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Antioxidant effects of whey protein on muscle C2C12 cells

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A B S T R A C T

In the present study, the *in vitro* scavenging activity of sheep whey protein against free radicals, as well as its reducing power were determined and compared with that of beef protein, soy protein and cow whey protein. Moreover, the possible protective effects of sheep whey protein from tert-butyl hydroperoxide (tBHP)-induced oxidative stress in muscle C_2C_{12} cells were determined by assessing oxidative stress markers by flow cytometry and spectrophotometry. The results showed that sheep whey protein scavenged DPPH$^{\cdot}$, ABTS$^{\cdot+}$ and OH$^{\cdot}$ radicals with IC_{50} values of 3.1, 4.1 and 1.8 mg of protein/ml. Moreover, the reducing power activity assessed with potassium ferricyanide of sheep whey protein was 1.3 mg/ml. As regards to the antioxidant effects in muscle cell line, sheep whey protein at 0.78, 1.56, 3.12 and 6.24 mg of protein/ml increased GSH levels up to 138%, lowered TBARS levels up to 25% and decreased ROS levels up to 41.4%.

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1. Introduction

Reactive oxygen species (ROS) are products of normal metabolism (Halliwell, 2001). ROS include free radicals, such as the superoxide radical ($O_2^{\cdot-}$), hydroxyl radical (OH$^{\cdot}$), peroxyl radical ($RO_2^{\cdot}$), as well as nonradical species, such as hydrogen peroxide (H_2O_2) (Halliwell, 2001). An amount of ROS is necessary for physiological processes, including phagocytosis (Dupré-Crochet, Erard, & Nüβe, 2013), intracellular signalling (Halliwell, 2001), cell proliferation, metabolism, apoptosis and muscle contraction (Linnane, Zhang, & Yarovaya, 2002). Some of the main endogenous sources of free radicals are the mitochondrial respiratory chain, inflammation, peroxisomes and the cytochrome P450 (Valko et al., 2007). Excessive production of ROS can lead to oxidative stress, a pathophysiological condition, implicated in oxidative damage of macromolecules (lipids, protein, DNA) (Halliwell, 2001; Mylonas & Kouretas, 1999), immune dysfunction (Schneider & Tiidus, 2007), muscle damage (Nikolaidis et al., 2007) and fatigue (Meeus, Nijs, Hermans, Goubert, & Calders, 2013).

Skeletal muscle is susceptible to ROS injury, even under physiological conditions, such as exercise (Nikolaidis et al., 2008). Skeletal muscle has the unique ability to increase the rate of oxygen usage during contraction. During intense activity, the high rate of O_2 consumption (VO_2) in skeletal muscles can cause incomplete oxygen reduction and electron leakage from the electron transfer chain, leading to the generation of ROS (Hood, Uguccioni, Vainshtein, & D'souza, 2011). Together with nitric oxide and its reaction products, these nitrogen and oxygen species cause oxidative stress leading to muscle damage. Both muscle damage and exercise-induced oxidative stress have been associated with muscle cell apoptosis (Phaneuf & Leeuwenburgh, 2001). At the same time, fatigue, which may be a symptom of skeletal muscle cell damage, has also been associated with oxidative stress (McClung et al., 2010). Due to the fact that oxidative stress may be responsible for muscle soreness and fatigue and even skeletal muscle damage, it has been suggested that antioxidant supplementation may relieve the problem (Kerasioti et al., 2012).

Muscle cells, like all eukaryotic cells, are equipped with antioxidant defence mechanisms to deal with ROS. The antioxidant mechanisms include enzymatic and non enzymatic. For example, important antioxidant enzymes are catalase, superoxide dismutase and glutathione peroxidase (Powers & Jackson, 2008). The non enzymatic mechanisms include antioxidant molecules such as glutathione, coenzyme Q_{10}, a-lipoic acid, selenium and bilirubin as

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well as exogenous antioxidants received from the diet (e.g. vitamin C, vitamin E, flavonoids) (Wu, Fang, Yang, Lupton, & Turner, 2004).

In an effort to increase antioxidant defense and protect the organism from the harmful effects of oxidative stress, there is an increased interest for natural sources of antioxidants. For example, whey is a popular dietary protein supplement augmenting antioxidant defense (Bartfay, Davis, Medves, & Lugowski, 2003; Kerasioti et al., 2012). It is considered as by-product of cheese manufacturing, but it is also described as functional food with nutritional applications (Marshall, 2004; Tseng et al., 2006). It is usually used as a food supplement especially by athletes for muscle growth. The components of whey include beta-lactoglobulin, alpha-lactalbumin, bovine serum albumin, lactoferrin, immunoglobulins, lactoperoxidase enzymes, glycomacropeptides and lactose (Walzem, Dillard, & German, 2002). Some of these components exhibit antioxidant properties. For example, alpha-lactalbulmin can chelate heavy metals, leading to the reduction of oxidative stress because of its iron-chelating properties (Ha & Zemel, 2003). Moreover, whey protein is rich in the sulphur-containing amino acids, cysteine and methionine, that enhance antioxidant defence through intracellular conversion to glutathione (Marshall, 2004).

In previous *in vivo* studies, we have shown that a cake containing sheep whey protein had antioxidant, as well as anti-inflammatory, activities in human beings, subjected to intense exercise (Kerasioti et al., 2012, 2013). The aim of the present study was to compare *in vitro* antioxidant activity of sheep whey protein with that of other proteins (i.e. beef protein, soy protein and cow whey protein) used as food supplements for muscle growth. Moreover, we investigated the effects of sheep whey protein against tert-butyl hydroperoxide (tBHP) – induced oxidative stress in mouse muscle cells C2C12.

2. Materials and methods

2.1. Chemicals, reagents and culture medium

Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), phosphate buffered saline (PBS) and trypsin were purchased from Gibco (Grand Island, NY). Tert-butyl hydroperoxide (tBHP), ethylenediaminetetraacetic acid (EDTA), 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid), diammonium salt (ABTS), hydrogen peroxide (H_2O_2) solution 30%, horseradish peroxidase (HRP), 2,2-diphenyl-1-picrylhydrazyl (DPPH), ferric chloride, 2-deoxyribose, nicotinamide adenine dinucleotide (NADH), nitroblue tetrazolium (NBT), phenazine methosulfate (PMS), mercury orange and 2,7-dichlorofluorescein diacetate (DCF-DA) were obtained from Sigma–Aldrich (St Louis, MO, USA). Trichloroacetic acid (TCA) and 2-thiobarbituric acid (TBA) were purchased from Merck (Darmstadt, Germany). Cell proliferation kit II (XTT) was purchased from Roche Diagnostics (Mannheim, Germany) and potassium ferricyanide was obtained from Applichem (Dresden, Germany).

