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Effect of Melatonin on Antioxidation in the PC12 Cell Line after Exposure to Ethanol

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ABSTRACT

Excessive ethanol consumption may increase the production of reactive oxygen species (ROS), which results in the oxidative damage of tissues. The purpose of this study was to evaluate the effects of melatonin on the antioxidative status in the PC12 cell line after exposure to ethanol. In this study, the PC12 cells were treated with the different concentrations of melatonin for 24 hr. Subsequently, the cells were incubated with a fresh medium containing 150 mM of ethanol for 4 hr. To observe the oxidative damage and evaluate the antioxidative status after acute exposure to ethanol, cell viability, percentage of lactate dehydrogenase released (% LDH released), glutathione (GSH) level, activities of superoxide dismutase (SOD) and glutathione reductase (GRx), and concentrations of the protein carbonyls were assayed. The results showed that cell viability and GSH were increased, whereas the ethanol-induced cytotoxicity and protein carbonyl concentration decreased when the PC12 cells were treated with 10, 100 and 1000 μM melatonin. In contrary, there was no significant difference in the activity of SOD in the ethanol-treated group. In addition, there were no significant differences in the activity of GRx in the groups with the treatment of 10 and 100 μM melatonin; however, GRx activity decreased in the cells treated with 1000 μM melatonin. In conclusion, melatonin of moderate concentration may be a protective agent to alleviate acute ethanol-induced oxidative damage by the generation of GSH in the PC12 cell line.

Key words: PC12 cell line, ethanol, melatonin, glutathione, protein carbonyl

INTRODUCTION

Excessive and prolonged ingestion of ethanol could result in the damage of human tissues, including liver[(1)], brain[(2)], nervous system[(3)], and digestive system[(4)]. In addition to the direct damages of ethanol, the ethanol-induced damage is related to its metabolic products[(5,6)]. ROS can cause damage to all types of biomolecules, including DNA, proteins, and lipids (lipid peroxidation). The carbonyl assay which is based on the fact that several ROS attack amino-acid residues in proteins to produce products with carbonyl groups is a general assay of oxidative protein damage. An elevation of the carbonyl compounds that are biomarkers of and leading to oxidative stress has been demonstrated in Down Syndrome (DS), Alzheimer's Disease (AD) brains, neurodegenerative diseases and aging[(7-9)].

It is well known that the intake of excess ethanol is toxic to human body; therefore, how to enhance the antioxidative capability for alcoholics is an interesting topic for us. Glutathione (GSH), a tripeptide product synthesized from cysteine, glutamate, and glycine, is a low-molecular-weight thiol reductant present in most cells. It is also well known that intracellular antioxidant GSH plays major roles in the maintenance of the redox status and defense of oxidative stress because it participates directly in the destruction of reactive oxygen species[(10)]. Numerous experimental studies have shown that the activity of antioxidant enzymes, for example SOD and GRx, exhibited various expressions with different conditions of oxidative stress[(11-14)]. A lot of research indicated that ethanol-induced damage could be reduced with appropriate antioxidants. It has been observed that certain hormone also has antioxidant ability. For example, melatonin, the primary hormone of the pineal gland, is both a potent free radical scavenger and a broad spectum antioxidant[(15,16)], in addition to its other well-known physiological functions. It has been speculated that primary function of melatonin may include protecting organisms from intrinsically or environmentally induced oxidative stress. Oner-Iyidogan *et al.*[(17)] indicated that melatonin might act as a protective agent against the testicular injury caused by ethanol consumption by enhancing the activity of antioxidative enzymes. In the

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study of El-Sokkary *et al.*[18], melatonin was shown to possess the ability to inhibit lipid peroxidation caused by chronic ingestion of ethanol. Roth *et al.*[19] indicated that the distinct expression from different concentrations of melatonin appeared in the growth or death of the PC12 cells.

