Evaluation of amitriptyline-induced toxicity in freshly isolated rat hepatocytes and the protective role of taurine"

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Abstract: Administration of amitriptyline, a most commonly used tricyclic antidepressant, is associated with anticholinergic side effects and rare but severe hepatotoxicity. It supposed that the intermediated metabolites of amitriptyline produced by CYP450 involved in hepatotoxicity but the exact mechanisms involved in hepatotoxicity are unknown. The aim of this study was to determine the mechanism of hepatotoxicity induced by amitriptyline and protective role of taurine in an *in vitro* model of isolated rat hepatocytes. Markers such as cell viability, reactive oxygen species (ROS) formation, lipid peroxidation, mitochondrial membrane potential, and hepatocytes glutathione content were evaluated every 60 minutes for 180 minutes. Our results showed that amitriptyline resulted in cytotoxicity characterized by the reduction in cell viability, an increase in ROS formation and lipid peroxidation, mitochondrial membrane potential collapse, and a reduction in cellular glutathione content. Our finding showed administration of taurine (1mM) effectively reduced the toxic effects of amitriptyline in isolated rat hepatocytes.
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1. Introduction

Amitriptyline, a tricyclic antidepressant, has been used for more than three decades (Wen et al, 2008). It has some adverse effects including anticholinergic side effects and rare but severe hepatotoxicity (Abernathy et al, 1975., Selim et al, 1999). One study showed that amitriptyline induced liver damage in 0.2% of patients who took amitriptyline continuously Health Me, 2012). It was investigated that reactive metabolite of amitriptyline causes hepatotoxicity, but the exact mechanism of initiation and propagation of cell damages is still poorly understood. It is proposed that the mechanism responsible for amitriptyline induced hepatotoxicity has attributed to the accumulation of aren oxides, due to an initial CYP450-catalyzed inactivation on an aromatic ring of amitriptyline(Wen et al, 2008), previous finding showed that CYP2D6 and CYP3A4 have important roles in oxidation of benzene nucleus in amitriptyline to an electrophilic epoxide.This metabolite reacts with water generating dihydrodiol or with glutathione and produces sulphydryl conjugate or attack cellular proteins to induced a toxicological response ,also epoxide intermediates could react directly with cellular proteins to trigger a toxicological effects (Wen et al, 2008).

Taurine is an essential amino acid with a sulfonic acid group; it has several physiological roles (Azuma et al, 2009, Heidari et al, 2012). Variety of experiments has been showed that taurine has protective effects against different chemically-induced hepatotoxicity (Azuma et al, 2009. Aruoma et al, 1988. Acharya et al, 2010). An investigation reported that taurine could act as a potent antioxidant in biological systems (Aruoma et al, 1988) Therefore; the protective role of taurine against different chemically-induced hepatotoxicity could be due to the antioxidant capability of this amino acid. Also taurine has the ability to scavenge the reactive oxygen species, diminish lipid peroxidation, and therefore stabilize biological membranes (Aruoma et al, 1988. Heidari et al, 2012)

The aim of our study was to investigate the protective role of taurine against cytotoxicity associated with amitriptyline. Cellular damage was estimated by measuring the percent of viable cells using the trypan blue exclusion test. The reactive oxygen species (ROS) formation and lipid peroxidation was studied; furthermore the effect of amitriptyline on intracellular glutathione levels was evaluated. Also, the effect of amitriptyline on hepatocytes mitochondria was evaluated.

Experimental

Chemicals

Amitriptyline was purchased from Sigma Aldrich chemical Co. Taurine was obtained from Acros chemical CO. Other reagents were obtained from Merck chemical CO. Amitriptyline and taurine were dissolved in water.

Animal treatment and hepatocytes preparation

Male Sprague-Dawley rats (250–300 g) were housed in ventilated plastic cages with 12h light photoperiod and an environmental temperature of 21-23°c with a 50-60% relative humidity. Animals were fed a normal chow diet and water *ad libitum*. The animals were handled and used according the animal handling protocol that approved by a local ethic committee in Tabriz University of medical sciences, Tabriz, Iran.