2.2. DPPH· radical scavenging assay

The DPPH radical scavenging activity of proteins was evaluated as described previously (Spanou et al., 2007). Briefly, a 1.0 ml freshly made methanolic solution of DPPH radical (100 μM) was mixed with the tested proteins dissolved in distiled water at different concentrations. The contents were vigorously mixed, incubated at room temperature in the dark for 20 min and the absorbance was read at 517 nm. In each experiment the tested protein alone in methanol was used as blank and the DPPH· alone in methanol was used as control. All experiments were carried out in triplicate and at least on two separate occasions. The DPPH· radical scavenging activity was calculated according to the equation:

$$\%\ \text{DPPH}^{\cdot}\ \text{radical scavenging activity} = [(Abs_{\text{control}} - Abs_{\text{sample}})/Abs_{\text{control}}] \times 100$$

where Abs_{control} and Abs_{sample} are the absorbance values of the control and the tested sample respectively.

2.3. ABTS·+ radical scavenging assay

ABTS·+ radical scavenging activity was measured as described previously (Spanou et al., 2007). The reaction was carried out in 1 ml final volume, containing 400 μl H_2O, 500 μl ABTS (1 mM), 50 μl H_2O_2 (30 μM) and 50 μl HRP (6 μM). Immediately after the addition of the enzyme the contents were mixed and incubated at room temperature in the dark for 45 min. After incubation, the tested proteins dissolved in distiled water (10 μl) at different concentrations were added, the contents were mixed and the absorbance was measured at 730 nm. In each experiment the samples without HRP were used as blanks, and the samples without protein were used as control. All experiments were carried out in triplicate and at least on two separate occasions. The ABTS·+ radical scavenging activity was calculated according to the equation:

$$\%\ \text{ABTS}^{\cdot+}\ \text{radical scavenging activity} = [(Abs_{\text{control}} - Abs_{\text{sample}})/Abs_{\text{control}}] \times 100$$

where Abs_{control} and Abs_{sample} are the absorbance values of the control and the tested sample respectively.

2.4. Hydroxyl (OH·) radical scavenging assay

Hydroxyl (OH·) radical scavenging activity was determined using the method of Chung, Osawa, and Kawakishi (1997). 75 μl of protein dissolved in distiled water at different concentrations were added to 450 μl sodium phosphate buffer (0.2 M, pH 7.4), 150 μl 2-deoxyribose (10 mM), 150 μl $FeSO_4$-EDTA (10 mM), 525 μl H_2O and 150 μl H_2O_2 (10 mM), and the samples were incubated at 37 °C for 4 h. After incubation, 750 μl TCA (2.8%) και 750 μl 2-thiobarbituric acid (1%) were added and the samples incubated at 95 °C for 10 min. The samples were cooled on ice for 5 min and centrifuged at 3000 rpm for 10 min at 25 °C. The absorbance was measured at 520 nm. In each experiment, the samples without H_2O_2 were used as blank and the samples without protein were used as control. All experiments were carried out in triplicate and at least on two separate occasions. The OH· radical scavenging activity was calculated according to the equation:

$$\%\ \text{OH}^{\cdot}\ \text{radical scavenging activity} = [(Abs_{\text{control}} - Abs_{\text{sample}})/Abs_{\text{control}}] \times 100$$

where Abs_{control} and Abs_{sample} are the absorbance values of the control and the tested sample respectively.

2.5. Superoxide ($O_2^{\cdot-}$) radical scavenging assay

Superoxide ($O_2^{\cdot-}$) radical scavenging activity was determined according to the method of Gülçin, Küfrevioglu, Oktay, and Büyükokuroglu (2004). Briefly, in 2.5 ml trishydroxymethylaminomethane hydrochloride (Tris–HCl) (16 mM, pH 8.0), 500 μl of NBT (300 μM), 500 μl of NADH (468 μM) and 250 μl of proteins dissolved in distiled water at different concentrations were added. The reaction was started by adding 500 μl PMS (60 μM). The samples were incubated for 5 min and centrifuged at 3000 rpm for 10 min at 25 °C. The absorbance was measured at 560 nm. In each experiment, the samples without PMS were used as blanks and the samples without protein were used as controls. All experiments were carried out in triplicate and at least on two separate

occasions. The superoxide ($O_2^{\cdot-}$) radical scavenging activity was calculated according to the equation:

$$\%\text{ superoxide }(O_2^{\cdot-})\text{ radical scavenging activity} = [(Abs_{control} - Abs_{sample})/Abs_{control}] \times 100$$

where $Abs_{control}$ and Abs_{sample} are the absorbance values of the control and the tested sample respectively.

2.6. Reducing power assay

Reducing power was determined according to Yen and Duh (1994). Briefly, the proteins were dissolved in phosphate buffer (0.2 M, pH 6.6) at different concentrations. An aliquot (2.5 ml) of the sample solution was added to 2.5 ml of potassium ferricyanide (1%) and incubated at 50 °C for 20 min. The samples were cooled on ice for 5 min. Then, 2.5 ml TCA (10%) were added and the samples were centrifuged at 3000 rpm for 10 min at 25 °C. To the supernatant (2.5 ml), 2.5 ml deionized water and 500 μl ferric chloride (0.1%) were added and the samples were incubated at room temperature for 10 min. The absorbance was measured at 700 nm. All experiments were carried out in triplicate and at least on two separate occasions.

2.7. Cell culture

C_2C_{12} muscle cells were a gift from Prof. Koutsilieris (National and Kapodistrian University of Athens, Greece). All cells were cultured in normal Dulbecco's modified Eagle's medium (DMEM, Gibko, UK), containing 10% (v/v) fetal bovine serum, 2 mM L-glutamine (Gibko, UK), 100 units/ml of penicillin, and 100 units/ml of streptomycin (Gibko, UK) in plastic disposable tissue culture flasks at 37 °C in 5% CO_2.