To observe the direct effect of ethanol on neuron cells, the neuron-like cell line or the cell more sensitive to ethanol is usually used as a neuron cell model to study ethanol-induced stress. In the studies by Pantazis *et al.*[20] and Sun *et al.*[21], the PC12 cells were regarded as a model for examining the effect of ethanol on iron-induced lipid peroxidation and cell death. Therefore, the PC12 cell line is a useful model system for ethanol-induced neurobiological and neurochemical studies. The effects of ethanol-induced damage due to chronic ingestion were more focused than those of acute and high dosage[22]. In the study by Pantazis *et al.*[20], the proliferation of the PC12 cell lines was inhibited by the treatment with 100, 200, 400 and 800 mg/dL of ethanol after four days. In the study by Li *et al.*[23], the reduction of the PC12 cell lines was more than 75% when the cells had been treated with ethanol at a concentration higher than 100 mM after 24 hr. The common denominator of all the studies mentioned above is that the period of treatment was longer than 24 hr. The objective of this study was to evaluate the effect of high concentration of melatonin on the antioxidative status in the PC12 cell line after acute exposure to ethanol.

MATERIALS AND METHODS

I. *Materials*

The ethanol medium was made up of absolute ethanol (Sigma, Chemical Co., St. Louis, MO, USA) dissolved in Dulbecco's modified Eagle's medium (DMEM) (Gibco, Grand Island, NY, USA). The 1×10^5 μM of melatonin (M.W. 232.2 g, Sigma, USA) was made up of 23.2 mg melatonin dissolved in 1.0 mL of dimethylsulfoxide (DMSO) (Gibco, USA) and then diluted to 10, 100 and 1000 μM melatonin by fresh DMEM.

II. *Cell Culture*

The PC12 cell line (American Type Culture Collection, CRL-1721, USA) derived from a transplantable rat pheochromocytoma was used for this study because of its documented sensitivity to ethanol-toxicity and neuron-like characteristic. The cells were grown routinely in T75 plastic tissue culture flasks in DMEM containing 10% heat-inactivated fetal bovine serum (FBS, Biological Ind. Haemek, Israel), 100 μg/mL of streptomycin sulfate, 0.25 μg/mL of amphotericin, 100 units/mL of penicillin, 1 mM of non-essential amino acid and 100 U/mL of insulin (Biological Ind., Haemek, Israel) and incubated in 85% humidified air and 5% CO_2 at 37°C.

III. *Designed Concentration of Melatonin*

Melatonin levels in blood are low during the day (about 0.04 μM) and gradually elevated at 6 p.m. Melatonin levels peak (about 0.5 μM) at about 2 a.m. in healthy young people and at about 3 a.m. in elderly people. At sunset, the cessation of light triggers neural signals that stimulate the pineal gland to begin releasing melatonin. This rise continues for hours, eventually peaks around 2 a.m. after which it steadily declines to the minimal levels by morning. Both the delay in timing and the decrease in intensity of the melatonin pulse are manifestations of the aging process[24]. There were documented studies indicating that Roth *et al.*[19] showed the melatonin at lower concentrations (1~10 nM) suppressed the growth of the PC12 cells whereas at higher concentration (10 μM) prevented cell death; Song and Lahiri[25] indicated that the production of beta-amyloid precursor protein in the PC12 cells could be inhibited by the higher concentration (3~4 mM) of melatonin. In addition, in the study of Bao *et al.*[26], they indicated that the 1-methyl-4-phenylpyridinium-induced death in the PC12 cells could be suppressed by 250 μM of melatonin. Therefore, higher concentration of melatonin was used in order to evaluate whether melatonin could promote the antioxidant ability on the PC12 cells. Ten, 100 and 1000 μM melatonin were selected for this study. Nevertheless, the above-mentioned higher concentration of melatonin could be treated only in the *in vitro* studies. The concentrations of melatonin of normal physiological situation have to be considered if the experiments are carried out *in vivo*.

IV. *Treatment of Cell Lines*

On the first day, 1.5 mL of cell suspension (about 1×10^6 cell/mL) from cell culture flasks was transferred to culture plates of 6 wells in order to adhere to the wall of the culture plate in the control and experimental group, respectively. On the second day, we removed the medium from the culture plate and then added the medium with the equivalent volume of different concentrations of melatonin to the culture plate in both the control and experimental group. On the third day, the medium of melatonin was removed after 24 hr of incubation and the cells were washed with PBS twice to avoid the remains of melatonin in the culture plate. Subsequently, the cells were incubated for 4 hr with a fresh medium containing 150 mM ethanol in the control groups and experimental groups, respectively. To reduce the interference of the vaporation of ethanol, we sealed the culture plate with a parafilm and punctured three small holes with a syringe.

