Pharmacological Study of the Possible Antidepressant Activity of Whey Protein Isolate in Mice

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Abstract: Depression is a serious psychiatric disorder and a leading cause of morbidity and mortality. Many synthetic antidepressant drugs show low response rates and even produce adverse side-effects. Nowadays new trends in the treatment of depression are directed towards the use of natural products with better response rates and fewer side-effects. Whey protein products have been used as functional foods with a number of health benefits. The present study aimed to evaluate the antidepressant-like effect of whey protein isolate in a chronic unpredictable stress model in mice. Main methods: depression-like behaviors were induced using the chronic unpredictable stress model (CUS) in mice; which depends on the subjection of the mice to different types of physical stressors for twenty four consecutive days. Whey protein isolate (WPI) (75, 150 and 300mg/kg bwt. p.o.) and fluoxetine (10mg/kg bwt. p.o.) were administered daily throughout the period of the investigation thirty minutes before exposure to the physical stressor. This method aimed to evaluate the ability of WPI to prevent the incidence of depression-like behaviors compared to fluoxetine. Key findings: Results revealed that WPI (300 mg/kg) showed a significant increase in the monoamines and the glutamate brain levels and inhibited GABA release. The results were comparable to those of fluoxetine. The antioxidant potential of WPI could contribute, in part, in ameliorating the depressive symptoms. Significance: Further investigations are recommended to study the mechanism of action of WPI on brain and evaluate its applicability to be used as an antidepressant drug.

Key words: Depression; whey protein isolate; Open field; Tail suspension; Forced swimming; brain neurotransmitters; malondialdehyde; reduced glutathione.

INTRODUCTION

Major depressive disorder (MDD) is a common, chronic, recurrent, life-threatening mental illness that affects up to 20% of the population across the world and considered a leading cause for morbidity and mortality (Yan et al., 2010). Although the pathophysiology of mood disorders remains poorly understood, recent evidence suggests that oxidative stress could contribute to this process (Drzyzga et al., 2009).

The brain is particularly vulnerable to reactive oxygen species (ROS) production because it metabolizes 20% of the total body oxygen and has a limited amount of antioxidant capacity (Frey et al., 2006). Antidepressant drugs are widely available in the pharmaceutical market (Shen and Liang 2007). However, because multiple pathogenic factors are involved in depression, many synthetic antidepressant drugs show low response rates and even produce adverse side-effects in depressed patients such as cardiotoxicity, hypertensive crises, sexual dysfunction and sleep disorders (Park et al., 2007). Therefore, it is desirable to seek antidepressants in natural products; such products are expected to show fewer side-effects (Mao et al., 2009).

Milk contains two primary sources of protein, the caseins and whey. After processing, the caseins are the proteins responsible for making curds, while whey remains in the aqueous environment (Marshall 2004; Yamaguchi et al., 2009). Whey has been used as a functional food with a number of health benefits. The biological components of whey demonstrate a range of immune-enhancing properties. In addition, whey has the ability to act as an antioxidant, antiinflammatory, antitumor, hypolipidemic, antiviral, antibacterial and chelating agent (Yamaguchi et al., 2009). Bovine whey proteins are rich in cysteine, the disulfide form of the amino acid cysteine. Cysteine is the rate-limiting amino acid for the synthesis of the beneficial antioxidant glutathione (GSH). Consumption of dietary whey proteins may provide a useful strategy to elevate the intracellular GSH level (Kent et al., 2003). Milk whey protein fractions are also rich in glutamine, tryptophan, phenylalanine and tyrosine (Marshall 2004). Glutamine plays a major role in the synthesis of the neurotransmitter glutamate in specialized excitatory neurons. Tryptophan on the other hand; is the precursor of serotonin (5-HT) while phenylalanine and tyrosine are the precursors of both norepinephrine (NE) and dopamine (DA) (Behar and Rothman 2001; Gordon et al., 2008; Gu et al., 2001; Kelleher et al., 2003; Lien 2003; Nieuwenhuizen et al., 2003). In the present study, depression was induced physically using the chronic unpredictable stress model.
(CUS) described by Mao et al., (2009) and aimed to evaluate the ability of whey protein isolate (WPI) to prevent the incidence of depression compared to fluoxetine (standard drug).