2.8. Cell viability assay

Cell viability was assessed using the XTT assay kit (Roche, Germany). Briefly, C_2C_{12} cells were subcultured into a 96-well plate with 1×10^4 cells per well in DMEM medium. After 24 h incubation, the cells were treated with increasing concentrations of proteins in serum-free DMEM medium for 24 h. 50 μl of XTT test solution, which was prepared by mixing 50 μl of XTT-labelling reagent with 1 μl of electron coupling reagent, was then added to each well. After 4 h of incubation, the absorbance was measured at 450 nm and also at 690 nm as a reference wavelength in a Bio-Tek ELx800 microplate reader (Winooski, VT, USA). Cell cultures in DMEM serum-free medium were used as a negative control. Also, the absorbance of each protein concentration alone in DMEM serum-free medium and XTT test solution was tested at 450 nm. The absorbance values shown by the proteins alone were subtracted from those derived from C_2C_{12} cell treatment with proteins. Data was calculated as percentage of inhibition by the following formula:

$$\text{inhibition }(\%) = [(\text{O.D.}_{control} - \text{O.D.}_{sample})/\text{O.D.}_{control}] \times 100$$

where $\text{O.D.}_{control}$ and O.D._{sample} indicated the optical density of the negative control and the tested compounds respectively. All experiments were carried out in triplicate and at least on two separate occasions.

2.9. Treatment of C2C12 cells

C2C12 cells were seeded in 25 cm^2 culture flasks for GSH, ROS and 75 cm^2 culture flasks for TBARS determination and incubated for 24 h at 37 °C in 5% CO_2. Then, the medium was removed and replaced with serum-free medium containing sheep whey protein at different concentrations (0–6.24 mg of protein/ml), followed by incubation for 24 h. The untreated cells were considered as controls. After incubation, the whey protein was removed and tBHP (0.3 mM) was added for 30 min. The cells were then trypsinized, collected and centrifuged twice at 300*g* for 10 min at 5 °C. After each centrifugation the supernatant was discarded and the cellular pellet was resuspended in PBS. After the second centrifugation the supernatant was used for the measurement of oxidative stress markers, namely GSH, ROS and TBARS.

2.10. Flow cytometric analysis of GSH and ROS levels

The intracellular GSH and ROS levels were assessed by flow cytometry using mercury orange and DCF-DA respectively. In particular, the fluorescent mercury orange binds directly to GSH, whilst DCF-DA within cells is deacetylated by esterases, and further converted to fluorescent DCF by oxidative action of ROS. A 400 μM stock solution of mercury orange was made up in acetone and stored at 4 °C, while a fresh 400 μM stock solution of DCF-DA was prepared in methanol. To assess the GSH and ROS levels, the cells were resuspended in PBS at 1×10^6 cells per ml and incubated in the presence of mercury orange (40 μM) or DCF-DA (10 μM) in the dark at 37 °C for 30 min. The cells were then washed, resuspended in PBS and submitted to flow cytometric analysis using a FACScan flow cytometer (Becton Dickinson, NJ, USA) with excitation and emission at 488 and 530 nm for ROS, and at 488 and 580 nm for GSH. Also, forward angle and right angle light scattering showing the cells size and cell internal complexity respectively were measured. Cells were analysed at a flow rate of 1000 events per second. Analyses were performed on 10,000 cells per sample and fluorescence intensities were measured on a logarithmic scale of four decades of the log of fluorescence. Data was analysed using BD Cell Quest software (Becton Dickinson). Each experiment was repeated at least three times.

2.11. Assessment of TBARS levels by spectrophotometry

For TBARS determination, a slightly modified assay of Keles, Taysi, Sen, Aksoy, and Akcay (2001) was used. 400 μl of cellular suspension or 400 μl of PBS for blank was mixed with 500 μl of 35% TCA and 500 μl of trishydroxymethylaminomethane hydrochloride (Tris–HCl) (200 mM, pH 7.4) and incubated for 10 min at room temperature. Afterwards, 1 ml of 2 M Na_2SO_4 and 55 mM thiobarbituric acid solution was added and the samples were incubated at 95 °C for 45 min. The samples were cooled on ice for 5 min and vortexed after adding 1 ml of 70% TCA. The samples were then centrifuged at 15,000*g* for 3 min and the absorbance of the supernatant was read at 530 nm. The assay requires more than 30 μg absolute amount of protein in the test sample. Total protein in cellular suspension was assayed using a Bradford reagent from Sigma–Aldrich. Calculation of TBARS concentration was based on the molar extinction coefficient of malondialdehyde.

2.12. Statistical analysis

Data was analysed by one-way ANOVA followed by Tukey's test for multiple pairwise comparisons. The level of statistical significance was set at $P < 0.05$. For all statistical analyses SPSS, version 13.0 (SPSS Inc., Chicago, Ill.) was used. Data is presented as mean ± SEM.

3. Results

3.1. Scavenging of free radicals

All tested proteins scavenged dose dependent DPPH, ABTS and OH radicals (Fig. 1A–C). However the superoxide radical was scavenged only by beef protein (Fig. 1D).

For each protein the IC_{50} showing the concentration that has the ability to scavenge 50% of radicals was evaluated. A lower IC_{50} value corresponds to a larger scavenging activity. The IC_{50} values in DPPH· radical scavenging assay were in the following order: beef protein > soy protein > sheep whey protein > cow whey protein. For $ABTS^{\cdot+}$ radical scavenging assay the order was: beef protein > soy protein > cow whey protein = sheep whey protein. For Hydroxyl (OH·) radical scavenging assay the order was: beef protein > soy protein > cow whey protein = sheep whey protein. Concerning the superoxide radical ($O_2^{\cdot-}$), only beef protein exhibited scavenging activity.

3.2. Reducing power

For the reducing power assay, it was determined the $RP_{0.5AU,}$ the concentration that produces an absorbance of 0.5 at 700 nm (Fig. 2). The $RP_{0.5AU}$ values in reducing power assay were in the following order: beef protein > sheep whey protein > soy protein > cow whey protein. For comparison purposes RP has been included in Table 1.

3.3. Effects of whey protein on GSH levels in C2C12 cells

For assessing the effects of sheep whey protein on GSH, ROS and TBARS levels in C2C12 cells, non-cytotoxic concentrations (0.78–6.24 mg of protein/ml) were used (Fig. 3).

The GSH levels were evaluated by flow cytometry using mercury orange for staining as described in 'Section 2'. Histograms demonstrating the cell counts versus fluorescence of mercury orange are shown in Fig. 4A. The mean fluorescent intensity was evaluated using the BD Cell Quest software and the values are presented as percentage of the control (untreated cells) (Fig. 5A). TBHP treatment decreased significantly GSH levels by 31.5% compared to the controls (Fig. 5A). However, treatment of C2C12 cells with whey protein, at concentrations of 0.78, 1.56, 3.12 and 6.24 mg of protein/ml, before tBHP administration increased GSH levels by 25.7%, 112.9%, 118.0% and 138.0%, respectively compared to tBHP treatment alone (Fig. 5A).