Collagenase perfusion via the portal vein was used to isolate rat hepatocytes which have been described in details before by Eghbal *et al* (Eghbal et al, 2008).The livers were perfused with different buffer solutions through the portal vein. The liver was perfused with collagenase after the removal of calcium ions (Ca^{2+}) with a chelator (EGTA 0.5 mM). So, the liver tissue was destructed by the collagenase enzyme in buffer solution and lead to easily isolation of hepatocytes during the next steps (Heidari et al, 2012). Isolated hepatocytes (10 mL, 10^{6} cells/mL) were suspended in the Krebs-Henseleit buffer (pH=7.4) in continuously rotating round bottom flasks, under an atmosphere of carbogen gas (95% O_2 and 5% CO_2) in a 37 °C water bath. Only the cells with viability of over the 85% were used (Heidari et al, 2012).

Cell viability

Hepatocytes viability was assessed by the trypan blue (0.1%, w/v) exclusion test microscopically (Moldeus et al, 1978). Only the cells with viability of over the 85% were used. Hepatocytes viability was determined every 60 minutes for 180 minutes. In all experiments, taurine was added 30 minutes before other agents. CYP450-inhibited hepatocytes were prepared by adding cimetidine 30 min before other reagent to flasks (Jamshidzadeh et al, 2007) and CYP450 induced by IP injection of β-naphtoflavone for 3 days (Madan et al, 2003).

Determination of reactive oxygen species (ROS)

To determine the extent of ROS formation by amitriptyline, 1.6 µl of 2.7- dichlorofluoresceine diacetate was added to hepatocytes incubate. DCFH-DA hydrolyzed to non-fluorescent DCFH in hepatocytes, DCFH reacted with ROS and became highly fluorescent. In 60,120,180 min 1 ml(10^{6}cell) of samples centrifuged at 3000g for 1 min, then the fluorescence of supernatant was detected fluorimetrically at excitation and emission wavelength of 490nm and 520nm respectively (Anoush et al,2009).

Determination of lipid peroxidation

Hepatocytes lipid peroxidation was detected by measuring of thiobarbituric acid reactive substance (TBARS) that formed during the decomposition of lipid hydro peroxides. 250µl of trichloroacetic acid (TCA, 70%w/v) was added to 1ml of hepatocytes suspension (106cell) and centrifuged at 3000g for 15 min, then 1 ml of thiobarbituric acid (0.8%w/v) added to supernatant and boiled for 20 min. The absorbance was measured at 532nm in a Ultrospec 2000 spectrophotometer (Smith et al, 1982).

Mitochondrial membrane potential assays

Rhodamine 123 (the fluorescent dye) accumulate in undamaged mitochondria by facilitated diffusion. The amount of Rhodamine 123 in media is increased when the mitochondrial membrane potential is reduced by a toxin. Every 60 min for 180 min 2ml samples of the cell suspension were taken and centrifuged at 1000 rpm for 1 min then the cell was resuspensed in 2 ml of krebs-Henseleit buffer containing 1.5 µM Rhodamine 123 and incubated at 37°c water bath. Then centrifuged at 3000 rpm for 1 min and hepatocytes were separated. The amount of Rhodamine 123 in media was determined by fluorimetrically using a Jusco FP-750 fluorescence spectrophotometer at 490 nm excitation and 520 nm emission wavelengths. The difference of capacity of mitochondria to take up the Rhodamine 123 between control group and treated group was expressed as percentage of control (Eghbal et al, 2004).

Cellular GSH/GSSG content

To determine the hepatocyte glutathione (GSH) content the method of Ellman was used (Reiner et al, 2002). A 1 ml aliquot of the cell suspension (10^{6} cells) was taken and 2 ml of 5% TCA was added and centrifuged. Then 0.5 ml of Ellman's reagent (0.0198% DTNB in 1% sodium citrate) and 3 ml of the phosphate buffer (pH 8.0) were added. The absorbance of the developed color was measured at 412 nm using a Biotech Pharmacia Ultrospec® 2000 spectro-photometer. Cell samples were reduced with potassium borohydride (KBH4) to prevent GSH oxidation during the experiment (Kleinman et al, 2000). The enzymatic recycling method was used to assess the hepatocyte oxidized glutathione (GSSG) level (Rahman et al, 2006), where cellular GSH content was covalently bonded to 2-vinylpyridine at first. Then the excess 2-vinylpyridine was neutralized with thriethanolamine, and GSSG was reduced to

GSH using the glutathione reductase enzyme and NADPH. The amount of GSH formed was measured as already described for GSH using the Ellman reagent (0.0198% DTNB in 1% sodium citrate) (Heidari et al, 2012).