V. *Assay of Cell Viability*

Cell viability was assessed by trypan blue dye exclusion. In all experiments, the medium was gently removed from the well after 4 hr of ethanol exposure. The number of cells was counted by using a light microscope

on a hemocytometer slide. The cell numbers per well were determined by counting the cells of a given well three times, and the mean was calculated. Cell viability was expressed as the percentage of the cell number according to the following formula: Cell viability (%) = (cell numbers in the groups treated with melatonin at different concentrations ÷ cell numbers in the group without melatonin treatment) × 100.

VI. *Assay of Cytotoxicity*

Cytotoxicity was estimated by measuring the activity of the released LDH. After the PC12 cells were incubated with agents for 4 hr as shown above, the medium, which contained few detached cells, was centrifuged at 50 ×g for 5 min. The supernatant was used for the assay of the LDH activity on an autoanalyzer (model: Vitros 750, Johnson & Johnson). Total LDH in cells was determined after lysing cells with 0.1% triton X-100 in DMEM. Cytotoxicity was expressed as the percentage of the LDH released according to the following formula: % LDH released = [(LDH in medium) ÷ (LDH in medium + LDH in survival cells)] × 100[27].

VII. *Assay of GSH*

The 100 μL of cell suspension in an amber microcentrifuge tube was mixed with 0.1% MPA by the 1:2 volumetric ratio, and then it was centrifuged at 500 × g for 10 min after being adequately mixed by a vortex mixer. The supernatant was assayed on the capillary electrophoresis analyzer (P/ACE MDQ, Beckman Coulter) after being filtered with a 0.2 μm syringe set. The analysis was performed at a constant temperature (28°C) with 300 mM borate running buffer (pH 7.8) equipped with a UV absorbance detector set to 200 nm[28].

VIII. *Assay of SOD and GRx Activity*

Cells from the wells for viability assessment were mixed homogenously and were centrifuged at 50 ×g for 5 min. After the supernatant was removed, the cell pellet was remixed with 200 μL of PBS and then 100 μL of cell suspension was assayed for the activity of SOD, GRx and protein analysis, and another 100 μL for the measurement of GSH. One hundred microliter of cell suspension was mixed with 0.1% triton X-100 in the 1:2 volumetric ratio, and then centrifuged at 500 ×g for 10 min after being adequately mixed by a vortex mixer. The supernatant was assayed for the activity of SOD and GRx on Synchron CX 5 (Beckman Coulter). The activities of SOD and GRx were assayed with commercial assay kits (Randox Laboratory Ltd. Antrim, UK).

IX. *Assay of Protein Carbonyl*

The protein carbonyl content was measured according to the method described by Levine *et al.*[29]. Protein carbonyl derivatives readily reacted with 2,4-dinitropheny lhydrazine (2,4-DNPH) to form hydrazone derivatives that could be measured spectrophotometrically. The 150 μL supernatant of assaying SOD and GRx was reacted with 600 μL of 10 mM DNPH (in 2.5 M HCl) for 1 hr with occasional mixing, and TCA precipitates were washed three times with 1 mL of ethanol/ethyl acetate (1:1). Pellets were broken up mechanically. The final pellet was dissolved in 1 mL of 6 M guanidine hydrochloride and 0.5 M potassium phosphate, pH 2.5, and A_{370} were measured. A blank with the protein reacted with 2 M HCl containing no DNPH was carried for each sample and its absorbance was subtracted. The carbonyl content was determined as nmol/mg protein using $\varepsilon_{370} = 22000\ M^{-1}\ cm^{-1}$.

X. *Statistical Analysis*

Statistical results were analyzed with one-way ANOVA to assess the significance of differences between groups. Data were expressed as mean ± standard deviation (S.D.), statistically significant at $p < 0.05$. Each test was carried out in triplicate.