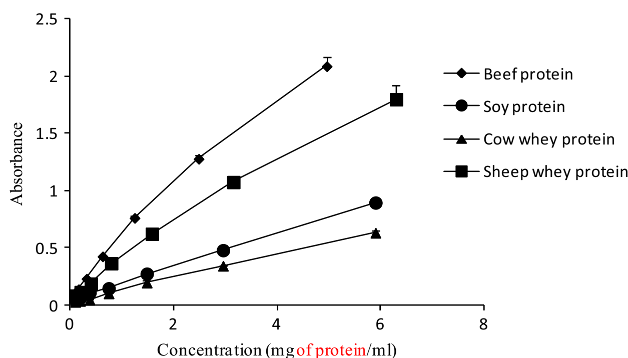

Fig. 2. Reducing power of beef protein, soy protein, whey protein and commercial whey protein. Values are presented as mean ± SEM.

3.4. Effects of whey protein on ROS levels in C2C12 cells

ROS levels were evaluated by flow cytometry using DCF-DA for staining. Histograms demonstrating the cell counts versus fluorescence of DCF-DA are shown in Fig. 4B. The mean fluorescence intensity values were evaluated using the BD Cell Quest software and are expressed as percentage of the control (untreated cells) (Fig. 5B). The administration of tBHP did not affect ROS levels compared to the control. However, treatment of C2C12 cells with whey

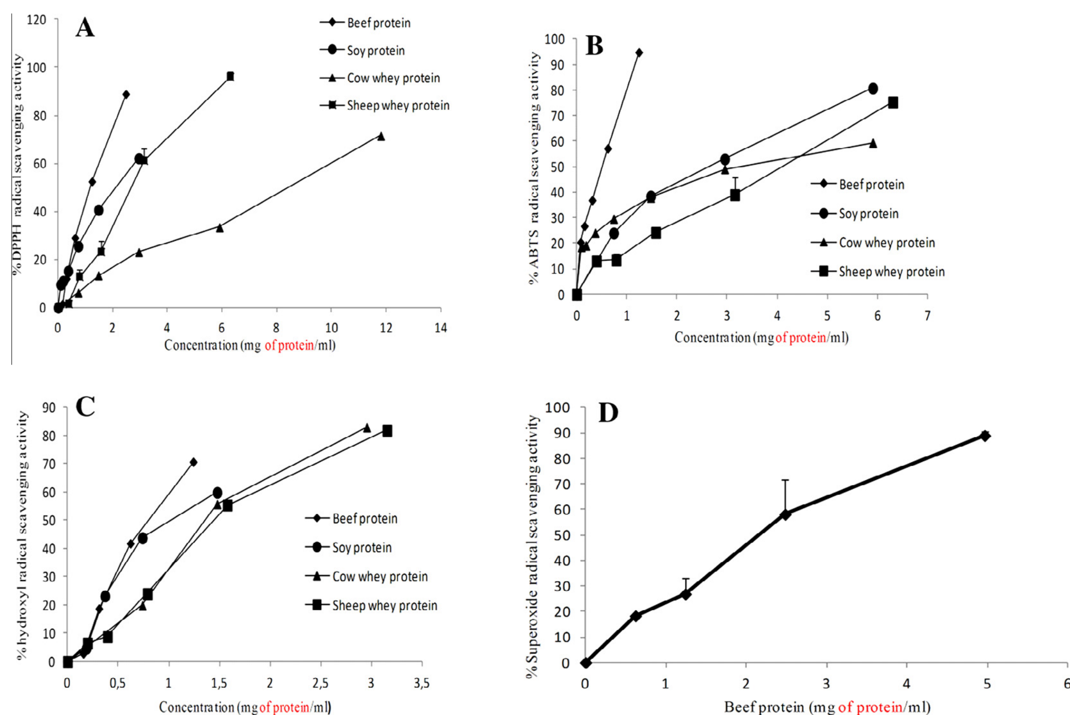

Fig. 1. (A) % DPPH· radical scavenging activity of beef protein, soy protein, whey protein and commercial whey protein. (B) % $ABTS^{\cdot+}$ radical scavenging activity of beef protein, soy protein, whey protein and commercial whey protein. (C) % Hydroxyl (OH·) radical scavenging activity of beef protein, soy protein, whey protein and commercial whey protein. (D) % Superoxide ($O_2^{\cdot-}$) radical scavenging activity of beef protein. Values are presented as mean ± SEM.

Table 1
Free radical scavenging activity against DPPH·, ABTS·+, OH· and $O_2^{\cdot-}$ radicals as well as reducing power of beef protein, soy protein, sheep whey protein and cow whey protein.

Proteins	DPPH·[a]	ABTS·+[a]	OH·[a]	$O_2^{\cdot-}$[a]	Reducing power[a]
	IC50 (mg of protein/ml)	IC50 (mg of protein/ml)	IC50 (mg of protein/ml)	IC50 (mg of protein/ml)	$RP_{0.5AU}$
Beef protein	1.3 ± 0.17*	0.6 ± 0.03*	0.85 ± 0.07*	2.5 ± 0.3*	0.9 ± 0.04*
Soyprotein	2.2 ± 0.44*	3.1 ± 1.4*	1.1 ± 0.47*	–	3.1 ± 1.7*
SWP	3.1 ± 0.09*	4.1 ± 0.21*	1.8 ± 0.28*	–	1.3 ± 0.06*
CWP	8.2 ± 0.77*	3.9 ± 0.13*	1.7 ± 0.14*	–	4.8 ± 0.03*

SWP: sheep whey protein. CWP: cow whey protein.
[a] Values are presented as mean ± sd of at least three independent experiments.
* $P < 0.05$.

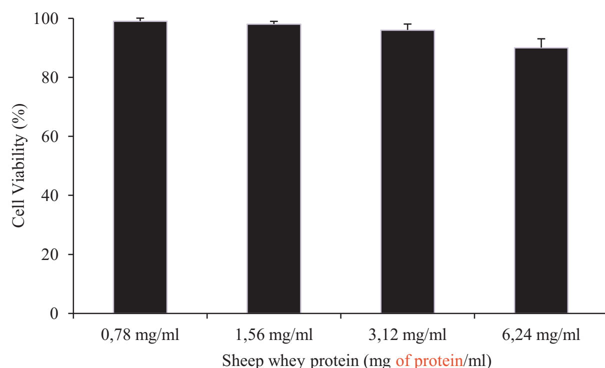

Fig. 3. Effects of sheep whey protein on viability of C2C12 cells.

protein, at concentrations of 1.56, 3.12 and 6.24 mg of protein/ml, before tBHP administration decreased significantly ROS levels by 12.8%, 16.4% and 41.3% respectively, compared to tBHP treatment alone (Fig. 5B).

