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Effects of Alcohol-Induced Human Peripheral Blood Mononuclear Cell (PBMC) Pretreated Whey Protein Concentrate (WPC) on Oxidative Damage

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Excessive alcohol consumption can induce apoptosis in a variety of tissues and influence the antioxidant status in peripheral blood mononuclear cells (PBMC). This paper investigates the effects of whey protein concentrate (WPC) pretreated in PBMC on the apoptosis and antioxidant status after the treatment of alcohol. The results show that the percentages of apoptotic cells in the alcohol-treated group were higher than those in the group without alcohol treatment. Additionally, there was higher glutathione (GSH) peroxidase (GPx) activity when the PBMC were treated with 300 mg/dL of alcohol. With regard to the activity of GSH reductase (GRx), there was higher activity in the group pretreated with WPC than in the group with the treatment of alcohol only. On the contrary, the levels of GSH were reduced after the treatment of alcohol, but there was a higher level of GSH in the group pretreated with WPC. In this study, it was found that the increased level of GSH in PBMC might not be attributed to the effect of GRx because there was still a higher level of GSH in the group with the treatment of WPC and BCNU (a GRx inhibitor) in this study. The results indicated that PBMC pretreated with WPC might ameliorate alcohol-induced effects such as imbalance of the antioxidant status.

KEYWORDS: Whey protein concentrate; antioxidant; apoptosis; alcohol; peripheral blood mononuclear cells

INTRODUCTION

Excessive alcohol consumption can result in a spectrum of abnormalities in many organs and tissues, including the immune system (*1*−*3*). Acute and chronic alcohol treatments are both associated with immunodepressive effects. The study by Souza et al. indicated the alcohol treatment could induce apoptosis of immune cells in gut-associated lymphoid tissue (*4*). Alcohol consumption also induces apoptosis in a variety of tissues (*5*) and involves various mechanisms such as activating specific intracellular death-related pathways leading to bax-dependent caspase-3 activation and causing a dramatic increase in cytosolic calcium influx (*6*).

Several studies have shown that alcohol-induced damage could be reduced by the adequate supplement of antioxidants (*7, 8*). We have already described the oxidative stress in the PC12 cell line caused by high doses of alcohol and that damage could be decreased by the supplement of the moderate concentrate of whey protein concentrate (WPC) and melatonin (*9, 10*). WPC is prepared in a special fashion to preserve the native forms of the cysteine-rich protein in whey and functions as a cysteine donor system in cells, and WPC is also an excellent food resource because of their relatively high protein concentration, excellent nutritional quality, and exceptional functional characteristics (*11*). WPC has been demonstrated to selectively manipulate glutathione (GSH) levels in normal or cancer cells

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and has been shown to be a potent modulator of cellular immune functions (*12*).

GSH, an endogenous antioxidant, has also been used as an indicator of lipid peroxidation severity. Because the conversion of GSH to its oxidized form (GSSG) by GSH peroxidase (GPx) generates an electron for use in stabilizing free radicals, it is generally believed that increases in free radical production occur in conjunction with decreases in GSH levels (*13*). To reduce the damage induced by free radicals, the antioxidant enzymes play important roles in organisms. Among the antioxidant enzymes, GPx is one of the most important antioxidant enzymes in humans. The biochemical function of GPx is to reduce lipid hydroperoxides to their corresponding alcohols and to reduce free hydrogen peroxide to water. Moreover, GSH reductase (GRx) can catalyze the recycling of reduced GSH from GSSG. It is believed that this system is responsible for the maintenance of the GSH/GSSG redox status, which is an important component of cellular antioxidant defense mechanisms (*14*). In addition, it has been shown that a lower intracellular level of GSH is associated with various disorders in lymphocyte functions (*15*). However, only limited information is available about the mechanisms of alcohol-induced apoptosis of immune cells. Therefore, the investigation of the potential mechanisms involved in the alcohol-induced apoptosis and oxidative stress is the purpose of our study.