RESULTS AND DISCUSSION

I. *Assessment of the Cell Viability after Melatonin Treatment*

To establish LC_{50} as an *in vitro* model for evaluation, viability of the cells and ethanol-induced cytotoxicity were assayed by the treatment with 45, 95, 140, 220, 380, 460 mM of ethanol after 2, 4, 8 hr in culture plates with 96, 48, 6 wells, respectively. In this study, the viability of cells was 52.5% in 140 mM of the ethanol group, approximately matching our request for LC_{50} after ethanol treatment for 4 hr. As the result, we adopted the 150 mM of ethanol as LC_{50}. In Randall and Peter's study[30], human astroglia cells were utilized to examine the expression of inducible nitric-oxide synthase after the acute exposure to 50 and 200 mM ethanol. Although the concentration of ethanol was far above physiological levels, we still adopted it in order to establish a uniform condition for the evaluation model in the consequent experiments.

In this work we investigated the effect of melatonin on cellular viability of the PC12 cells. The results showed that exposure of the PC12 cells to ethanol induces a reduction on cell viability shown as Figure 1(A). As shown in Figure 1(A), there was not significant difference ($p > 0.05$) on the viability of cells in groups without ethanol treatment even though the concentrations of melatonin had been increased. But in the groups with ethanol treatment, the viability of cells with added melatonin increase more than the groups without added melatonin, especially the groups treated with 100 and 1000 μM of melatonin ($p < 0.01$). However, in the groups with higher concentrations of melatonin, the viability of cells showed no significant difference in the

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ethanol-treated groups compared with the groups without ethanol treatment. This study provides further evidence for the toxicity of ethanol in the PC12 cell line. With the cell models, we demonstrated that melatonin significantly increase cell viability and reduce the damage in the cells exposed to ethanol. In addition, although a significant increase after the addition of melatonin in cell viability was observed in the PC 12 cells, we also had to consider the physiological concentrations of melatonin to examine if higher concentrations of melatonin were applied to animal models.

II. *Assessment of Cytotoxicity*

The ethanol-induced toxicity was evaluated by determining the % LDH released upon incubation of the PC12 cells for 4 hr with 150 mM ethanol. As shown in Figure 1(B), the % LDH released of the ethanol-treated groups was apparently greater than the groups without ethanol treatment. But in the ethanol-treated groups, the % LDH released showed significant decrease ($p < 0.01$) after treatment of melatonin, especially in the 100 μM melatonin group. The % LDH released in 10 and 100 μM of the melatonin-treated groups showed no significance ($p > 0.05$) between the groups with ethanol or without ethanol. From the aforementioned results, the pretreatment of melatonin could prevent cells from the damages of acute ethanol-induced free radicals; the phenomenon might attribute to the neutralization of free radicals by melatonin on cell membranes, and this result was similar to the document by Garcia *et al.*[31] which stated moderate treatment of melatonin could destroy the free radicals on cell membranes to maintain fluidity of the membrane. Meanwhile, the results also showed that the treatment of excessively high concentrations of melatonin might elevate cytotoxicity, as shown in Figure 1(B). For the results mentioned above, although the addition of melatonin to cells could reduce the ethanol-induced toxicity, the appropriate concentration of melatonin has to be considered if it is to be adopted for clinical application.

III. *Assessment of GSH*

To evaluate the role of GSH in ethanol toxicity after treatment of melatonin, the PC12 cells were treated with different concentrations of melatonin for 24 hr. We found that the concentrations of GSH increased only after treatment with 100 and 1000 μM melatonin in the groups without ethanol treatment ($p < 0.05$ and $p < 0.01$). However, there were all significant increases ($p < 0.01$) after treatment with melatonin in groups with ethanol treatment shown as Figure 2(A). Kilanczyk and Bryszewska[32] have reported that exposure to 100~1000 μM of melatonin could cause a significant increase in the GSH level in human diabetic skin fibroblasts. GSH plays an important role in the protection of cells against ethanol toxicity. It had been demonstrated that an intracellular GSH level decreases after ethanol