Statistical analysis

The results are shown as the Mean±SEM for at least three independent experiments. Statistical analysis for the control and experimental groups was performed by a one-way ANOVA (analysis of variance) test. A $P < 0.05$ was considered as a significant difference.

Results

Our results showed that amitriptyline toxicity in rat hepatocytes was concentration-dependent, As shown in table 1, incubation of hepatocytes with 40 µM of amitriptyline led to the death of 50% of the cells in 2h (LC50=400 µM). An optimum effective dose of taurine that provided suitable protection was found 1mM. Taurine was added to hepatocytes 30 minutes before adding amitriptyline .As table 2 shows, taurine prevented cell death induced by amitriptyline effectively ($p<0.05$).

To investigate the mechanism by which taurine protected hepatocytes against amitriptyline-induced toxicity Markers such as ROS formation, lipid peroxidation, cellular glutathione content, and mitochondrial membrane potential were assessed.

As table 3-1 illustrates, amitriptyline at 1h, 2h and 3h of sampling leads to an increase of ROS formation. Pre-treatment of hepatocytes with 1mM of taurine decreases ROS formation significantly ($p<0.05$). According to table3-2 ROS formation was increased significantly in hepatocytes that CYP450 were induced by β-naphtoflavone.

As shown in table 4, amitriptyline caused lipid peroxidation in isolated rat hepatocytes and the amount of TBARS increased greatly. Pre - incubation of hepatocytes with 1mM of taurine prevented TBARS production ($p<0.05$).

According to figure 1, in MMP test, in comparison between the groups receiving amitriptyline and the control group, the degree of permeability of Rhodamine to the mitochondria has decreased drastically. It shows the toxic effects of amitriptyline in mitochondrial membrane potential. Taurine has significant effects on the prevention of the toxicity of mitochondria.

According to figure 2, amitriptyline caused a significant reduction in cellular glutathione content. GSH content of amitriptyline -exposed cells was notably increased in the taurine-supplemented groups ($p<0.05$).

According to figure 3, amitriptyline significantly increases GSSG levels of hepatocytes. GSSG levels of amitriptyline -exposed cells were notably diminished in the taurine-supplemented groups ($p<0.05$).

Discussion

In this study, the toxic effects of amitriptyline on the isolated rat hepatocytes were found concentration - dependent. In our study, the diminution of the toxicity of amitriptyline by cimetidine (CYP450 inhibitors) and the rise of ROS generation in cells that CYP450 were induced by β-naphtoflavone showed that metabolites produced by CYP450 enzymes are involved in the amitriptyline hepatotoxicity. These findings confirm the previous study (Wen et al, 2008).

Reduced GSH play an important role in hepatocytes defense (Jamshidzadeh et al, 2007). The result from the current study showed that GSH levels were diminished by incubation of hepatocytes with amitriptyline and the levels of GSSG were increased, amitriptyline leads to an increase of ROS formation, GSH react with ROS, and GSSG levels were increased, Also according to previous study P450 catalyzed oxidation of the benzene nucleus in amitriptyline to an electrophilic epoxide (Wen et al, 2008). Amitriptyline oxidation product formed GSH conjugation and GSH levels decreased. Taurine can rise the Glutathione savings and decreases GSSG that is confirm with the last test about the level of ROS (taurine is a potent ROS scavenger and GSH increased). This finding also supports the previous studies regarding the antioxidant effects of taurine (Aruoma et al, 1988. Heidari et al, 2012).

