Protective Role of Melatonin and Taurine Against Carbamazepine-induced Toxicity in Freshly Isolated Rat Hepatocytes

Mohammad Ali Eghbal*,**; Shohreh Taziki*,**,*** & Mohammad Reza Sattari****


SUMMARY: Carbamazepine is widely used in a broad spectrum of psychiatric and neurological disorders. Idiosyncratic hepatotoxicity is a well-known adverse reaction associated with carbamazepine. Hepatotoxicity is rare, but a real concern when initiating therapy. It was found that oxidative stress is a potential mechanism for carbamazepine-induced hepatotoxicity. Present study evaluated the hepato protective role of taurine and melatonin against carbamazepine-induced hepatotoxicity. Hepatocytes were prepared by the method of collagenase enzyme perfusion via portal vein. Cells were treated with 400 $\mu$M carbamazepine, 1mM taurine, and 1mM melatonin. Cell death, reactive oxygen species formation, lipid peroxidation, and mitochondrial membrane depolarization were assessed as toxicity markers and the effects of taurine and melatonin administration on them were investigated. Our results showed that carbamazepine induced oxidative stress; increased ROS formation and lipid peroxidation products and also decreased mitochondrial membrane potential ($\Delta$Ψm). Carbamazepine caused a decrease in cellular glutathione content and an elevation in oxidized glutathione levels. Our investigation showed that preincubation of hepatocytes with taurine (1 mM) could alleviate oxidative damages induced by carbamazepine; melatonin was also a good antioxidant to protect hepatocytes against cytotoxicity induced by carbamazepine. It may be concluded that taurine and melatonin are effective antioxidants to prevent carbamazepine-induced hepatotoxicity. Following our findings, further studies are suggested on the antioxidant effects of taurine and melatonin in patients receiving carbamazepine.

KEY WORDS: Carbamazepine; Hepatotoxicity; Oxidative stress; Taurine; Melatonin.

INTRODUCTION

Carbamazepine, one of the aromatic antiepileptic drugs, has been extensively used for treatment of psychiatric and neurological disorders. It was previously reported that aromatic antiepileptic drugs are potentially hepatotoxic. Hepatotoxicity associated with carbamazepine is rare but considerable. A transient increase of liver enzymes occurred in 25-61% of patients administered carbamazepine (Ahmed & Siddiqi, 2006; Forbes et al., 1992). The so-called hepatotoxicity progresses usually during the first six weeks after the onset of treatment (Santos et al., 2008a). In some investigations, it has been shown that reactive metabolites of carbamazepine could be involved in carbamazepine-induced hepatotoxicity. It is assumed that the accumulation of the arene oxide metabolites could be the responsible mechanism for the cellular damage caused by this drug (Bavdekar et al., 2004). Accumulation of arene oxide metabolite leads to direct mitochondrial toxicity, and also could result in immune response (Santos et al., 2008b).

Previous study showed that both the mitochondrial dysfunction and the oxidative stress caused by the metabolites of carbamazepine-induced hepatotoxicity (Santos et al., 2008a). To prevent carbamazepine-induced toxicity, no particular protective agents have been reported. Taurine is an essential amino acid containing a sulfonic acid group with several physiological roles. Taurine is a potent antioxidant and is able to scavenge reactive oxygen species (ROS), attenuate lipid peroxidation and stabilize biomembranes (Nandhini et al., 2005; Schaffer et al., 2009). There are many reports on taurine’s protective effects against hepatotoxicities induced by different chemicals such as acetaminophen, carbon tetrachloride and retinol (Gaull et
al., 1985; Nakashima et al., 1982; Waters et al., 2001). Melatonin is a hormone found naturally in the body. Previous studies demonstrated that melatonin has protective effect against oxidative damages caused by free radical species in cells or tissues (Hong et al., 2009). It efficiently scavenges the hydroxyl radical and possibly the peroxy radical (Parmar et al., 2002). The hepatoprotective effect of melatonin against non-alcoholic fatty liver has been demonstrated in vivo (Pan et al., 2006). Based on our medical article databases, there are few studies on the hepato-protective role of antioxidants against carbamazepine-induced hepatotoxicity. In the present study, we investigated the protective roles of taurine and melatonin against cytotoxicity induced by carbamazepine towards freshly isolated rat hepatocytes. Cell death, ROS formation, lipid peroxidation and mitochondrial damage were considered as toxicity markers and the effects of taurine and melatonin on them were studied. Furthermore, the levels of cellular reduced and oxidized glutathione were measured to evaluate the ability of taurine and melatonin in preventing carbamazepine induced hepatotoxicity.

