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Research Article

Cytoprotective Effects of Melatonin Against Amitriptyline-Induced Toxicity in Isolated Rat Hepatocytes

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Abstract

***Purpose*:** Amitriptyline, one of the commonly used tricyclic antidepressants, caused rare but severe hepatotoxicity in patients who received it continuously. Previous findings showed that the intermediate metabolites of amitriptyline produced by CYP450 are involved in hepatic injury. Melatonin is an antiaging and antioxidant hormone synthesized from pineal gland. The aim of present study was to evaluate the protective role of melatonin in an *in vitro* model of isolated rat hepatocytes.

Methods: Markers such as cell viability, reactive oxygen species formation, lipid peroxidation, mitochondrial membrane potential, and hepatocytes glutathione content were evaluated every 60 minutes for 180 minutes.

Results: Present results indicated that administration of 1mM of melatonin effectively reduced the cell death, ROS formation and lipid peroxidation, mitochondrial membrane potential collapse, and reduced cellular glutathione content caused by amitriptyline.

Conclusion: Our results indicated that melatonin is an effective antioxidant in preventing amitriptyline-induced hepatotoxicity. We recommend further *in vivo* animal and clinical trial studies on the hepatoprotective effects of melatonin in patients receiving amitriptyline.

Introduction

Melatonin is produced in the vertebrate pineal gland, the retina and possibly some other organs. This hormone plays several physiological roles such as regulation of sleep. Also there are many reports that melatonin's antioxidant action contributes to the protection of the organism from carcinogenesis and neurodegenerative disorders. It acts as a potent antioxidant by mechanisms such as stimulation of antioxidant enzymes, increasing the efficiency of mitochondrial oxidative phosphorylation and reducing free radical generation and direct free radical scavenging.[1-4] There are many reports on melatonin's protective effects against different chemicals-induced hepatotoxicity.[5,6]

It has been reported that amitriptyline increased liver enzymes in patients that received it continuously.[7] It is assumed that the accumulation of the aren oxide metabolites could be the responsible mechanism for the cellular damage caused by this drug.[8-10] It was previously reported that amitriptyline induced oxidative stress, it increased ROS formation and lipid peroxidation also amitriptyline increased GSH consumption in isolated rat hepatocytes.[9] In addition, a previous study showed that amitriptyline caused mitochondrial membrane potential reduction.[9] Based on our medical article data bases, there are few studies on the hepatoprotective role of antioxidants against amitriptyline-induced hepatotoxicity. In the present study, we investigated, for the first time, the protective roles of melatonin against cytotoxicity induced by amitriptyline towards freshly isolated rat hepatocytes.

Trypan blue exclusion test was used to measuring the percent of viable cells. The possibility of reactive oxygen species formation and lipid peroxidation was assessed and the effect of amitriptyline on intracellular glutathione was evaluated. In addition, the effect of amitriptylin on hepatocyte mitochondria was studied.

Materials and Methods

Chemicals

Amitriptyline, melatonin (N-Acetyl-5-methoxytryptamine) and Collagenase were obtained Sigma Aldrich chemical Co (St. Louis, USA). Other reagents were purchased from Merck chemical CO (Darmstadt, Germany).

Amitriptyline was dissolved in water and melatonin was dissolved in methanol. The amount of solvent in media was less than 20µl. The solvents had no effects on toxin or antioxidant effects. Male Sprague-Dawley rats (250–

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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.

The rat liver was perfused with different buffer solutions through the portal vein. Hepatocytes were isolated from liver using collagenase perfusion technique which has been described in details before by Eghbal et al.[9] The collagenase enzyme in buffer solution destructed the liver tissue and lead to easily isolated of hepatocytes during the next steps.[9] 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.