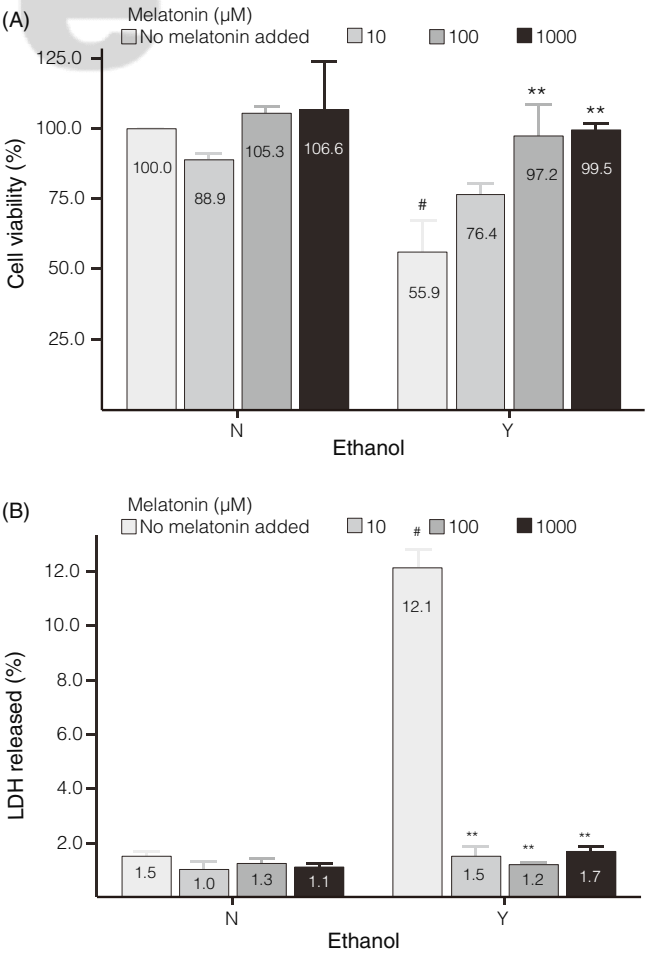

Figure 1. Change of cell viability (A), % LDH released (B) in the PC12 cell line after exposure to 150 mM of ethanol for 4 hr in the culture plate with 6 wells. N: non-treatment with ethanol, Y: treatment with ethanol. Data represent the mean ± S.D. of at least three experiments, each conducted in triplicate (#$p < 0.05$, compared to groups without the addition of melatonin in the groups without ethanol; **$p < 0.01$, compared to groups without the addition of melatonin in the groups with ethanol).

treatment, since the ROS produced by ethanol metabolism can bind to the SH group and decrease the GSH level[33]. In this study, we found that the level of GSH was remarkably decreased after acute exposure to ethanol. However, the treatment of melatonin could enhance the production of GSH in both groups, especially in the ethanol-treated groups but the mechanism of enhancing the production of GSH was unknown. At first, we attempted to assay the GSH and GSSG levels to evaluate whether melatonin could promote the production of GSH in the PC12 cells after the treatment of melatonin. We measured the GSH and GSSG levels in cell suspension samples by the capillary electrophoresis analyzer but the level of GSSG could not be obtained because it was either undetectable or too low. So we did not display the ratio of GSH/GSSG to evaluate the antioxidant capacity in the PC12 cells after the treatment of melatonin. Furthermore, it has been reported that the ratio of GSH/GSSG in the culture cells after the exposure of ethanol (about

93~144:1) was higher than that in the blood (about 10:1)[34], which indicates that the level of GSSG in culture cells might be very low so that the ratio of GSH/GSSG was higher.