MATERIAL AND METHODS

Animals:
Adult male albino mice, weighing 20-25 g each, purchased from the National Research Center (NRC, Giza, Egypt). All animals received human care in compliance with the guidelines of the animal care and use committee of the NRC. The animals were kept in a quiet place and were allowed free access to water and food throughout the period of investigation. Experiments were performed according to the National Regulations on Animal Welfare and Institutional Animal Ethical Committee (IAEC).

Drugs:
Fluoxetine Hydrochloride (Prozac 20mg dispersible tablets, Lilly, Spain). The tablets were freshly suspended in distilled water prior to oral administration.
Whey protein isolate (WPI) was provided as a generous gift from [Davisco Foods International Inc.]. It was provided as a pure powder for ingestion. The powder was freshly dissolved in distilled water just before oral administration.

CUS Model:
Mice were randomly assigned into six groups of ten individuals: normal control receiving oral distilled water ingestion, depressed control receiving oral distilled water ingestion, standard group receiving fluoxetine (10mg/kg p.o.) (Brocardo et al., 2008), whey protein groups receiving WPI at dose levels of (75, 150 and 300 mg/kg p.o.) [WPI doses were chosen by a dose response curve experiment previously conducted in our laboratory (data not shown in the paper)]. Normal control group was housed in a separate room, did not have any contact with the stressed animals and was undisturbed except for the necessary procedures such as routine cage cleaning. All the other groups were subjected to a CUS procedure for a period of 24 days, where the mice were subjected each day to only one of six physical stressors 30 min. after the corresponding drug ingestion. The CUS procedure was performed as described by Mao et al., (2009) with a slight modification. Briefly, the CUS consisted of a variety of unpredictable stressors namely; 24-hrs. of water deprivation, 6-min. swimming in cold water (at 8°C), 1-min. of tail pinching (1 cm from the end of the tail), 2-hrs. of restraint, 24-hrs. in a soiled cage (200ml water in 100 g sawdust bedding) and overnight illumination. One of these stressors (in random order) was given every day for 24 days.

Behavioral Tests:
Twenty-four hrs. after the last stressor behavioral tests were performed; these include: the open-field test, the tail suspension test and the forced swimming test. Behavioral observations were recorded by an observer who was blind to the treatments.

Open Field Test:
The open field test was carried out in a square wooden arena (40 cm x 40 cm x 40cm high) with red walls and white smooth polished floor divided by black lines into 16 equal squares. The test was performed under white light in a quiet room. Each mouse was placed at the same corner square and observed during 5 min. The floor and walls were cleaned after testing each mouse. The following parameters were recorded during the 5 min. observation period: latency: time taken by each animal till it starts moving in the arena, ambulation frequency: number of squares crossed by the animal, rearing frequency: number of times the animal stood stretched on its hind limbs with or without forelimb support and grooming frequency: number of face scratching and washing with the hind limbs and licking of the forelimbs (kim et al., 2005; Kwak et al., 2009; Van den Buuse and de Jong 1989).

Tail Suspension Test:
The tail suspension test is based on the observation that a mouse suspended by the tail shows alternating periods of agitation and immobility. The test was performed as described by Stéru et al., (1985). Each mouse was suspended by its tail from a steel bar using an adhesive tape placed approximately 1cm. from the tip of the tail, the distance between the floor and the tail was about 30 cm. (Kwak et al., 2009). The total duration of immobility during a 6-min. test was measured (El Yacoubi et al., 2001). Mice were considered immobile only when they hung passively in a completely motionless state.

Forced Swimming Test:
The forced swimming test was performed according to the method described by Porsolt et al., (1977). In this method, each mouse was forced to swim in a restricted space from which it can’t escape. Each mouse was
placed for 6 min. in a cylindrical transparent water tank with a diameter of 18 cm and height of 25 cm. The water level was about 15 cm and its temperature was maintained at 25±1°C. The total duration of immobility of each animal in the last 4 min. was recorded. The tank was emptied and washed with fresh water flush after each mouse.

**Biochemical Analysis:**

**Tissue Sampling:**

Twenty-four hours after completion of the behavioral tests, mice were sacrificed by decapitation. Brains were isolated and each brain was washed with cold sterile physiological saline, blotted between two damp filter papers and stored at ≈80°C until use for biochemical analysis.