Cell viability

Hepatocytes viability was measured by the trypan blue (0.1%, w/v) exclusion test microscopically.[11,12] Hepatocytes viability was determined every 60 minutes for 180 minutes to evaluate the effect of amitriptyline on cell viability, determining LC_{50} (lethal concentration 50%) dose of amitriptyline and testing the protective effects of melatonin against cell death induced by amitriptyline.[9,13-15]

Reactive oxygen species formation

To determine the extent of ROS generation by amitriptyline, 1.6 µl of 2.7- dichlorofluoresceine diacetate was added to hepatocytes flasks. DCFH-DA hydrolyzed to non-fluorescent DCFH in hepatocytes, DCFH reacted with ROS and became highly fluorescent. In 60,120,180 min 1 ml(10^6cell) 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.[16-20]

Lipid peroxidation Measurement

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 (10^6cell) 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 an Ultrospec 2000 spectrophotometer.[21,22]

Mitochondrial membrane potential

Mitochondrial membrane potential was evaluated as a marker of toxicity induced by amitriptyline. To determining mitochondrial membrane potential Rhodamine 123 (the fluorescent dye) was used. This dye accumulates in intact mitochondria by facilitated diffusion. When the mitochondrial membrane potential is reduced by a toxin the amount of Rhodamine 123 in media is increased. One mL sample was taken from the cell suspension at programmed time points, and centrifuged at 1000 rpm for 1 minute. The cell was resuspensed in 2 ml of krebs-Henseleit buffer containing 1.5 µM Rhodamine 123 and incubated at 37°C water bath for 10 minutes. Hepatocytes were separated by centrifugation (3000 rpm for one minute) and the amount of rhodamine 123 appearing in the incubation medium were determined fluorimeterically at 490 nm excitation and 520 nm emission wavelengths using a Jasco® FP-750 spectrofluorometer.[1,23]

Determination of Hepatocytes GSH/GSSG content

To determine the hepatocyte glutathione (GSH) content the method of Ellman was used.[24] 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.[25] The enzymatic recycling method was used to assess the hepatocyte oxidized glutathione (GSSG) level.[26] 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).[2,27]

Statistical analysis

Results are given 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 followed by a Tukey's post hoc test. A $P < 0.05$ was considered as a significant difference.

Results

Trypan blue exclusion test was used to determine the ability of hepatocytes to maintain their viability with amitriptyline alone or in combination with melatonin.

Previous finding showed that amitriptyline caused cell death in a concentration dependent manner. The concentration in which amitriptyline caused 50 % cell death (LC_{50}) was found to be 40µM.[9] Our findings indicated that an effective dose of melatonin that provided suitable protection was 1mM (Table 1). Melatonin administration effectively prevented cell death induced by amitriptyline ($P<0.05$) (Table 1).

Markers such as ROS formation, lipid peroxidation, cellular glutathione content, and mitochondrial membrane potential were measured to study the

mechanism by which melatonin protected hepatotoxicity induced by amitriptyline.

Table 1. Protective effect of melatonin against amitriptyline induced toxicity in isolated rat hepatocytes

Cytotoxicity (% Trypan blue uptake)			
Incubate	time(min):60	120	180
Control (only hepatocytes)	21±1	24±1	28±1
+Methanol 10µl	23±2	26±1	30±2
+Melatonine 1mM	20±3	22±2	24±3
+ Amitriptyline 40µm	41±3*	52±3*	59±2*
+ Melatonin 1mM	21±3 **	30±2**	37±2**
+Amitriptyline 200µM	100	100	100
+Amitriptyline 100µM	100	100	100
+Amitriptyline 20µM	32±3	40±5	50±2

Isolated rat hepatocytes (10^6 cells/mL) were incubated at 37°C in rotating round bottom flasks with 95 % O_2 and 5 % CO_2 in Krebs-Henseleit buffer (pH 7.4).
The results shown represent Mean±SE for three separate experiments.
*: Significantly different as compared to control group (P<0.05).
**: Significantly different from amitriptyline-treated group (P<0.05)

According to Figure 1, when hepatocytes were treted with melatonin (1 mM), the amount of ROS formed by amiytriptyline was suppressed drastically (P<0.05).