The direct relationship between the rise of ROS formation and the increase in TBARS levels and GSH decrease confirms the existence of the process of oxidative stress in hepatotoxicity due to amitriptyline. Pre-incubation of hepatocytes with the 1mM of taurine decreased the death rate of hepatocytes significantly. Taurine reduced ROS generation and in lipid peroxidation test, decreased TBARS as well. These findings are in line with the previous studies regarding the radical scavenger effects of taurine (Aruoma et al, 1988, Heidari et al, 2012).

In the present study we strongly suggest that the implication of mitochondrial toxicity in the hepatotoxicity associated with amitriptyline. In MMP test, taurine have significant effects on the prevention of the change of mitochondrial membrane potential.

1. Conclusion

All in all, the results indicate that toxicity induced by amitriptyline in isolated rat hepatocytes is associated with oxidative stress. Taurine can prevent amitriptyline induced liver damage. Following our findings, further studies are suggested on the antioxidant effects of taurine in patients receiving amitriptyline.

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Table 1: amitriptyline induced Cytotoxicity in isolated rat hepatocytes
%cytotoxicity

	Time(min)		
	60	**120**	**180**
control	21±1	24±2	28±2
Amitriptyline 2mM	100	100	100
Amitriptyline 1mM	100	100	100
Amitriptyline 0.5mM	100	100	100
Amitriptyline 200µM	100	100	100
Amitriptyline 100µM	100	100	100
Amitriptyline 40µM	48±3*	55±2*	60±4*
Amitriptyline 20µM	32±3	40±2	50±2

Table 2:prevention of amitriptyline hepatotoxicity by Taurine and cimetidine (CYP450 inhibitor)
Cytotoxicity %

	Time(min)		
	60	**120**	**180**
Amitriptyline 40µm	41±3*	52±3*	59±2*
+Taurine 1mM	29±3**	31±2**	34±1**
+Cimetidine 2mM	25±3**	26±2**	28±3**

Results are mean ±SEM of at least three different experiments.
*significantly different from control group ($p<0.05$)
** Significantly different from amitriptyline treated group ($P<0.05$)

Table 3-1: Effect of amitriptyline on ROS formation and the prevention effects of Taurine and cimetidine(CYP450 inhibitor)
fluorescence unit

	Time(min)		
Control	**60**	**120**	**180**
Amitriptyline 40µm	159±4*	175±3*	202±4*
+Taurine 1mM	60±3**	80±5**	95±3**
+Cimetidine 2mM	122±5**	138±8**	169±5**

Results are mean ±SEM of at least three different experiments.
*significantly different from control group ($p<0.05$)
** Significantly different from amitriptyline treated group ($P<0.05$)

Table 3-2: effect of amitriptyline on ROS formation (Hepatocytes were induced by β-naphtoflavone)
fluorescence unit

	Time(min)		
	60	**120**	**180**
Control	191±3	198±4	228±5
Amitriptyline 40µm	356±5*	386±4*	732±10*
+Taurine 1mM	162±6**	166±6**	185±7**

Results are mean ±SEM of at least three different experiments. *significantly different from control group ($p<0.05$).
** Significantly different from amitriptyline treated group ($P<0.05$)

Table 4: the effect of amitriptyline on lipid peroxidation and protective effects of Taurine TBARS µm/10^6 cell

	Time(min)		
	60	**120**	**180**
Amitriptyline 40µm	0.26±0.030*	0.33±0.020*	0.48±0.040*
+Taurine 1mM	0.12±0.011**	0.22±0.017**	0.28±0.018**
+cimetidine 2mM	0.17±0.010**	0.22±0.020**	0.28±0.020**

Results are mean ±SEM of at least three different experiments.
*significantly different from control group (p<0.05)
** Significantly different from amitriptyline treated group (P<0.05)