Human peripheral blood mononuclear cells (PBMC) were used widely to investigate the alcohol-induced abnormality of immune functions (*16*). However, few studies evaluated the effects of WPC on the alcohol-induced antioxidant status and apoptosis in PBMC. Our previous study indicated that the activity of GRx was elevated after the supplement of WPC in PC12 cells treated with high dosages of alcohol (*9*). Therefore, we used PBMC, pretreated with WPC, to study the effects of WPC on the antioxidant status and apoptosis by alcohol on PBMC. Moreover, we also elucidate whether the elevated GSH by the supplement of WPC may be involved in the contribution of GRx by carmustine [1,3-bis(2-chloroethyl)-1-nitrosourea; BCNU] to selectively inhibit the GRx activity.

MATERIALS AND METHODS

Materials. The alcohol medium was made up of absolute alcohol (Sigma Chemical Co., St. Louis, MO) dissolved in serum-free medium (Biotarget-1, Biological Industries Ltd., Kibbutz Beit Haemek, Israel). The WPC (Immunocal, Immunotec Research Ltd., Quebec, Canada) contained about 90% whey protein isolate, <1.5% lactose, <0.5% fat, and <5.0% moisture, with a solubility index of 99% at pH 4.6. WPC was freshly prepared before each experiment. BCNU was obtained from Sigma Chemical Co. A cell preparation tube (CPT) with sodium citrate and Ficoll Hypaque solution (BD, Franklin Lakes, NJ) was used for the collection of PBMC. Reagents for the apoptosis assay, including Annexin V-FITC and propidium iodide (PI), were obtained from Clontech Laboratories, Inc. (ApoAlert, A Takara Bio Co., Mountain View, CA). The activities of GPx and GRx were assayed with commercial assay kits (RANSOD, GSH reductase).

Preparation of WPC and BCNU. Preparation of WPC was the same as in our previous study (*9*). Briefly, we followed the instructions of Immunocal to design the concentrations of WPC in this experiment. In the instructions from Immunocal, the daily suggested dose in adults was 10−50 g, and we imitated the ratio of the blood volume in a person of 60 kg; 50 mg of WPC was dissolved in a fresh 5 mL serum-free medium (approximately 10 mg of WPC/mL of medium). Therefore, concentrations of 10 mg/mL of WPC were used in this experiment. WPC was freshly prepared before each experiment. WPC powder (200 mg) was resuspended in a 20 mL of serum-free medium and centrifuged at 12000*g* for 10 min at room temperature. The supernatant was filtered on a 0.2 μm filter. Doroshenko et al. (*17*) indicated cells would be injured when they were exposed to >10 μM BCNU or incubated for 2−3 h. Therefore, 10 μM BCNU was used in this experiment. Stock solution of BCNU (20 mM in H_2O) was kept aliquoted at −20 °C until use.

Cell Preparation and Treatment. The PBMC were obtained from healthy subjects free of immune-related diseases by CPT. This study was approved by the Institutional Review Board of the Kaohsiung Veterans General Hospital, and informed consent was obtained from each participant. The viability of cells determined by the trypan blue exclusion test (*18*) was >90%, and then approximately 1×10^6/mL PBMC were transferred into a cell culture plate with six wells. The PBMC grown in the cell culture plate with serum-free medium containing 100 μg/mL streptomycin sulfate, 0.25 μg/mL amphotericin, and 100 units/penicillin were incubated at 37 °C in a humidified atmosphere of 85% air and 5% CO_2. On the first day, the PBMC suspensions from the cell culture plate were treated with 10 mg of WPC/mL of the medium. On the second day, 10 μM BCNU was added into the cell suspension and then incubated at 37 °C for 1 h. Subsequently, the cell suspensions were transferred to a 15 mL sterile centrifugal tube and centrifuged at 1200*g* for 10 min at room temperature to remove the supernatant. To avoid the remaining WPC in cell pellets, PBS was added to wash the cell pellets. Finally, the cell suspensions were divided into two groups, and then one of the cell suspensions was replaced with 300 mg/dL of the alcohol medium for 2 h. To study the effects of alcohol on the apoptosis and antioxidants, we divided the PBMC into two groups, group I (with alcohol treatment) and group II (without alcohol treatment); in addition, to evaluate the effects of WPC, we divided again groups I and II into four subgroups: group a (no WPC or BCNU), group b (BCNU only), group c (WPC only), and group d (both BCNU and WPC).