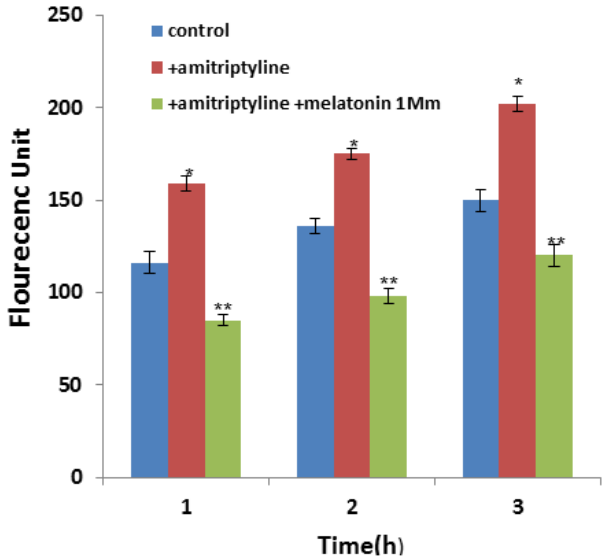

Figure 1. Protective effect of melatonin against amitriptyline induced ROS formation in isolated rat hepatocytes
Isolated rat hepatocytes (10^6 cells/mL) were incubated at 37 °C in rotating round bottom flasks with 95 % O_2 and 5 % CO_2 in Krebs-Henseleit buffer (pH 7.4).
The results shown represent Mean ± SE for three separate experiments.
*: Significantly different as compared to control group (P<0.05).
**: Significantly different from amitriptyline-treated group (P<0.05)

Previous findings showed that a notable amount of thiobarbituric acid reactive substances (TBARS) was formed in amitriptyline-treated rat hepatocytes as compared to the control group.[9] This indicates lipid peroxidation induced by cytotoxic concentrations of the drug. As shown in Figure 2 incubation of hepatocytes with 1mM of melatonin, prevented lipid peroxidation induced by amitriptyline (P<0.05).

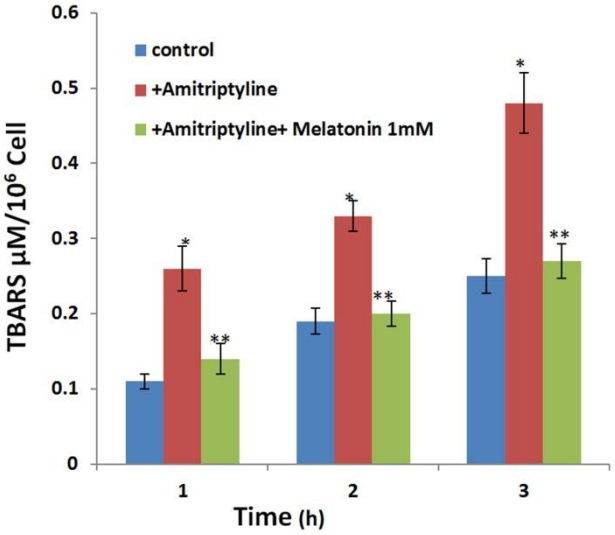

Figure 2. Protective effect of melatonin on lipid peroxidation induced by amitriptyline
Isolated rat hepatocytes (10^6 cells/mL) were incubated at 37 °C in rotating round bottom flasks with 95 % O_2 and 5 % CO_2 in Krebs-Henseleit buffer (pH 7.4).
The results shown represent Mean ± SE for three separate experiments.
*: Significantly different as compared to control group (P<0.05).
**: Significantly different from amitriptyline-treated group (P<0.05)

Previous findings showed that the LC_{50} dose of amitriptyline (40 µM) caused a reduction in mitochondrial membrane potential as measured by rhodamine 123 test.[9] This indicates that mitochondria could be a target for amitriptyline.[9] Melatonin was administered with amitriptyline to investigate if it could alleviate mitochondrial injury induced by amitriptyline. In comparison between the groups receiving amitriptyline alone and the group receiving amitriptyline in combination with 1mM melatonin, the degree of permeability of Rhodamine to the mitochondria was increased by melatonin significantly. It was found that melatonin effectively prevented mitochondrial depolarization caused by amitriptyline (Table 2).
Previous findings indicated that hepatocytes glutathione content was diminished notably when hepatocytes were treated with amitriptyline (40µm).[9] According to Table 3, comparing the group receiving only amitriptyline to the group receiving amitriptyline and 1mM melatonin, GSH content of cells was conspicuously increased in the melatonin-supplemented groups (P<0.05).