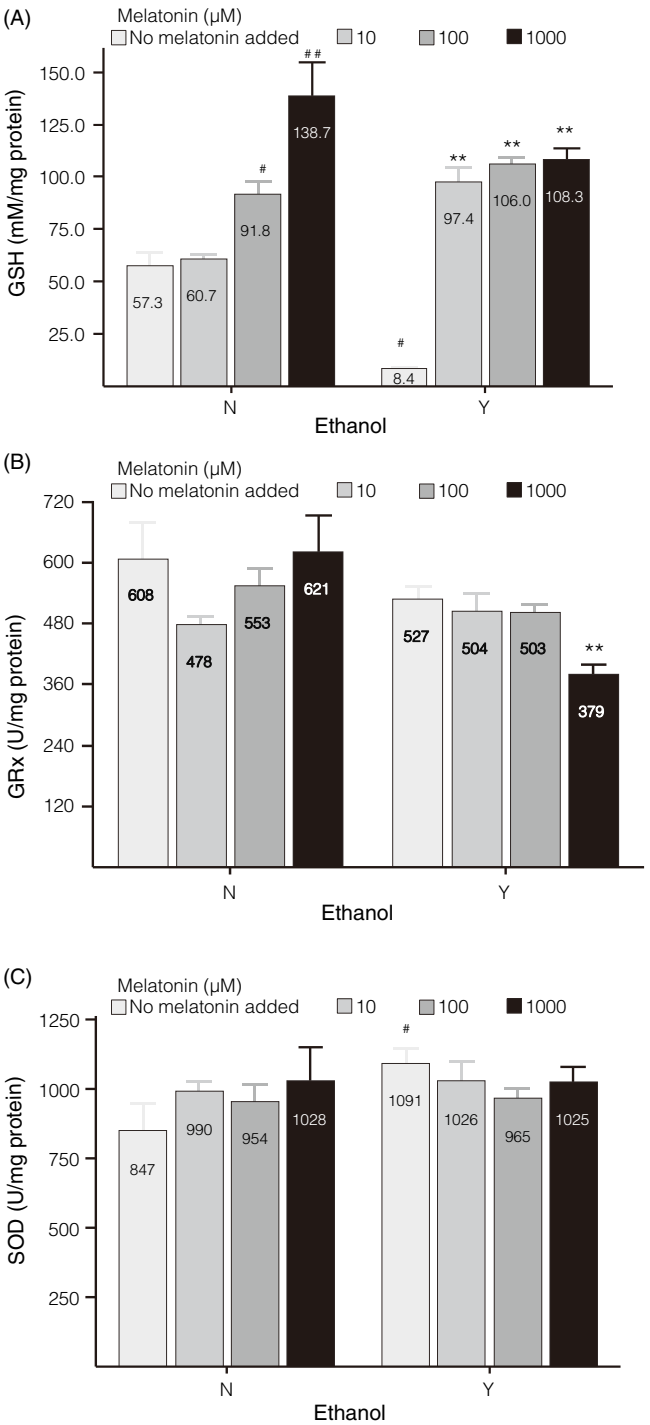

Figure 2. Change of GSH (A), GRx (B), SOD (C), in the PC12 cell line after exposure to 150 mM of ethanol for 4 hr in the culture plate with 6 wells. N: non-treatment with ethanol, Y: treatment with ethanol. Data represent the mean ± S.D. of at least three experiments, each conducted in triplicate (#$p < 0.05$ and # #$p < 0.01$ respectively, compared to groups without the addition of melatonin in the groups without ethanol; **$p < 0.01$, compared to groups without the addition of melatonin in the groups with ethanol).

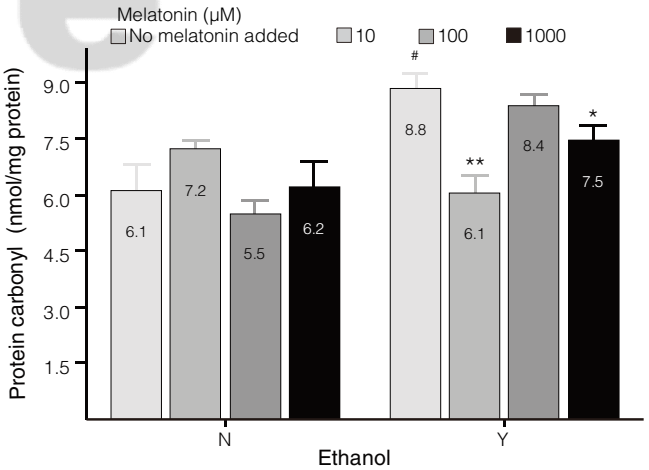

Figure 3. Change of protein carbonyl in the PC12 cell line after exposure to 150 mM of ethanol for 4 hr in the culture plate with 6 wells. N: non-treatment with ethanol, Y: treatment with ethanol. Data represent the mean ± S.D. of at least three experiments, each conducted in triplicate (#$p < 0.05$, compared to groups without the addition of melatonin in the groups without ethanol; *$p < 0.05$ and **$p < 0.01$ respectively, compared to groups without the addition of melatonin in groups with ethanol).