Assessment of Early and Late Apoptosis. In apoptotic cells, the membrane phospholipid phosphatidylserine (PS) is translocated from the inner to the outer leaflet of the plasma membrane, thereby exposing PS to the external cellular environment. PS translocation precedes other apoptotic events, thus allowing early detection of apoptosis. Annexin V is a phospholipid-binding protein that has a high affinity for PS and binds to cells with exposed PS and thus serves as a marker for flow cytometric analysis of cells that are undergoing apoptosis (*19*). The pellets of PBMC were washed with PBS and resuspended in 200 μL of 1× binding buffer (pH 7.5); 5 μL of Annexin V and 10 μL of PI were added into cell suspensions at room temperature. The mixture was then incubated at room temperature for 15 min in the dark. The samples suspended in 500 μL of PBS were assayed with flow cytometry (Coulter EPICS XL Flow Cytometer, Beckman Coulter). In this study, the cells that stained positive for Annexin V-FITC, but not for PI, were in the early stage of apoptosis. Cells that stained positive for both Annexin V-FITC and PI were in the late stage of apoptosis.

Assay of GRx and GPx Activity. Cell suspensions were centrifuged at 1200*g* for 5 min. The supernatant was removed, and the cell pellet was remixed with 200 μL of PBS. Among the cell suspensions, 100 μL was assayed for the assay of the activity of GRx and GPx. In brief, the 100 μL cell suspension was mixed with 0.1% Triton X-100 in 1:2 volumetric ratios and then centrifuged at 1200*g* for 10 min after being adequately mixed by a vortex mixer. The supernatant was assayed for the activity of GPx and GRx on an autoanalyzer (Synchron CX 7, Beckman Coulter) (*9*). The activities of GPx and GRx for each group were expressed as units per 10^6 cells.

Assay of GSH and GSSG. The 100 μL cell suspension, as indicated above, in an amber microcentrifuge tube was mixed with 0.1% MPA in a 1:2 volumetric ratio, and then it was centrifuged at 1200*g* for 10 min after being adequately mixed by a vortex mixer. The supernatant was assayed on a 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) and a UV absorbance detector set to 200 nm (*20*).

Statistical Analysis. Statistical results were analyzed with SPSS 10.0 one-way ANOVA and Student's *t* test to assess the significance of differences between groups. Data were expressed as mean ± standard deviation (SD), with $p < 0.05$ being considered to be significant. Each test was carried out in triplicate.