MATERIAL AND METHOD

Collagenase, carbamazepine and melatonin were purchased from Sigma Aldrich Chemical Co. (St. Louis, USA). Taurine, 4-2-hydroxyethyl-1-piperazine-ethanesulfonic acid (HEPES), triethanolamine, oxidized glutathione (GSSG), and 2-vinyl pyridine were obtained from Acros (New Jersey, USA). Other reagents were obtained from Merck Chemical Co. (Darmstadt, Germany). Taurine was prepared in water and melatonin and carbamazepine were dissolved in methanol. The amount of solvents in media was less than 20 µl/10ml. The solvents have no effects on toxin or antioxidants effects.

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

We isolated the hepatocytes from rat livers using collagenase perfusion technique which has been described in details before by Eghbal et al. (2004). First, through the portal vein, the liver parenchyma was perfused with different buffer solutions. So, the collagenase enzyme in buffer solution destructed the liver tissue and lead to easily isolation of hepatocytes during the next steps (Eghbal et al.). Isolated hepatocytes (10 ml, 10^6 cells/1 ml) were suspended in Krebs–Henseleit buffer (PH=7.4) containing 12.5 mM HEPES in continuously rotating 50 ml rounded bottomed flasks at 37°C water bath under an atmosphere of carbogen gas (95% O2 and 5% CO2). Only the cells with viability of over the 85% were used. According to another study, carbamazepine as a parent compound was not able to induced oxidative stress. Therefore, activation of carbamazepine by the liver microsomal enzymes was a necessary step in the production of oxidative stress (Santos et al., 2008a). So, in this study, Cytochrome P450 enzymes were induced by Intra-peritoneal injection of Phenobarbital (10 mg/kg) to the rats for 3 days before the isolation of hepatocytes (Madan et al., 2003). Hepatocyte viability was determined microscopically by plasma membrane intactness as determined by trypan blue (0.1%/w/v) exclusion test (Moldeus et al., 1978). Hepatocyte viability was determined every 60 minutes for 180 minutes.

To determine the extent of ROS generated by carbamazepine, 1.6 µl of 2-7-dichlorofluorescein diacetate (DCFH-DA) was added to hepatocytes. DCFH-DA hydrolyzed to non-fluorescent DCFH in hepatocytes, DCFH reacted with ROS and became highly fluorescent. In 60,120,180 minutes 1 ml (10^6 cells) of sample centrifuged at 3000 g for 1 minute, then the fluorescence of supernatant was detected fluorimetrically at excitation and emission wavelengths of 490 nm and 520 nm respectively (Anoush et al., 2009).

Hepatocytes lipid peroxidation was detected by measuring thiobarbituric acid reactive substance (TBARS) such as malondialdehyde (MDA) that formed during the decomposition of lipid hydroperoxides. 250 µl of trichloro acetic acid (TCA, 70% w/v) was added to 1 ml (10^6 cells) of hepatocyte suspension and centrifuged at 3000 g for 15 minutes, then 1 ml of thiobarbituric acid (0.8% w/v) was added to the supernatant and boiled for 20 minutes. The absorbance was measured at 532 nm using an UltrospecR 2000 spectrophotometer (Smith et al., 1982). TBARS formation was expressed as mM TBARS/10^6 cells -1.