3.5. Effects of whey protein on TBARS levels in C2C12 cells

The results showed that tBHP treatment increased significantly TBARS levels by 29% compared to control. Pre-treatment of cells with whey protein at 0.78–6.4 mg of protein/ml before tBHP administration led to a significant decrease in TBARS levels by 21.7%, 15%, 25.5% and 24% respectively (Fig. 5C).

4. Discussion

The overproduction of free radicals in organisms can cause oxidative stress, a pathophysiological condition which is associated with several diseases (Valko et al., 2007). Living organisms are equipped with endogenous antioxidant defense mechanisms to cope with free radicals and oxidative stress, but also exogenous sources of antioxidants are important.

Whey protein is a by-product of cheese manufacturing which is widely used as a food supplement, especially by athletes for muscle growth. However, apart from whey protein's effects on muscle regeneration, we and others have shown that sheep whey protein possess antioxidant activity (Kerasioti et al., 2012; Xu, Liu, Xu, & Kong, 2011). In the present study we compared the *in vitro* antioxidant activity of sheep whey protein with other proteins used frequently as food supplements for muscle growth. Moreover we investigated the effects of sheep whey protein on the redox status of muscle cells.

At first, it was examined the scavenging activity of sheep whey protein against DPPH·, ABTS·+, OH· and $O_2^{\cdot-}$ radicals. Then the scavenging activity of sheep whey protein was compared with that of beef protein, soy protein and cow whey protein. The protein content of beef, soy, cow whey and sheep whey protein was 62, 73.8, 73.8 and 78 g/100 g respectively. Regarding the DPPH· radical, sheep whey protein showed dose dependent scavenging activity and it was more effective than cow whey protein. However, beef protein was the most effective among all the tested proteins. In a previous study, it was found that whey protein at a concentration of 1 mg/ml, inhibited DPPH· radical by 70%. This whey protein was more effective than ours maybe due to its hydrolysis (Kamau & Lu,

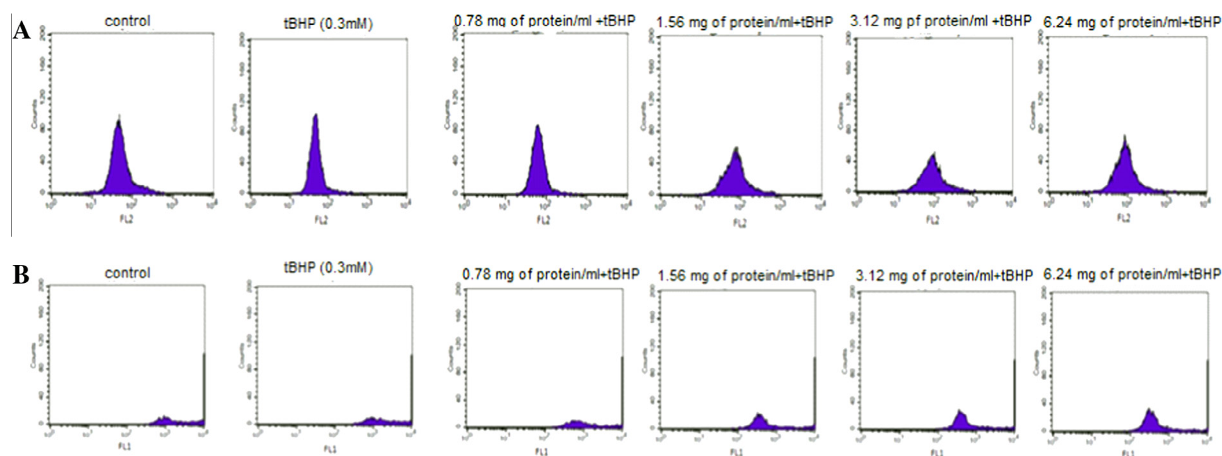

Fig. 4. Flow cytometry analysis in C2C12 cells. (A) The histogram of cell counts versus fluorescence of 10,000 cells analysed by the flow cytometer for the detection of GSH. FL2 represented the detection of fluorescence using 488 and 580 nm as the excitation and emission wavelength respectively. (B) The histogram of cell counts versus fluorescence of 10,000 cells analysed by the flow cytometer for the detection of ROS. FL1 represented the detection of fluorescence using 488 and 530 nm as the excitation and emission wavelength respectively. For ROS and GSH measurement, C2C12 cells were incubated with 10 μM DCF-DA and 40 μM mercury orange respectively for 30 min at 37 °C. The cells were then washed, suspended in PBS and analysed by flow cytometry, as described in Section 2.