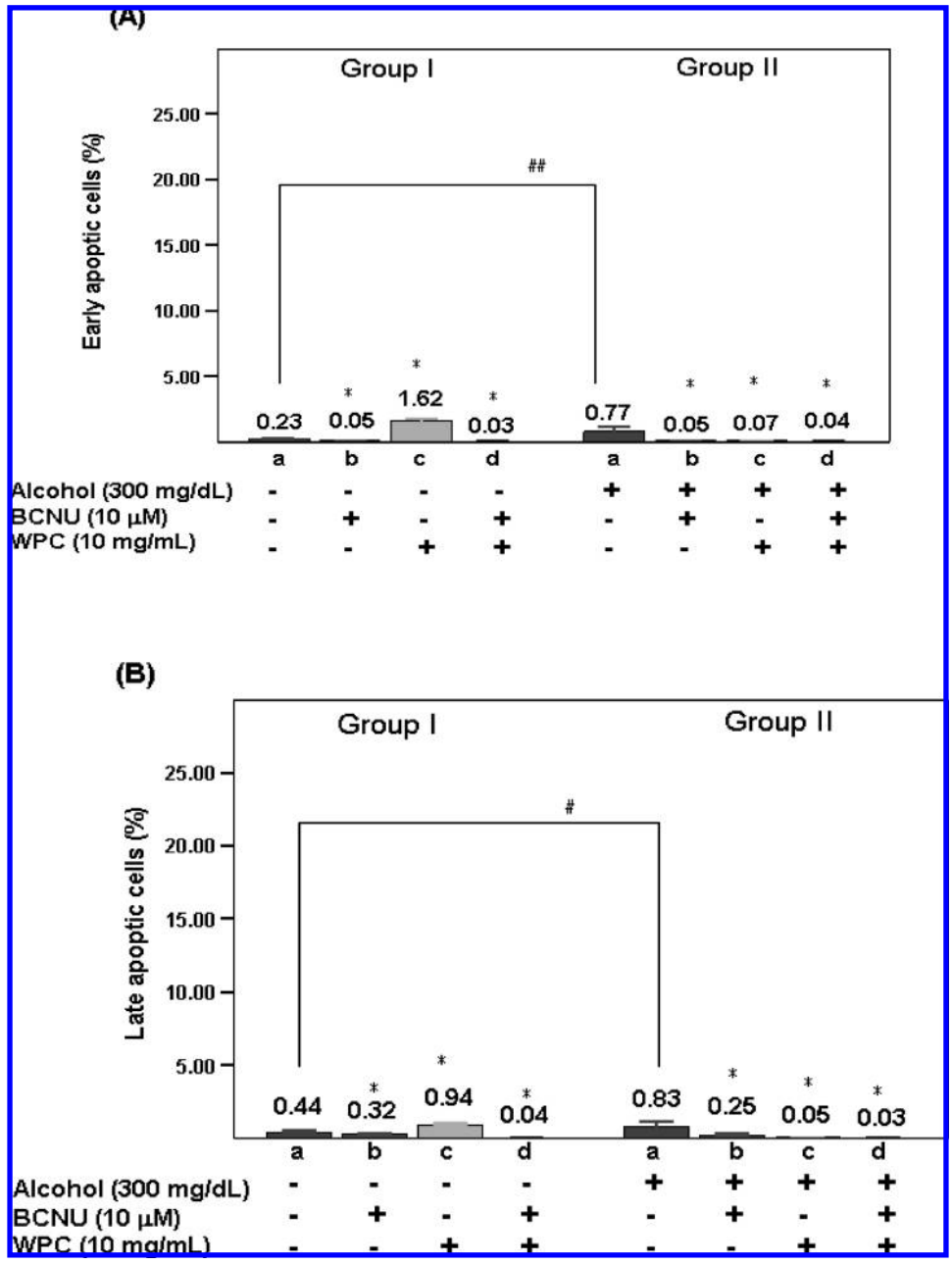

Figure 1. Changes of early apoptosis (**A**) and late apoptosis (**B**) in the PBMC: group I, without alcohol treatment; group II, treated with alcohol. Data represent the mean ± SD of at least three experiments; each was conducted in triplicate (*, $p < 0.05$, compared with the control group with or without alcohol treatment; #, $p < 0.05$ and ##, $p < 0.01$, compared with the group without alcohol treatment).

RESULTS

Effects of WPC on Apoptosis in PBMC Treated with Alcohol. For the observation of alcohol-induced apoptosis, we found that the percentages of apoptotic cells were very low among all groups, although there were significant differences in group IIa when compared with group Ia (**Figure 1**). Furthermore, we evaluated the effects of WPC on the apoptosis of the PBMC after the treatment with 300 mg/dL of alcohol. There were lower percentages of early apoptosis in the group with WPC compared with the controls in group II (**Figure 1A**). Additionally, there were higher percentages of early apoptosis in group Ic when compared with group Ia; however, there were lower percentages of early apoptosis in group IIc when compared with group IIa. Simultaneously, the effects of WPC on late apoptosis were similar to those effects on early apoptosis (**Figure 1B**). Besides, the percentage of early apoptotic cells without any treatment (group Ia) is 0.23%, but the percentage of apoptotic cells with the treatment of BCNU decreased to a low value in all cases (groups Ib, Id, IIb, and IId) irrespective of whether it was the treatment of alcohol or WPC.