**Determination Of Brain Amino Acids And Monoamines:**

Each brain tissue was weighed and homogenized in 75% aqueous HPLC grade methanol (10% w/v) (Arafa et al., 2010). The homogenate was spun at 4000 r.p.m. for 10 min. and the supernatant was divided into two halves; the first was dried using vacuum (70 millipore) at room temperature and used for GABA (gamma-aminobutyric acid) and glutamate determination, whereas the second half was used for monoamine determination.

Brain glutamate and GABA were detected by high performance liquid chromatography (HPLC) using the precolumn PTC derivatization technique according to the method of Heinrikson and Meredith (1984). Brain monoamines were detected by HPLC according to method described by Pagel et al., (2000).

**Determination of MDA and GSH:**

Each brain tissue was homogenized in ice-cold saline (20% w/v) (Mansour et al., 2002). The homogenate was divided into 2 portions for the determination of malondialdehyde (MDA) and reduced glutathione (GSH) levels. The level of MDA was determined in mice brain homogenate according to the method of Ruiz-Larea et al., (1994). The level of GSH was determined in mice brain homogenate according to the method of Ellman (1959) modified by Bulaj et al., (1998).

**Statistical Analysis:**

Statistical analysis was performed by one way analysis of variance (ANOVA) followed by the Least Significant Difference test (LSD test) Data was expressed as means±SEMs. where any two groups were considered significantly different if the difference between their mean values was more that the corresponding LSD value stated for each experiment at p < 0.05 (Howell 2002).

**Results:**

**Effect of Whey Protein Isolate on the Latency Time, Ambulation, Rearing and Grooming Frequencies:**

The results are graphically illustrated in figures (1a and 1b). Depression-like behavior induced by the CUS model could be clearly demonstrated by the significant decrease in the activity of all mice where the ambulation, rearing and grooming frequencies decreased to 57.2 %, 34.94 % & 42.86 % of normal control values while the latency time increased to 453.6 % of the normal control value. Fluoxetine (10mg/kg) treatment restored the normal value of the latency time and the ambulation frequency, it significantly increased the rearing frequency up to 237.93% compared to the depressed group value and increased the grooming frequency up to 134.01 % of the normal control value. WPI (75mg/kg) and WPI (150mg/kg) significantly decreased the latency time to 65.818% & 37.228% and elevated the rearing frequency up to 220.69 % and 235.17% respectively compared to the depressed group Values. Both doses normalized the ambulation and the grooming frequencies. WPI (300mg/kg) on the other hand, normalized all the four parameters and gave results that were comparable to those of fluoxetine (10 mg/kg).

The (OFT) was performed 24hrs. after the last drugs ingestion Statistical analysis was carried out by ANOVA – one way followed by LSD multiple comparisons test. Each vertical bar represents mean of percent from the normal control, n=10 mice/group at p> 0.05. @: significantly different from the normal group.* : significantly different from the depressed group.

The (OFT) was performed 24hrs. after the last drugs ingestion Statistical analysis was carried out by ANOVA – one way followed by LSD multiple comparisons test. Each vertical bar represents mean of percent from the normal control, n=10 mice/group at p< 0.05. @: significantly different from the normal group.* : significantly different from the depressed group.

**Effect Of Whey Protein Isolate On The Immobility Duration In The Forced Swimming Test (FST):**

The results are graphically illustrated in Figure (2). Application of the CUS model on mice produced a significant increase in the immobility duration (98.5±8.69 seconds) compared to the normal control value which was only 3.2±0.42 seconds. Fluoxetine (10mg/kg) reinstated the immobility duration back to the normal value.
WPI (75 & 150 mg/ kg) resulted in a significant decrease in the immobility duration (44.57% & 22.74%) respectively compared to the depressed group value while WPI (300mg/kg) normalized the immobility duration.

**Fig. 1a:** Effect of WPI on the latency time in the open field test (OFT).

**Fig. 1b:** Effect of WPI on the ambulation, rearing and grooming frequencies in the open field test (OFT).

**Fig. 2:** Effect of WPI on the immobility duration in the forced swimming test (FST).
The (FST) was performed 24hrs. after the last drugs ingestion. Statistical analysis was carried out by ANOVA – one way followed by LSD multiple comparisons test. Each vertical bar represents mean of percent from the normal control, n=10 mice/group at p< 0.05. @: significantly different from the normal group.* : significantly different from the depressed group.