To determine the Mitochondrial membrane potential (MMP); Rhodamine 123 (the fluorescent dye) accumulate in intact mitochondria by facilitated diffusion. When the MMP is altered by a toxin, there is no facilitated diffusion and the amount of Rhodamine 123 in media is increased. Two ml samples of the cell suspension were taken every 60 minutes for 180 minutes and centrifuged at 1000 g for 1 minute then the cell was suspended in 2 ml of Krebs-Henseleit buffer containing 1.5 µM Rhodamine 123 and incubated at 37°C water bath. Then centrifuged at 3000 g for 1 minute and hepatocytes were separated.
of Rhodamine 123 in media was determined using a Jusco FP-750 fluorescence spectrophotometer at 490 nm excitation and 520nm emission wavelengths. The difference of capacity of mitochondria to uptake the Rhodamine 123 between control group and treated group was expressed as percentage of control (Eghbal et al.; Heidari et al., 2012).

The hepatocyte glutathione (GSH) content was determined by the method of Elman (Riener et al., 2002). A 1 ml aliquot of cell suspension (10^6 cells/ml) was taken and treated with 2 ml of trichloroacetic acid (5% w/v) and centrifuged at 3000 g for 2 minutes. 0.5 ml Elman reagent (0.0198% DTNB in 1% sodium citrate) and 3ml of phosphate buffer (pH=8.0) were added to supernatant. The color that developed was read at 412 nm using an Ultrospec 2000 R spectrophotometer (Pharmacia Biotech). The samples were reduced with potassium borohydride (KBH4) to prevent the auto oxidation of Glutathione during the experiment (Kleinman, W. A. & Richie, 2000). The enzymatic recycling method was used to assess the hepatocyte oxidized glutathione (GSSG) level, 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 Elman reagent (0.0198% DTNB in 1% sodium citrate). Absorbance was recorded at 412 nm after 60, 120 and 180 minutes (Heidari et al.; Rahman et al., 2006). The amount of GSSG was determined from a standard curve.

Difference between control and treated groups was expressed as percentage of control. Results represent the Mean±SEM of at least three independent experiments. Statistical significance of difference between control and treatment groups was determined using one way analysis of variance (ANOVA) followed by a Tukey’s post hoc test. The minimal level of significance was (P < 0.05).

RESULTS

Carbamazepine toxicity in rat hepatocytes was concentration-dependent. As shown in Figure 1, incubation of hepatocytes with 400 µM of carbamazepine led to the death of 50% of the cells in 2h (LC50=400 µM). An optimum effective dose of taurine that provided appropriate protection was found 1 mM. Hepatocytes were treated with taurine 30 minutes before adding carbamazepine. It was found that taurine effectively prevented cell death induced by carbamazepine. Melatonin at the concentration of 1mM caused a significant decline in hepatocytes death (p<0.05).

As Figure 2 illustrates, carbamazepine 400 µM caused an increase in ROS formation and pre-incubation of hepatocytes with 1mMtaurine,decreasedROS formation conspicuously. Incubation of hepatocytes with 1 mM melatonin, also decreased ROS formation significantly (p<0.05).

![Fig. 1. Protective effect of taurine (1 mm) and melatonin (1 mm) against cell death induced by carbamazepine(400 µm) in isolated rat hepatocytes. Results are mean ±SEM of at least three different experiments. * significantly different from control group (p<0.05) ** significantly different from carbamazepine-treated hepatocytes (P<0.05)
As shown in Figure 3, carbamazepine increased the amount of lipid peroxidation biomarkers meaningfully after 120 minutes. Pre-incubation of hepatocytes with 1 mM taurine prevented TBARS production significantly (p<0.05), and incubation of hepatocytes with 1 mM melatonin, also reduced production of TBARS (p<0.05).

According to Figure 4, the effect of carbamazepine on the mitochondria as the energy-producing and key organelle of hepatocytes was evaluated. It was found that carbamazepine caused a reduction in MMP. It indicates the toxic effects of carbamazepine towards mitochondria. Taurine attenuated the reduction in MMP caused by carbamazepine. Also in the
presence of 1 mM melatonin, the mitochondrial toxicity declined dramatically (p<0.05) (Fig. 4).

As illustrated in Figure 5, carbamazepine exposure significantly diminished GSH levels of hepatocytes, a result that is in line with the results of the tests of ROS formation and Lipid peroxidation. Glutathione contents of carbamazepine-exposed cells were notably increased in the melatonin- and ortaurine-supplemented groups (p<0.05).