Table 2. protective effect of melatonin on mitochondrial toxicity induced by amitriptyline

% Control			
Incubation time (min): Incubate	60	120	180
Control (only hepatocytes)	100±0	94±2	85±3
+ Amitriptyline 40µm	60±3*	52±1*	43±3*
+ melatonin 1mM	80±3 **	68±4 **	60±3**

Isolated rat hepatocytes (10^6 cells/mL) were incubated at 37°C in rotating round bottom flasks with 95 % O_2 and 5 % CO_2 in Krebs-Henseleit buffer (pH 7.4).
The results shown represent Mean ± SE for three separate experiments.
*: Significantly different as compared to control group (P<0.05).
**: Significantly different from amitriptyline-treated group (P<0.05)

Table 3. Effect of melatonin on GSH consumption in presence of amitriptyline

% Control			
Incubation time (min): Incubate	60	120	180
Control (only hepatocytes)	100±0	84±3	75±3
+ Amitriptyline 40µm	65±2*	54±3*	45±3*
+ melatonin 1mM	90±1 **	76±2 **	65±3**

Isolated rat hepatocytes (10^6 cells/mL) were incubated at 37°C in rotating round bottom flasks with 95 % O_2 and 5 % CO_2 in Krebs-Henseleit buffer (pH 7.4). The results shown represent Mean±SE for three separate experiments.
*: Significantly different as compared to control group (P<0.05).
**: Significantly different from amitriptyline-treated group (P<0.05)

According to previous results, the amount of oxidized glutathione (GSSG) was increased in hepatocytes treated with 40µm amitriptyline.[9] According to Table 4, GSSG levels of amitriptyline-exposed cells were notably diminished in the melatonin-supplemented groups (P<0.05).

Table 4. Effect of melatonin on GSSG level in presence of amitriptyline

% Control			
Incubation time (min): Incubate	60	120	180
Control (only hepatocytes)	100±0	110±2	131±6
+ Amitriptyline 40µm	123±3*	143±2*	190±2*
+ melatonin 1mM	81±4**	115±6**	135±5**

Isolated rat hepatocytes (10^6 cells/mL) were incubated at 37°C in rotating round bottom flasks with 95 % O_2 and 5 % CO_2 in Krebs-Henseleit buffer (pH 7.4). GSSG level was measured as described by Irfan Rahman *et al* (2006)
The results shown represent Mean±SE for three separate experiments.
*: Significantly different as compared to control group (P<0.05).
**: Significantly different from amitriptyline-treated group (P<0.05)

Discussion

We have recently demonstrated that the cytotoxicity induced by amitriptyline was accompanied with ROS formation, lipid peroxidation, and mitochondrial depolarization. Moreover, reduction in cellular glutathione content was occurred. These results confirm the existence of the process of oxidative stress in hepatotoxicity due to amitriptyline.[9] Present results showed that hepatocytes supplementation with 1mM melatonin reduced cell death induced by amitriptyline and diminished the consequences of amitriptyline induced toxicity such as ROS generation, TBARS production and mitochondrial collapse. The protective effects of melatonin against amitriptyline could be due to its capability in scavenging reactive metabolites and conjugating with them. These findings are in line with the previous studies regarding the radical scavenger effects of melatonin.[3,4] Melatonin can rise the glutathione saving and decreases GSSG formation during amitriptyline toxicity notably (Table 3, 4). This might indicate the importance of melatonin in alleviating oxidative stress and scavenging reactive species to prevent the glutathione consumption as one of the main cellular defense mechanisms against toxic insults. This finding also supports the previous studies regarding the antioxidant effects of melatonin.[3,4] Mitochondrial depolarization can cause energy crisis and releasing of apoptotic signaling molecules, which could finally encounter cell death.[27] Administrations of 1mM melatonin provide protection against amitriptyline-induced mitochondrial collapse. There may be other functions of melatonin, yet undiscovered, which enhance its ability to protect against molecular damage by oxygen and toxic reactants. Protective effect of melatonin against amitriptyline-induced mitochondrial damage is through its activity in attenuating oxidative stress and scavenging reactive species.

Conclusion

According to our results, it could be concluded that melatonin is an effective protective agent in preventing amitriptyline-induced hepatotoxicity. We recommend further clinical trial studies on the antioxidant effects of melatonin in patients receiving amitriptyline. Also, the protective effect of melatonin against amitriptyline-induced toxicity proposes this agent as the subject of further studies for preventing different xenobiotics-induced liver damages, especially those accompanied by oxidative stress.

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Ethical Issues

The procedures carried out on the animals (rats) in accordance with the principiles for the care and use of laboratory animals, approved by the Ethics Committee in Tabriz university of Medical Sciences.

Conflict of Interest

The authors report no conflicts of interest.

Abbreviation

ROS, reactive oxygen species; MMP, mitochondrial membrane potential; TBARS, Thiobarbituric acid reactive substance

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