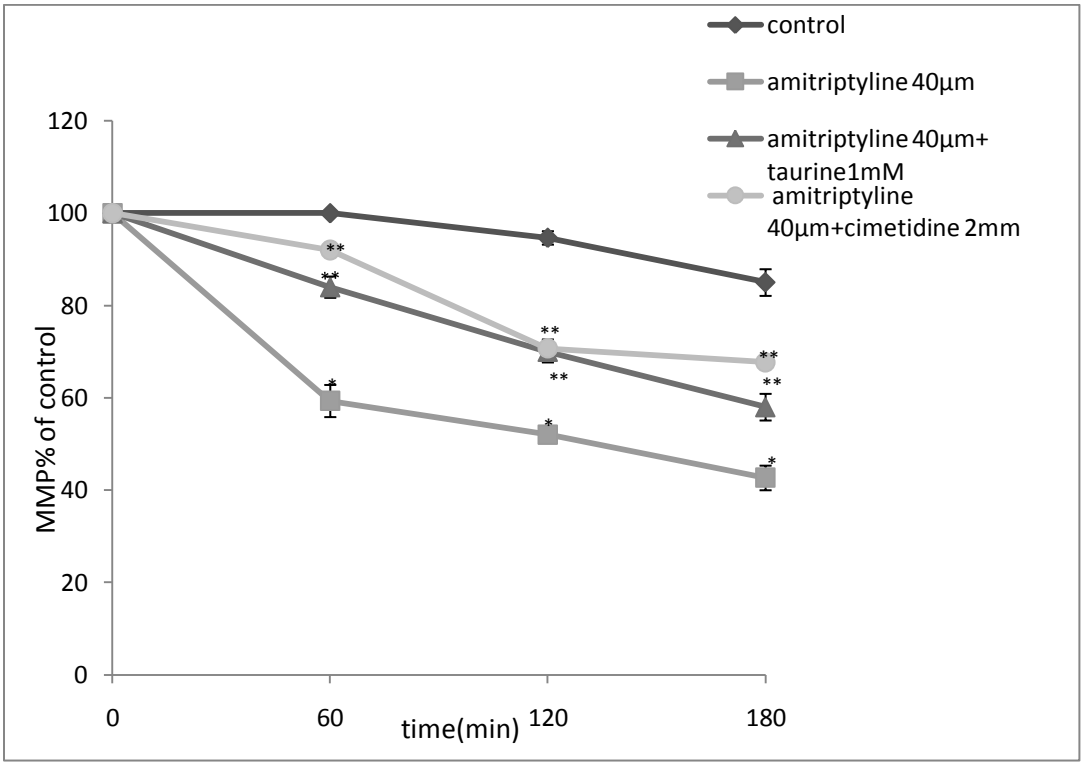

Fig.1.Modulating amitriptyline-induced mitochondrial membrane potential toxicity by taurine and Cimetidine (P450 inhibitor). Means ±SEM for three separate experiments are given.
* Significant as compared to control hepatocyte (P<0.05). ** Significant as compared to amitriptyline-treated hepatocyte (P<0.05).