IV. *Assessment of the Activities of GRx and SOD*

The GSH for enhancing the defense system in cells could be produced by reducing the GSSG (an oxidized form of GSH) to GSH through the action of GRx. To evaluate the role of GRx in toxicity, the activity of GRx was assayed and the results are showed in Figure 2(B). After treatment with the different concentrations of melatonin, no significant change appeared in the activity of GRx in the groups without ethanol treatment. When the cells were treated with lower than 1000 μM (10 and 100 μM) melatonin in the groups with ethanol treatment, there were also no significant changes in the activity of GRx. However, the concentration of melatonin up to 1000 μM had a significantly lower effect ($p < 0.01$) on the activity of GRx in the groups with ethanol treatment. The weak activity of GRx after the treatment of the higher concentration of melatonin might be caused by the fact that free radicals had been neutralized by melatonin, and no more GSH was needed through the reduction of GSSG by GRx to diminish the free radicals. Hence, the elevated GSH after the treatment of melatonin did not result from the increased activity of GRx in this study. In addition, the activity of SOD showed significant increase after the treatment of ethanol compared with the groups without ethanol treatment. We suggested that the production of superoxide after exposure to ethanol must be removed by SOD. The activity of SOD showed no significant change after treatment of melatonin in both groups (Figure 2C), indicating the treatment of melatonin did not stimulate the activity of SOD in this study.

The activities of the antioxidant enzymes after the addition of melatonin showed various characteristics. More studies on how to influence the mechanisms of the

antioxidant enzymes by melatonin are still required.

V. *Assessment of Protein Carbonyls*

To evaluate whether melatonin influenced the ethanol-induced oxidative damage or not, we measured the malondialdehyde (MDA) and protein carbonyl levels by the colorimetric method in the PC12 cell. The protein carbonyl levels exhibited an increased trend after exposure to the ethanol, but the levels of MDA could not be detected in this study. The reasons for undetectable levels of MDA might be attributed to the fact that the period of ethanol treatment was too short so that the levels of MDA could not be accumulated for detection by the colorimetric method. Therefore, we adopted protein carbonyl level as the marker of oxidative damage after acute ethanol treatment.

Figure 3 shows that when melatonin treatment was not administered, protein carbonyl concentrations were higher in the ethanol-treated groups than in the groups without ethanol. As shown in Figure 3, the concentrations of protein carbonyl did not show any significant difference even though different concentrations of melatonin were added to the groups without ethanol treatment. In groups with ethanol treatment, however, the concentrations of protein carbonyl showed significant decreases ($p < 0.01$ and $p < 0.05$) after the treatment of 10 and 1000 μM melatonin. Protein carbonyl assay was used as an indicator of protein damage by free radical reactions *in vitro* and in a variety of pathologies. In this study, the levels of protein carbonyl increased significantly after the treatment with ethanol, and the treatment of moderate melatonin could decrease the production of protein carbonyl. Protein carbonyl levels did not decrease after the treatment with the higher concentration of melatonin, while various concentrations of melatonin had different effects to oxidize protein in the PC12 cell lines. Thus, the cytotoxicity of ethanol might be due to the protein damage (carbonyl formation). Many previously documented reports indicated that protein damage could be reduced by melatonin by several mechanisms, including (1) modification of the neural response to 3-nitropropionic acid which could induce oxidative stress in some brain regions[35], (2) reduction of the effectiveness on cells by alkyl peroxyl radicals[36], and (3) scavenging of oxygen free radicals and stimulation of antioxidant enzyme activity[37]. In this study, the antioxidant melatonin with a moderate concentration interacted with the oxidant and protected cells during ethanol exposure by diminishing ethanol-induced cytotoxic effects and reducing protein carbonyl generation. With regard to the mechanisms that melatonin reduced the ethanol-induced protein damages, we will conduct more research in the future.

CONCLUSIONS

In summary, we have shown that melatonin increases intracellular of GSH and decreases cytotoxicity of ethanol to PC12 cells at high concentration. Furthermore, the results showed that melatonin, at a moderate concentration, may serve as a protective agent to alleviate acute ethanol-induced damage in the PC12 cell lines. Hence, we believe that the application of melatonin against the ethanol-induced oxidative damage needs more *in vivo* studies to elucidate how melatonin exerts the cytoprotective effect in appropriate concentration. We are currently extending our *in vitro* studies to investigate the potential mechanism of the protection of melatonin during acute ethanol treatment in the PC12 cell line.

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