Effects of WPC on the Activity of GPx and GRx in PBMC Treated with Alcohol. To investigate whether the activities of antioxidant enzymes may be influenced by the addition of WPC, we assayed the activities of GPx and GRx. As shown in **Figure 2A**, there was higher activity of GPx in group Ic compared with group Ia, and the effect was not influenced by the addition of BCNU; however, similar phenomena could not be observed in group II. After the treatment of alcohol (shown as **Figure 2A**, group II), we found that the activity of GPx was increased. As shown in **Figure 2B**, the GRx activity showed higher activities in groups with the supplement of WPC in either group I or II. The results are consistent with our previous study (*9*). However, the effects of WPC on GRx were reduced by the treatment of BCNU. WPC, as mentioned above, is a cysteine donor in cells;

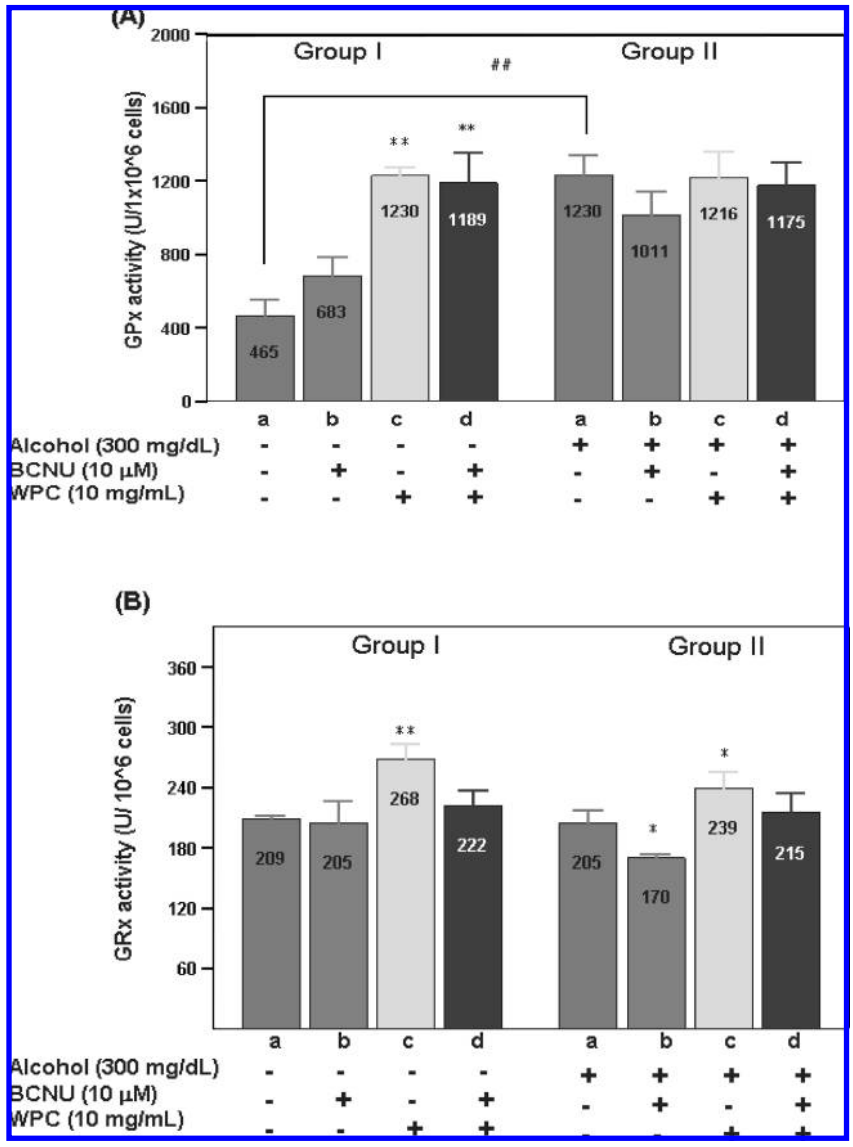

Figure 2. Activities of GPx (**A**) and GRx (**B**) in PBMC: group I, without alcohol treatment; group II, treated with alcohol. Data represent the mean ± SD of at least three experiments; each was conducted in triplicate (∗, $p < 0.05$, and ∗∗, $p < 0.01$, compared with the control group with or without alcohol treatment; ##, $p < 0.01$, compared with the group without alcohol treatment).