Carbamazepine exposure significantly increased the GSSG levels in hepatocytes (Fig. 6). Treating rat hepatocytes with taurine (1 mM) significantly reduced the amount of GSSG formed after carbamazepine administration (p<0.05). Also, GSSG levels in carbamazepine-exposed cells were notably diminished in the melatonin (1 mM) treated groups (p<0.05).
DISCUSSION

In this study, we investigated oxidative stress biomarkers such as ROS formation, lipid peroxidation, MMP, and the levels of protein sulfhydryls in isolated rat hepatocytes in the absence or presence of an antioxidant. We established cytoprotective strategies concerning the hepatotoxicity associated with carbamazepine therapy. The toxicity was investigated in hepatocytes induced by phenobarbital (CYP450 inducer), because of the probability that the intermediate areneoxides formed in biotransformation might be the causes of the toxicity (Bavdekar et al.; Kalapos, 2002; Madden et al., 1996; Shear & Spielberg, 1988). The results of our study showed that oxidative stress was involved in hepatotoxicity induced by carbamazepine and its metabolites. This is in agreement with the previous findings (Santos et al., 2008a, 2008b; Aycicek & Iscan, 2007). Exposure to carbamazepine dramatically increased ROS production, enhanced oxidative stress and induced cell death in hepatocytes. Reactive oxygen species may be involved in the progressive course of this hepatotoxicity. Therefore, we evaluated whether taurine, an endogenous antioxidant, could limit the extent of liver injury. In this study we found that the cell viability was improved upon taurine treatment. Pre-incubation of hepatocytes with taurine could significantly decrease ROS formation. Taurine is an amino acid with a sulfonic acid group, and probably reacts with reactive oxygen species; complex formation between sulfonic acid group (SO3 –) to free ion species has been reported (Trachtman et al., 1992). Hence, the reactive oxygen species formed during carbamazepine metabolism were scavenged by taurine, this may have had a role in its protective effects in carbamazepine cytotoxicity. Lipid peroxidation is usually one of the consequences of ROS formation and oxidative stress in biological systems (Benzie et al., 1996).

Taurine could protect hepatocytes from lipid peroxidation induced by carbamazepine after 2 h of incubation. The role of taurine in attenuating the lipid peroxidation induced by carbamazepine might be due to its effects in modulating the oxidative stress caused by this drug. It is believed that taurine’s ability to stabilize cell membranes may be attributed to several events, one of them is prevention of lipid peroxidation (van Gelder, 1990). The effect of carbamazepine and its metabolite on the mitochondria as the energy-producing and key organelle of hepatocytes was evaluated. It was found that carbamazepine decreased MMP, that is in line with the previous finding demonstrating the mitochondrial toxicity induced by carbamazepine and its metabolites. It is noteworthy that mitochondrial dysfunction is generally accompanied by oxidative stress; a key regulator of mitochondria-mediated cell death in hepatocytes (Santos et al., 2008b).
One of the pathways for disruption of mitochondrial function involves ROS formation and GSH oxidation. ROS formation and GSH depletion increases permeability transition pore (PTP) formation; PTP opening can also lead to the release of cytochrome C, ultimately causing apoptotic cell death (Eghbal et al.). Our results showed that taurine attenuated the reduction in MMP caused by carbamazepine. This effect may be due to the ability of taurine in scavenging the reactive metabolites produced during carbamazepine metabolism. High concentrations of GSH are present in most living cells. It is believed that GSH is involved in responses to various oxidative stresses. GSH serves several vital functions, including detoxifying electrophiles, maintaining the essential thiol status of proteins, scavenging free radicals, modulating critical cellular processes such as DNA synthesis, and immune function. Severe oxidative stress may overcome the ability of the cell to reduce GSSG to GSH, leading to the accumulation of GSSG within the cytosol (Lu, 1999). In this study, carbamazepine decreased GSH and increased GSSG levels. Our results showed that taurine could prevent oxidation of glutathione. Taurine is a ROS scavenger and decreases the level of free radicals. Therefore, in the supplemented group with taurine, less GSH was oxidized to GSSG, and GSSG accumulation was prevented. With regard to melatonin, incubation of hepatocytes with the 1mM of melatonin decreased the death rate of hepatocytes significantly. Both ROS formation and TBARS production were reduced in the presence of melatonin. These findings confirm previous studies regarding the antioxidant effects of melatonin. Melatonin is an efficient scavenger of ROS which are abundantly produced in mitochondria (Parmar et al.). Hence, the reactive oxygen species formed during carbamazepine metabolism were scavenged by melatonin; this might have a role in its protective effects in carbamazepine cytotoxicity. A reduction in cellular glutathione by carbamazepine or its metabolite leaves hepatocytes defenseless against different stresses such as ROS formation. This may have a role in carbamazepine-induced hepatotoxicity. Melatonin can react with free radicals or reactive metabolites produced during carbamazepine metabolism, and prevent glutathione consumption in hepatocytes. Preventing the depletion of glutathione reservoirs could be another mechanism by which melatonin reduces the toxicity of carbamazepine or its reactive metabolites in hepatocytes. In vitro experiments showed that melatonin could prevent oxidative damage in mitochondria induced by t-butyl hydro peroxide. Previous findings documented that melatonin can stimulate complexes 1 and 4 activities. It was reported that ATP production elevated in the presence of melatonin (Martín et al., 2002). Therefore, we evaluated whether melatonin could prevent the decline of MMP induced by carbamazepine. Our data showed that melatonin supplementation could prevent the decrease of MMP. It might have occurred as a consequence of the ROS scavenging effect of melatonin (El Idrissi et al., 2003).