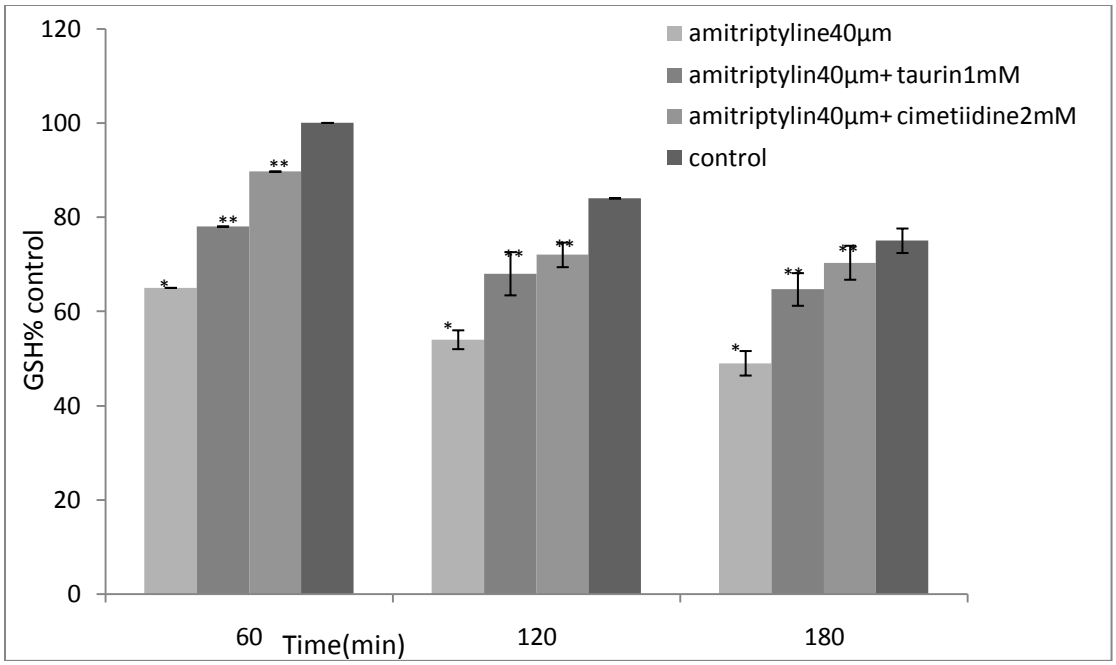

Fig.2. Amitriptyline decreased GSH levels by taurine and Cimetidine (P450 inhibitor).Means ±SE for three separate experiments are given.
* Significant as compared to control hepatocyte (P<0.05).
** significant as compared to amitriptyline-treated hepatocyte(P<0.05).

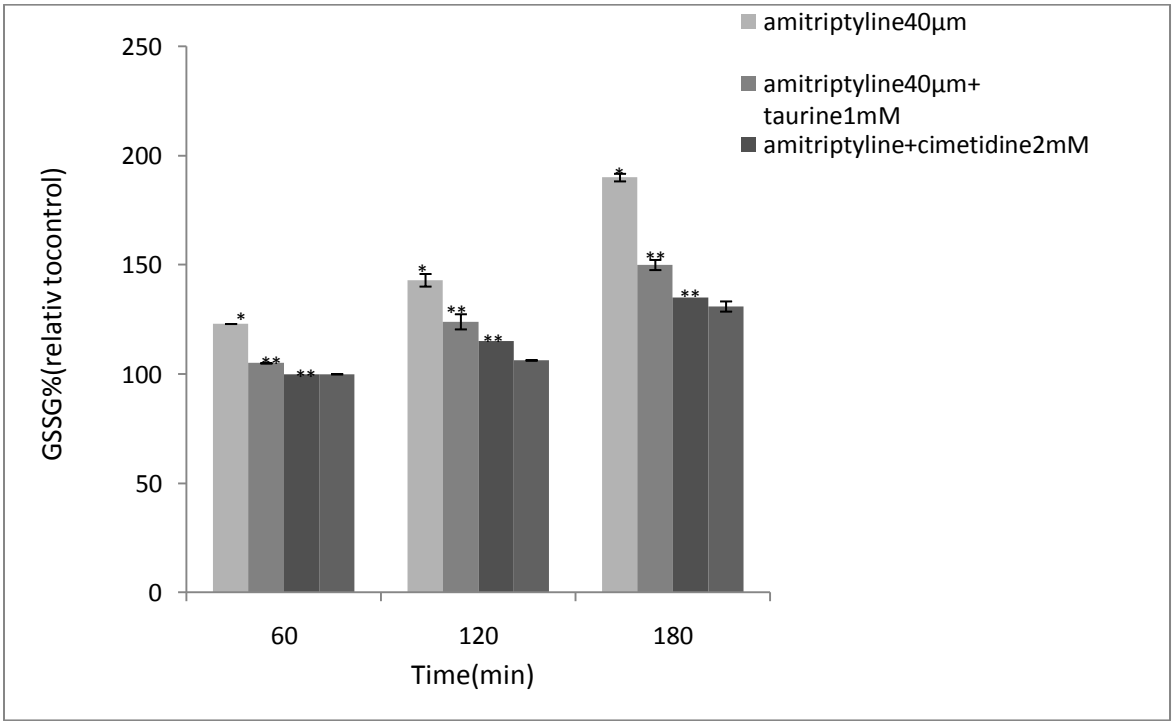

Fig.3. Modulating amitriptyline increased GSSG levels by taurine and cimetidine. Means ±SEM for three separate experiments are given.
* Significant as compared to control hepatocyte (P<0.05). ** Significant as compared to amitriptyline-treated hepatocyte (P<0.05).