thus, it can act as a precursor of GSH. However, whether the elevated level of GSH after the supplement of WPC was contributed by the GRx or not is unclear. Therefore, we used BCNU, an effective inhibitor of GRx, to prevent the reduction of GSSG to GSH (*17*). In addition, as shown in **Figure 2B** (group II), there was lower activity of GRx in group IIb. On the other hand, we found the level of GSH did not decrease after the addition of 10 μM BCNU in **Figure 3A** (group I or II). The level of GSH in groups with the addition of both BCNU and WPC was higher than that of the groups with the addition of WPC only in **Figure 3A**.

Effects of WPC on the Level of GSH and GSSG in PBMC Treated with Alcohol. To determine whether the levels of GSH and GSSG of PBMC with the supplement of WPC or BCNU may be influenced by the treatment of alcohol, we assayed the levels of GSH and GSSG in PBMC. In **Figure 3A**, there was only a small increase in the level of GSH in group Ib,d when compared with group Ia, but the increase was significantly different. In addition, the levels of GSH were also elevated after the treatment of BCNU or WPC in group II. We also found that the level of GSH was markedly decreased after the treatment of alcohol; however, the supplement of WPC could elevate the level of GSH in PBMC. **Figure 3B** shows that the levels of GSSG were increased after the addition of BCNU in groups I and II. This result is in accordance with the action of BCNU to inhibit the activity of GRx; however, there was no significant difference in group Ic compared with group Ia. We also found that there was a higher level in group Id than in group Ia. Similar results were observed in group II. In addition, the level of GSSG was reduced after the treatment of alcohol (group Ia vs group IIa).

DISCUSSION

Excessive alcohol consumption is associated with alterations in many immune responses including PBMC dysfunction, which may be due to alcohol-induced modifications in cytokine production (*16*). In this study, we used the higher alcohol concentration, 300 mg/dL of alcohol, to imitate the PBMC in blood streams with high alcohol concentration (*21*) because apoptosis could not be detected by the 160 mg/dL treatment of alcohol in PBMC (*22*). Besides, the peak time of blood alcohol concentration after alcohol intake is approximately 1−2 h (*23*); thus, we adopted 2 h as the incubation time for the treatment of PBMC with 300 mg/dL of alcohol.