CONCLUSION

According to our study results, it could be concluded that taurine and melatonin are effective antioxidants in prevention of carbamazepine-induced hepatotoxicity. However, there was no statistically significant difference in hepatoprotective effects between melatonin and taurine. We recommend further clinical trial studies on the antioxidant effects of taurine and melatonin in patients receiving carbamazepine.

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RESUMEN: La carbamazepina es ampliamente utilizada en un gran espectro de trastornos psiquiátricos y neurológicos. La hepatotoxicidad idiosincrásica es una conocida reacción adversa asociada con la carbamazepina. La hepatotoxicidad es rara, pero es una preocupación real al iniciar el tratamiento. Se ha reportado que el estrés oxidativo es un potencial mecanismo para la hepatotoxicidad inducida por carbamazepina. El presente estudio evaluó la función hepato-protectora de la taurina y melatonina contra la hepatotoxicidad inducida por carbamazepina. Los hepatocitos se prepararon por el método de perfusión de la enzima colagenasa a través de la vena porta. Las células fueron tratadas con 400 µM de carbamazepina, 1 mM de taurina, y 1 mM de melatonina. La muerte celular, formación de especies reactivas de oxígeno (ERO), peroxidación de lípidos, y despolarización de la membrana mitocondrial fueron evaluadas como marcadores de toxicidad, junto con investigar los efectos de la taurina y melatonina administrada en ellos. Nuestros resultados mostraron estrés oxidativo inducido
por carbamazepina, con aumento de las ERO, formación de productos de la peroxidación lipídica y disminución del potencial de membrana mitocondrial ($\Delta\Psi_m$). La carbamazepina causó una disminución en el contenido celular de glutatión y una elevación de los niveles de glutatión no-oxidado. Se observó que la preincubación de los hepatocitos con taurina (1 mM) podría aliviar los daños oxidativos inducidos por carbamazepina; además la melatonina también fue un buen antioxidante para proteger a los hepatocitos. Se puede concluir que tanto la taurina y melatonina son antioxidantes eficaces para prevenir la hepatotoxicidad inducida por carbamazepina. Tras nuestros resultados, se sugiere estudiar los efectos antioxidantes de la taurina y melatonina en pacientes tratados con carbamazepina.

**PALABRAS CLAVE:** Carbamazepina; Hepatotoxicidad; Estrés oxidativo; Taurina; Melatonina.

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Correspondence to:
Mohammad Ali Eghbal
Department of Pharmacology & Toxicology
Faculty of Pharmacy
Tabriz University of Medical Sciences
Tabriz
IRAN

Tel: +98 411 337 2250
Fax: +98 411 334 4798

Email: m.a.eghbal@hotmail.com

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