy period similar to conditions in the absence of ethanol. In the 21^{st} day (Table 4) enzymatic changes were determined to be significant (P<0.01) similar to 14^{th} day. Also concerning bilirubin changes were evaluated to be significant (P<0.01) in comparison of groups 2 and 3, while no significant difference was observed (P=0.33) between bilirubin changes in group 3 compared to control group. Therefore, the results at this stage as well indicate the positive effect of silymarin in prevention of ethanol misuse consequences.

According to previous studies, it is established that acute or chronic hepatic toxicity caused by alcohol is mainly the result of free radicals production (5). Classic pathway of ethanol metabolism is through its decomposition by alcohol dehydrogenase enzyme and its conversion into acetaldehyde (the most significant alcohol metabolite which causes hepatic damage). In this decomposition, free radicals are produced by the changes in levels of $NADH/NAD^+$ and the resulting activation of Xanthine oxidase. Induction of CYP_2E_1 enzyme in hepatic microsomes in turn leads to production of hydroxyethyl radicals. Through reaction of aldehyde oxidase with acetaldehyde or NADH, ethanol causes the production of oxygen radicals (28,29). It also causes the production of nitrogen free radicals by induction of nitric oxide synthase (iNOS) enzyme in inflamed cells (30). Additionally, ethanol is hown to reduce the level of hepatic glutathione (31,32). Silymarin and its derivatives have antioxidant effect on free radicals (33). Also, silymarin attenuates the decreasing effect of ethanol on glutathione (34). In a study on patients who have suffered from chronic disease of alcoholic liver, 6-month silymarin treatment with dose of 420 mg/day caused considerable recovery of superoxide dismutase activity in red blood cells and lymphocytes and also the amount of sulfonyl free groups and activity of glutathione peroxidase was recovered to normal level (35). Ethanol leads to considerable accumulation of triglycerides in liver, and silymarin treatment reduces it (35). Silymarin decreases the increased level of lipid peroxidation in liver caused by ethanol (34). Ethanol consumption results in a remarkable increase in production of TNF-α by the liver, and this effect of ethanol is as well attenuated by silymarin (34). It has been shown that, the activity of CYP_2E_1 enzyme in both groups which received ethanol and ethanol + silymarin increases approximately two times compared to control group. Therefore, protective activity of silymarin against ethanol's toxicity is independent of the activity of CYP_2E_1 hepatic enzyme (34). Hence, silymarin's property of reducing acute hepatic damages caused by ethanol might be related to its antioxidant characteristics, decreased lipid peroxidation, and cytokine production.

In our study, in the group which received only ethanol, increase in level of the enzymes ALT and ALP was the initial results, which implies rapid effect of ethanol on parenchyma tissue and hepatic ducts. As ALP is produced in duct cells, its increase in hepatic parenchymal diseases is usually subsequent to other hepatic enzymes. In this study a considerable increase was observed in level of this enzyme at subsequent stages due to its synthesis and according to alcohol's role. In acute hepatic diseases, due to its hepatic specificity, the increase in ALT level is higher than that of AST; but AST is a more superficial enzyme compared to ALT and generally increases prior to ALT.

Additionally due to AST existence in other tissues, its increase is not specific for hepatic diseases. In the current study the ALT's increase occurred sooner, which may imply the destructive effect of acute alcohol consumption on liver and effusion of intercellular ALT content (36). Except in case of cirrhosis, pattern of increase in level of hepatic enzymes in acute and chronic alcohol diseases is by overcoming ALT. The exception lies in cirrhosis and alcoholic liver disease. Any type of cirrhosis is expressed by dominance of AST versus ALT. Also in alcoholic liver disease the increase of AST is more than twice compared to ALT, which is specific to alcoholic liver disease. The results of this experiment confirm the above mentioned pattern and the increase in level of AST was higher than that of ALT (36). In the case of bilirubin, its increase through cholestasis mechanism requires damage to at least 75% of the level which is responsible for bilirubin production. So in spite of the presence of hepatic damage in acute consumption of alcohol, bilirubin has not been observed to increase (37). Differences in changes at level of indicators among the members of a group can be attributed to intra-personal differences, since the types of inflammatory factors and cytokines released in response to alcohol are different in individuals and are dependent to genetic polymorphism (38,39).

To the best of our knowledge, no study similar to ours has been performed and the results of the current research are the first report in this field. However, in mentioning almost similar studies, it should be noted that a study in 2003 for evaluation of hepatic fibrosis caused by alcohol in baboons from primates family indicated that silymarin inhibits the oxidative damage caused by alcohol, prevents the increase in hepatic lipids and circulated ALT, and delays the progress of hepatic fibrosis caused by alcohol in primates (40). In 2006, another study for investigation of silymarin's protective effects on mouse revealed that acute consumption of ethanol was associated with raised ALT, while silymarin treatment was able to lower this increase in ALT compared to the group which had received ethanol (34). These studies are to a great extent in concordance with results of present research.

It is noteworthy that, reaching useful effects of most drugs requires their long-term administration. The present study, however, showed that silymarin's protective effects are expressed from the first day of treatment. This is of much significance concerning alcoholic damage to fetus, as many mothers are unaware of their pregnancy during the initial weeks of their pregnancy; so if the appearance of drug's therapeutic effects requires long time, useful effects of drug will not be expressed at initial months of pregnancy which are critical for growth and development of the fetus (41). Consistent with obtained results, higher doses of silymarin which have been proved to have no risk for mother and fetus may lead to better results. Also in further experiments the minimum effective dose can be studied for maximum protective effect, as well as the maximum dose which has no risk for mother and fetus. Nonetheless, because of fetus sensitivity and serious effects of drugs on physical and behavioral development of children in future, further studies on all organs and prospective studies on born infants are necessary to confirm that this drug has no risk for pregnant mother and fetus.

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