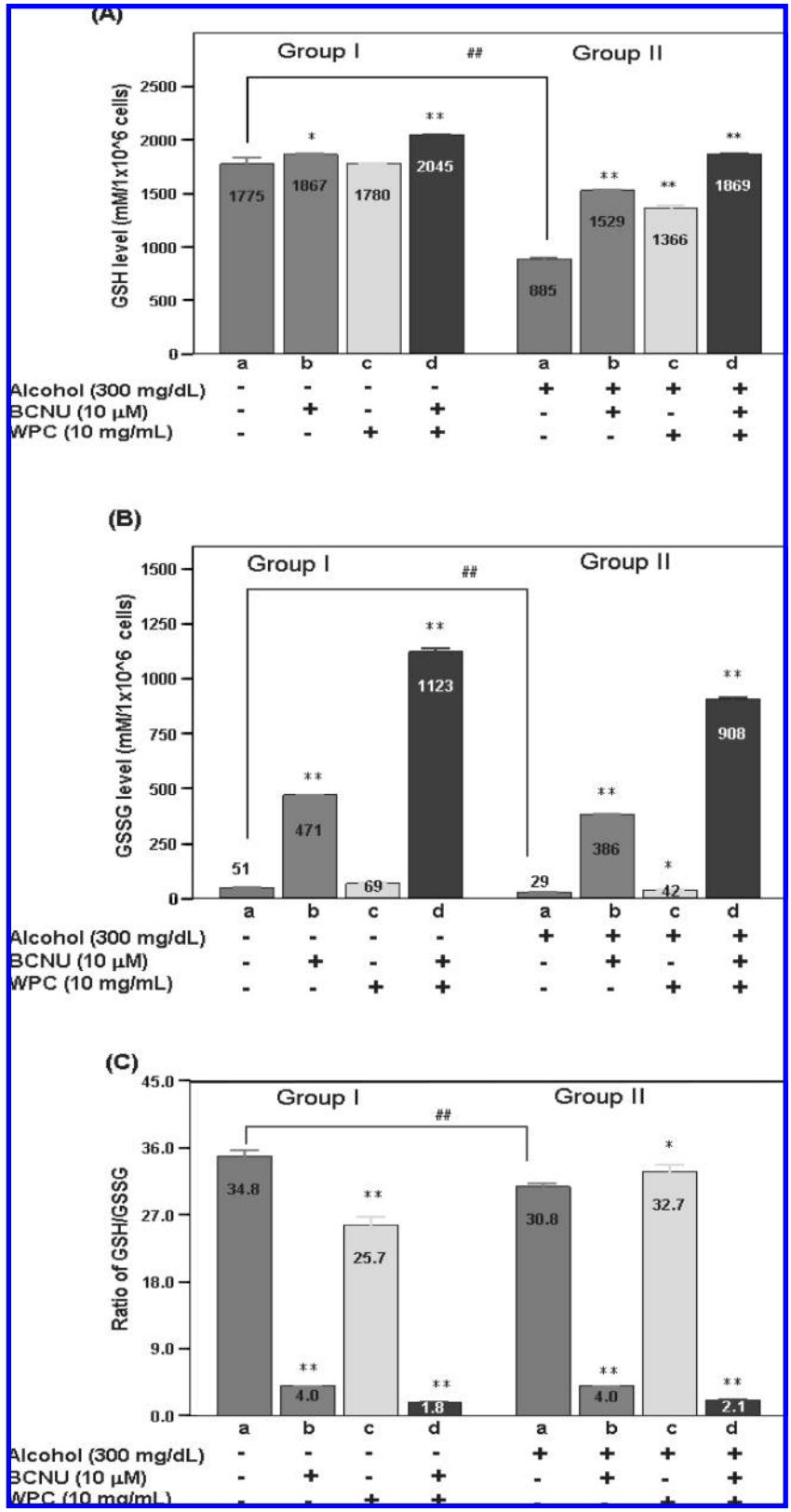

Figure 3. Levels of GSH (**A**), GSSG (**B**), and GSH/GSSG (**C**) in PBMC: group I, without alcohol treatment; group II, treated with alcohol. Data represent the mean ± SD of at least three experiments; each was conducted in triplicate (∗, $p < 0.05$, and ∗∗, $p < 0.01$, compared with the control group with or without alcohol treatment; ##, $p < 0.01$, compared with the group without alcohol treatment).

In this study, we found that the percentages of apoptotic cells were very low among all groups (<1.62%); thus, it is hard to interpret whether WPC is harmful on the basis of low percentages of apoptotic cells (1.62% for early apoptotic cells and 0.94% for late apoptotic cells). Additionally, we found that the percentages of apoptotic cells when they were treated with BCNU, regardless of whether the cells were treated with or without alcohol, were also low in early apoptosis or late apoptosis. The phenomenon was similar with WPC.