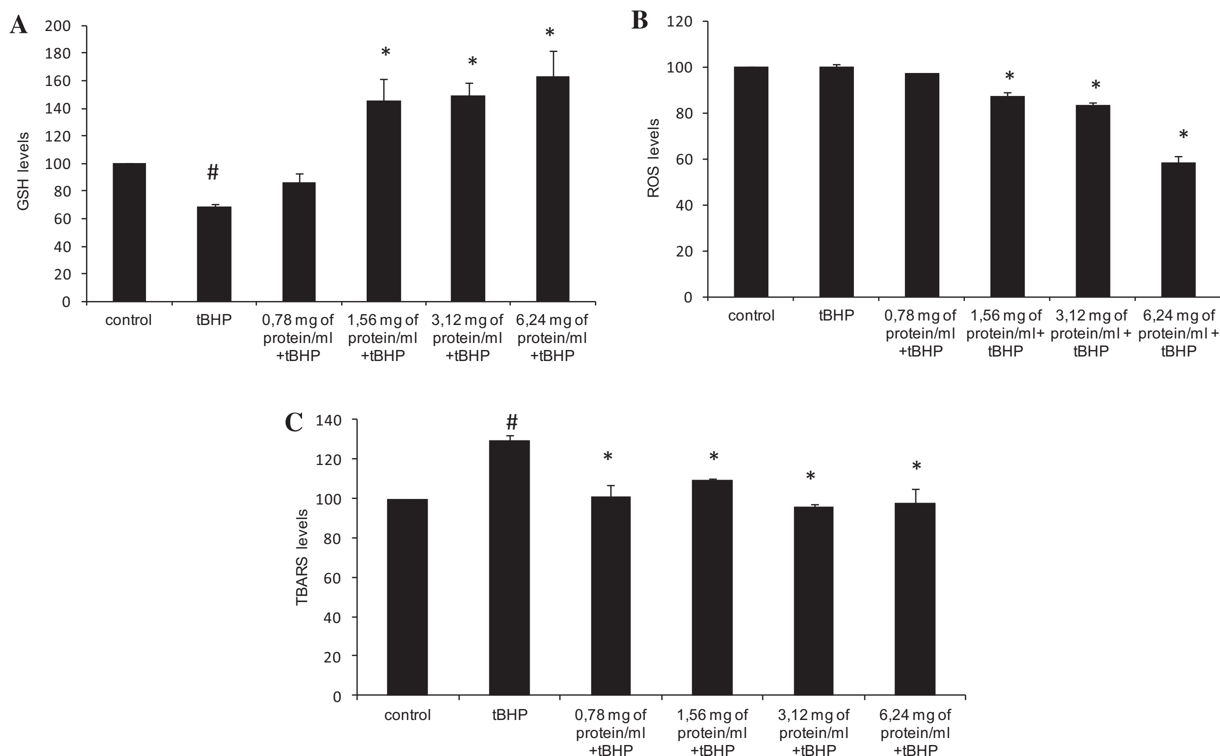

Fig. 5. Effects of whey protein on GSH levels (A), ROS levels (B) and TBARS levels (C) in C2C12 myoblasts. Cells were studied under three conditions: under normal conditions (control), under treatment with tBHP (0.3 mM) for 30 min and under the combination of whey protein (0.78–6.24 mg of protein/ml) for 24 h and tBHP (0.3 mM) for 30 min. GSH and ROS levels were assessed by flow cytometry and the mean fluorescence intensity is presented. TBARS levels were evaluated by spectrophotometer. All values are presented as the mean ± SEM of 3 experiments. * Statistically significant compared to tBHP alone ($P < 0.05$). # Statistically significant compared to control ($P < 0.05$).

2011). Moreover, in ABTS$^{\cdot+}$ assay, sheep whey protein showed dose-dependent scavenging activity. Cow whey protein exhibited similar action with sheep whey protein against ABTS$^{\cdot+}$ radicals. However, like the DPPH assay, beef protein had the highest scavenging activity against ABTS$^{\cdot+}$ radicals. Apart from DPPH and ABTS$^{\cdot+}$ radicals, the free radical scavenging activity of proteins against hydroxyl and superoxide radicals which are present in living organisms were also examined. In particular, hydroxyl (OH$^{\cdot}$) radicals are highly reactive and can damage all types of macromolecules (carbohydrates, lipids, amino acids). These radicals react with nucleotides in DNA and cause strand breakage leading to carcinogenesis, mutagenesis and cytotoxicity (Halliwell, 2001). The sheep whey protein scavenged OH$^{\cdot}$ radical with an IC_{50} value similar to that of cow whey protein. Like DPPH and ABTS assays, beef protein had the lowest IC_{50} value against OH$^{\cdot}$. Superoxide ($O_2^{\cdot-}$) radicals have been observed to kill cells, inactivate enzymes, and degrade DNA, cell membranes, and polysaccharides. These radicals may also play an important role in the peroxidation of unsaturated fatty acids and possibly other susceptible substances (Halliwell, 2001). In the superoxide scavenging assay, beef protein was shown to be not only the most potent as demonstrated in the other assays, but it was the only one that scavenged $O_2^{\cdot-}$ radicals. The fact that only one of the tested proteins scavenged $O_2^{\cdot-}$ radicals may be explained by the low rate of reaction of this radical with amino acids as well as by its short half-life (Halliwell & Gutteridge, 1999).

Apart from the free radical scavenging activity, the reducing power of the tested proteins was also examined. The reducing power of a substance is associated with its antioxidant activity, since it indicates that the substances are electron donors and can reduce the oxidised intermediates of the lipid peroxidation processes, so that they can act as primary and secondary antioxidants (Chanda & Dave, 2009). The sheep whey protein at increasing concentrations showed increased reducing power. Like the free radical scavenging assays, beef protein exhibited the highest reducing power activity. As it seems from the above results, beef protein was the most effective of all with IC_{50} values ranging from 0.6–2.5 mg/ml. The high radical scavenging activity of beef protein may be due to its amino acid content. It has been suggested that proteins exhibiting high antioxidant activity have a high content of amino acids that are very labile to oxidation, and so can potentially scavenge radicals before they are able to attack and damage other macromolecules (Elias, Kellerby, & Decker, 2008). Amino acids that are very oxidizable are those with sulfur groups (cysteine and methionine) or aromatic side chains (tryptophan, tyrosine, and phenylalanine) from which hydrogen is easily removed (Elias et al., 2008).

As mentioned above, since sheep whey protein exhibits antioxidant activity, we investigated its potential protective activity against tBHP-induced oxidative stress in muscle cells. Cells are equipped with antioxidant defence mechanisms to deal with the excessive production of ROS. For example, glutathione (GSH), the most abundant non-protein thiol source in the cells, is an important intracellular antioxidant that protects against oxidative stress. About 20% of intracellular GSH is located in the mitochondria where it helps protect from ROS produced as by-products of the electron transport chain (Livingstone & Davis, 2007). GSH exerts its antioxidant action by donating a hydrogen atom to a variety of radicals (Yu, 1994), serving as a substrate for glutathione peroxidase (GPX) to eliminate H_2O_2 (Meister & Anderson, 1983) and reducing other antioxidants in the cell such as vitamin E (Reddy, Scholz, Thomas, & Massaro, 1982). GSH levels after incubation with tBHP only, decreased significantly by 31.5%. It has been reported that tBHP oxidises GSH through the action of glutathione peroxidase (GP_x) and leads to increased levels of GSSG (Lima, Fernandes-Ferreira, & Pereira-Wilson, 2006). Treatment of cells with sheep whey protein before tBHP administration led to an increase

in GSH levels compared to tBHP alone treatment. Our results are consistent with those of previous studies. In particular, Xu et al. (2011) showed that treatment of C2C12 cells with 0.5 mg/ml whey protein, under the influence of hydrogen peroxide (H_2O_2), increased GSH levels by 341% compared to H_2O_2 alone treatment. In another study, administration of 0.1, 1 and 10 mg/ml of whey protein, before ethanol exposure in pheochromocytoma cell line (PC12), increased GSH levels by 20%, 43% and 98% respectively (Tseng et al., 2006). The effect of whey protein on GSH levels is associated with its high content in cysteine. Cysteine is one of the three amino acids of GSH and determines the rate of GSH biosynthesis (Lu, 1999). There is a competition for the use of cysteine either for GSH production or for protein synthesis, with the second to be favoured when the levels of the amino acid in the cell are low. Therefore, if a cysteine rich protein, like whey protein, is taken through the diet, then biosynthesis of GSH will be induced. Furhermore, whey protein appears to induce the synthesis of glutathione peroxidase which converts GSSG into GSH (Xu et al., 2011).