For the antioxidant enzymes, this higher GPx activity may be a compensatory response for the protection of the PBMC from the damage of high doses of alcohol; however, this result

Table 1. Summary of Effects of Additives (BCNU and WPC) on Antioxidant Enzymes and the GSH/GSSG Status in PBMC with Alcohol Treatment

	antioxidant enzymes		GSH/GSSG		
additive	GPx	GRx	GSH	GSSG	GSH/GSSG
Group I (without Alcohol)			versus Control Group (Group Ia)		
b BCNU	–	–	↑	↑	↓
c WPC	↑	↑	–	–	↓
d BCNU + WPC	↑	–	↑	↑	↓
Group II (with Alcohol)			versus Control Group (Group IIa)		
b BCNU	–	↓	↑	↑	↓
c WPC	–	↑	↑	↑	↑
d BCNU + WPC	–	–	↑	↑	↓
Group I versus Group II					
a no WPC or BCNU	↑	–	↓	↓	↓
b BCNU	↑	↓	↓	↓	–
c WPC	–	–	↓	↓	↑
d BCNU + WPC	–	–	↓	↓	↑

was different from the studies of Kocak-Toker et al. and Baily et al. (*24, 25*). Kocak-Toker et al. (*25*) indicated the activity of GPx remained unchanged in the rats with acute alcohol consumption; however, they showed the activity of GPx was reduced in the hepatocytes of rats with chronic alcohol consumption. Furthermore, we found that the activity of GPx in group I with the supplement of WPC was increased. In other words, the supplement of WPC might enhance the antioxidant capacity in PBMC.

BCNU is an anti-neoplastic agent that is widely used in the chemotherapy of brain tumors and has also been shown to inhibit glutathione reductase (GRx) (*17*). GRx is responsible for the reduction of GSSG to GSH. It is believed that this system is responsible for the maintenance of the GSH/GSSG redox status, which is an important component of cellular antioxidant defense mechanisms (*26*). Therefore, maintaining a high cellular GSH/GSSG ratio can provide optimal protection against oxidant-induced cell damage. In our previous study, we found that the activity of GRx and the level of GSH were increased by the moderate supplement of WPC in the PC12 cell line with alcohol treatment (*9*), but the present study showed that the elevated level of GSH in PBMC might not be attributed to the GRx; thus, more studies are needed to investigate if the phenomenon is associated with different cell types. On the whole, the activities of GPx and GRx were elevated by the supplement of WPC; thus, the antioxidant capacity of PBMC might be elevated by the supplement of WPC, and the effect of alcohol on GPx was more significant than that of GRx in this study. As mentioned above, the supplement of WPC seems to reduce the alcohol-induced damage through the elevated GSH.

The reduced level of GSSG in **Figure 3B** (group IIa) seems to contradict the decreased level of GSH shown in **Figure 3A**. We think that the reduced level of GSSG might be the unchanged activity of GRx after the treatment of alcohol (**Figure 2B**, group IIa); thus, the level of GSSG was not increased. However, further studies are required to investigate the potential mechanism involved in the reduced level of GSSG after the treatment of alcohol in PBMC. In **Figure 3C**, there were reverse results in the ratio of GSH/GSSG compared with those of GSSG (**Figure 3B**). The elevated level of GSSG may be attributed to the addition of BCNU.

GSH, an endogenous antioxidant, could attenuate the alcohol-induced adverse effects by directly scavenging the free radicals derived from the metabolism of alcohol. In this study, there was indeed a marked decrease in the level of GSH in the control group after the treatment of alcohol; as a result, the decreased levels of GSH might be attributed to the increased activity of GPx (**Figures 2A** and **3A**). The imbalance of the alcohol-induced antioxidant status in PBMC, such as the changes in the activity of antioxidant enzymes and the depletion of GSH, might be major causes of cell injury. WPC has been shown to be an effective and safe cysteine donor for GSH replenishment during GSH depletion in immune deficiency states; moreover, several studies have indicated WPC could be used in pharmaceutical and medical industries because WPC, which contains IgA, IgG, albumin, lactoferrin, and lactoperoxidase, is highly valuable (*27*). Therefore, the supplement of WPC has been a suggested strategy for the treatment of patients with immune-related diseases (*28, 29*).

In conclusion, WPC offers the source of GSH to increase the antioxidant capacity in PBMC. In this study, the results indicated that the alcohol-induced effects in PBMC might be ameliorated or differently influenced by the supplement of WPC (**Table 1**). How the function of antioxidants can be elevated to enhance immunity against alcohol-induced damage has become an important topic in alcohol-related studies.

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