Moreover, the effects of sheep whey protein on TBARS levels was studied (a marker of lipid peroxidation). Treatment of C2C12 cells with tBHP increased significantly TBARS levels by 29%. TBHP reacts with Fe^{2+} leading to the formation of tert-butyl-hydoperoxyl ($tBO^{\cdot}$) radicals. Both the generated $tBO^{\cdot}$ radicals and the interaction of tBHP with GSH have been associated with lipid peroxidation (Alia, Ramos, Mateos, Bravo, & Goya, 2005; Lima et al., 2006). The treatment of muscle cells C2C12 with sheep whey protein decreased tBHP-induced increase of TBARS levels. Interestingly, in a previous study, we have shown that a cake containing sheep whey protein decreased plasma TBARS levels in athletes after intense exercise (Kerasioti et al., 2012). Moreover, Xu et al. (2011) showed that in C2C12 cells whey protein at 0.5 mg/ml inhibited by 67% hydrogen peroxide-induced increase of malondialdehyde (MDA) levels, a marker of lipid peroxidation.

Regarding the ROS levels, tBHP treatment had no effect. This may be due to neutralization of free radicals by the antioxidant mechanisms of the cell (e.g. GSH) and/or to reaction with other molecules. Thus, although tBHP produces free radicals, their "free form" cannot be observed. For example, we observed a decrease in GSH and an increase in lipid peroxidation after tBHP treatment indicating that free radicals reacted with GSH and lipids. Treatment of C2C12 cells with sheep whey protein before tBHP administration led to a decrease in ROS levels up to 41.30% compared to tBHP treatment alone. Moreover, in another study, whey protein isolate (pWPI) and whey protein native hydrolysates (nWPI) at 2 mg/ml inhibited H_2O_2-induced ROS formation by 76% and 32.5% respectively in a human colonic adenocarcinoma (Caco-2) cell line (Piccolomini, Iskandar, Lands, & Kubow, 2012).

In conclusion, the present results showed that sheep whey protein was effective to scavenge free radicals and to protect muscle cells from oxidative stress-induced damage. Therefore, the results support our findings from previous *in vivo* studies suggesting that a supplement in a cake form containing sheep whey protein protected athletes from exercise-induced oxidative stress (Kerasioti et al., 2012). Moreover, the present results provide an explanation for the cellular mechanisms through which the sheep whey protein may exert its protective activity against oxidative stress, since it was observed an increase in GSH levels and a decrease in lipid peroxidation (i.e. TBARS levels) and ROS levels in C2C12 cells. Thus, the findings of the present study suggest that the use of sheep whey protein as food supplement for athletes may have not only effects on muscle growth but also antioxidant enhancement. Of course, more research is needed as concerns the molecular mechanisms through which whey protein acts as an antioxidant. For example, it has been shown that whey protein increases a number of antioxidant enzymes (Sukkar & Bounous, 2004), and thus we currently perform experiments to find out the molecular pathways involved in these effects.

Conflict of interest

The authors declare that there are no conflicts of interest.

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References

Alia, M., Ramos, S., Mateos, R., Bravo, L., & Goya, L. (2005). Response of the antioxidant defense system to *tert*-butyl hydroperoxide and hydrogen peroxide in a human hepatoma cell line (HepG2). *Journal of Biochemical and Molecular Toxicology, 19*, 119–127.

Bartfay, W. J., Davis, M. T., Medves, J. M., & Lugowski, S. (2003). Milk whey protein decreases oxygen free radical production in a murine model of chronic ironoverload cardiomyopathy. *Canadian Journal of Cardiology, 191*, 1163–1168.

Chanda, S., & Dave, R. (2009). *In vitro* models for antioxidant activity evaluation and some medicinal plants possessing antioxidant properties: An overview. *African Journal of Microbiology Research, 3*, 981–996.

Chung, S. K., Osawa, T., & Kawakishi, S. (1997). Hydroxyl radical scavenging effects of species and scavengers from brown mustard (*Brassica nigra*). *Bioscience, Biotechnology and Biochemistry, 61*, 118–123.

Dupré-Crochet, S., Erard, M., & Nüβe, O. (2013). ROS production in phagocytes: Why, when, and where? *Journal of Leucocute Biology, 94*(4), 657–670.

Elias, R. J., Kellerby, S. S., & Decker, E. A. (2008). Antioxidant activity of proteins and peptides. *Critical Reviews in Food Science and Nutrition, 48*(5), 430–441.

Gülçin, I., Küfrevioglu, O. I., Oktay, M., & Büyükokuroglu, M. E. (2004). Antioxidant, antimicrobial, antiulcer and analgesic activities of nettle (*Urtica dioica* L.). *Journal of Ethnopharmacology, 90*, 205–215.

Ha, E., & Zemel, M. B. (2003). Functional properties of whey, whey components, and essential amino acids: Mechanisms underlying health benefits for active people. *Journal of Nutritional Biochemistry, 14*, 251–258.

Halliwell, B. (2001). Free radicals and other reactive species in disease. In J. Wiley & Sons (Ed.), *Encyclopedia of life sciences*. Nature Publishing Group.

Halliwell, B., & Gutteridge, J. M. C. (1999). *Free radicals in biology and medicine* (3rd ed.). Oxford Science Publications (Chapter 2).

Hood, D. A., Uguccioni, G., Vainshtein, A., & D'souza, D. (2011). Mechanisms of exercise-induced mitochondrial biogenesis in skeletal muscle: Implications for health and disease. *Comprehensive Physiology, 1*, 1119–1134.

Kamau, S. M., & Lu, R. R. (2011). The effects of enzymes and hydrolysis conditions on degree of hydrolysis and DPPH radical scavenging activity of whey protein hydrolysates. *Current Research in Dairy Science, 3*, 25–35.

Keles, M. S., Taysi, S., Sen, N., Aksoy, H., & Akcay, F. (2001). Effect of corticosteroid therapy on serum and CSF malondialdehyde and antioxidant proteins in multiple sclerosis. *Canadian Journal of Neurological Science, 28*, 141–143.

Kerasioti, E., Kiskini, A., Veskoukis, A., Jamurtas, A., Tsitsimpikou, C., Tsatsakis, A. M., et al. (2012). Effect of a special carbohydrate-protein cake on oxidative stress markers after exhaustive cycling in humans. *Food and Chemical Toxicology, 50*, 2805–2810.

Kerasioti, E., Stagos, D., Jamurtas, A., Kiskini, A., Koutedakis, Y., Goutzourelas, N., et al. (2013). Anti-inflammatory effects of a special carbohydrate-whey protein cake after exhaustive cycling in humans. *Food and Chemical Toxicology, 13*, 61–66.

Lima, C. F., Fernandes-Ferreira, M., & Pereira-Wilson, C. (2006). Phenolic compounds protect HepG2 cells from oxidative damage: Relevance of glutathione levels. *Life Sciences, 79*, 2056–2068.

Linnane, A. W., Zhang, C., & Yarovaya, N. (2002). Human aging and global function of coenzyme Q10. *Annals of the New York Academy of Sciences, 959*, 396–411.

Livingstone, C., & Davis, J. (2007). Targeting therapeutics against glutathione depletion in diabetes and its complications. *British Journal of Diabetes & Vascular Disease, 7*, 258–265.

Lu, S. (1999). Regulation of hepatic glutathione synthesis: Current concepts and controversies. *FASEB Journal, 13*, 1169–1183.

Marshall, K. (2004). Therapeutic applications of whey protein. *Alternative Medicine Review, 9*, 136–156.

McClung, J. M., Deruisseau, K. C., Whidden, M. A., Van Remmen, H., Richardson, A., Song, W., et al. (2010). Overexpression of antioxidant enzymes in diaphragm muscle does not alter contraction-induced fatigue or recovery. *Experimental Physiology, 95*, 222–231.

Meeus, M., Nijs, J., Hermans, L., Goubert, D., & Calders, P. (2013). The role of mitochondrial dysfunctions due to oxidative and nitrosative stress in the chronic pain or chronic fatigue syndromes and fibromyalgia patients: Peripheral and central mechanisms as therapeutic targets? *Expert Opinion on Therapeutic Targets, 17*, 1081–1089.

Meister, A., & Anderson, M. E. (1983). Glutathione. *Annual Review of Biochemistry, 52*, 711–760.

Mylonas, C., & Kouretas, D. (1999). Lipid peroxidation and tissue damage. *In Vivo, 13*, 295–309.

Nikolaidis, M. G., Jamurtas, A. Z., Paschalis, V., Fatouros, I. G., Koutedakis, Y., & Kouretas, D. (2008). The effect of muscle-damaging exercise on blood and skeletal muscle oxidative stress: Magnitude and time-course considerations. *Sports Medicine, 38*, 579–606.

Nikolaidis, M. G., Kyparos, A., Hadziioannou, M., Panou, N., Samaras, L., Jamurtas, A. Z., et al. (2007). Acute exercise markedly increases blood oxidative stress in boys and girls. *Applied Physiology, Nutrition and Metabolism, 32*, 197–205.

Phaneuf, S., & Leeuwenburgh, C. (2001). Apoptosis and exercise. *Medicine & Science in Sports & Exercise, 33*, 393–396.

Piccolomini, A. F., Iskandar, M. M., Lands, L. C., & Kubow, S. (2012). High hydrostatic pressure pre-treatment of whey proteins enhances whey protein hydrolysate inhibition of oxidative stress and IL-8 secretion in intestinal epithelial cells. *Food & Nutrition Research, 56*.

Powers, S. K., & Jackson, M. J. (2008). Exercise-induced oxidative stress: Cellular mechanisms and impact on muscle force production. *Physiological Reviews, 88*, 1243–1276.

Reddy, C. C., Scholz, R. W., Thomas, C. E., & Massaro, E. J. (1982). Vitamin E dependent reduced glutathione inhibition of rat liver microsomal lipid peroxidation. *Life Sciences, 31*, 571–576.

Schneider, B. S., & Tiidus, P. M. (2007). Neutrophil infiltration in exercise-injured skeletal muscle: How do we resolve the controversy? *Sports Medicine, 37*, 837–856.

Spanou, C., Stagos, D., Tousias, L., Angelis, A., Aligiannis, N., Skaltsounis, A. L., et al. (2007). Assessment of antioxidant activity of extracts from unique Greek varieties of Leguminosae plants using *in vitro* assays. *Anticancer Research, 27*, 3403–3410.

Sukkar, S. G., & Bounous, G. (2004). The role of whey protein in antioxidant defense. *Rivista Italiana di Nutrizione Parenterale ed Enterale, 22*, 193–200.

Tseng, Y. M., Lin, S. K., Hsiao, J. K., Chen, I. J., Lee, J. H., Wu, S. H., et al. (2006). Whey protein concentrate promotes the production of glutathione (GSH) by GSH reductase in the PC12 cell line after acute ethanol exposure. *Food and Chemical Toxicology, 44*, 574–578.

Valko, M., Leibfritz, D., Moncol, J., Cronin, M. T. D., Mazur, M., & Telser, J. (2007). Free radicals and antioxidants in normal physiological functions and human disease. *International Journal of Biochemistry & Cell Biology, 39*, 44–84.

Walzem, R. L., Dillard, C. J., & German, J. B. (2002). Whey components: Millennia of evolution create functionalities for mammalian nutrition: What we know and what we may be overlooking. *Critical Reviews in Food Science & Nutrition, 42*, 353–375.

Wu, G., Fang, Y. Z., Yang, S., Lupton, J. R., & Turner, N. D. (2004). Glutathione metabolism and its implications for health. *Journal of Nutrition, 134*, 489–492.

Xu, R., Liu, N., Xu, X., & Kong, B. (2011). Antioxidative effects of whey protein on peroxide-induced cytotoxicity. *Journal of Dairy Science, 94*, 3739–3746.

Yen, G. C., & Duh, P. D. (1994). Scavenging effect of methanolic extracts of peanut hulls on free radical and active oxygen species. *Journal of Agricultural and Food Chemistry, 42*, 629–632.

Yu, B. P. (1994). Cellular defenses against damage from reactive oxygen species. *Physiological Reviews, 74*, 139–162.