Effect of Whey Protein Isolate on the Immobility Duration in the Tail Suspension Test (TST):

The results are graphically illustrated in Figure (3). The CUS model of depression produced a significant increase in the immobility duration (140.8±6.14 seconds) compared to the normal control value (29.9±2.91 seconds). Fluoxetine (10mg/kg) normalized the immobility duration. WPI (75 & 150 mg/ kg) resulted in a significant decrease in the immobility duration compared to the depressed group value (66.33% & 47.23% respectively). WPI (300mg/kg) reduced the immobility duration to 30.11% of the depressed group value and there was no significant difference between its result and the normal control.

![Fig. 3: Effect of WPI on the immobility duration in the tail suspension test (TST).](image)

Effect of Whey Protein Isolate on Brain Serotonin, Norepinephrine and Dopamine Contents:

The results are graphically illustrated in figure (4). The CUS model caused a significant decrease in the levels of serotonin, norepinephrine and dopamine to 44.83 %, 36.13% & 63.33% compared to the normal control levels respectively. Fluoxetine (10mg/kg) elevated the levels of serotonin, norepinephrine and dopamine up to 117.24 %, 94.45 % and 101.33% respectively compared to the normal control levels. WPI (75, 150 and 300 mg/kg) resulted in a significant increase in serotonin (130.77%, 138.46% & 146.15%) respectively compared to the depressed control level. On the other hand the three dose levels increased the norepinephrine content up to (146.91 % 156.74 % &165.45 %) and dopamine levels to (172% 172.67 % & 218.67 %) respectively compared to the normal control values.

Decapitation was performed 48hrs. after the last drugs ingestion. Statistical analysis was carried out by ANOVA – one way followed by LSD multiple comparisons test. Each vertical bar represents mean of percent from the normal control, n=10 mice/group at p< 0.05. @: significantly different from the normal group.* : significantly different from the depressed group.

Effect of Whey Protein Isolate on Brain GABA and Glutamate Contents:

The results are graphically illustrated in figure (5). The CUS model caused a significant increase in the level of GABA (202.94%) and a significant decrease in the level of glutamate (27.98 %) compared to the normal control levels. Fluoxetine (10mg/kg) resulted in a significant decrease in the level of GABA (71.01%) compared to the depressed group level and significantly elevated the glutamate level up to (193.76%) compared to the normal control level. Treatment with WPI (75 mg/kg) didn’t have any effect on either the GABA or the
glutamate levels and its results were not significant from those of the depressed control. On the other hand, although WPI (150 mg/kg) didn’t have any effect on the glutamate level, it decreased the GABA content down to the normal level. Meanwhile, ingestion of WPI (300 mg/kg) significantly decreased the GABA level down to 14.49 % compared to the depressed group level and significantly increased the glutamate level up to 224.39 % of the normal control level.

Fig. 4: Effect of WPI on brain serotonin, norepinephrine and dopamine contents.

Decapitation was performed 48hrs. after the last drugs ingestion. Statistical analysis was carried out by ANOVA – one way followed by LSD multiple comparisons test. Each vertical bar represents mean of percent from the normal control, n=10 mice/group at p< 0.05. @: significantly different from the normal group.* : significantly different from the depressed group.

**Effect of Whey Protein Isolate on Brain Malondialdehyde (MDA) and Reduced Glutathione (GSH) Levels:**

The results are graphically illustrated in figure (6). The CUS model caused a significant decrease in the GSH level to 47.53 % of the normal control value. On the other hand, it increased the level of MDA up to 124.56 % compared to the normal control level. Treatment with fluoxetine (10mg/kg) normalized the levels of both MDA and GSH. WPI (75mg/kg & 150mg/kg) didn’t have any significant effect on GSH compared to the
Depressed group but on the contrary both doses reduced the MDA level down to 76.38% and 59.24% of the depressed group level respectively. Meanwhile, ingestion of WPI (300 mg/kg) resulted in a significant increase in the GSH level that reached 92.36% of the normal control value and its effect was similar to that of fluoxetine, it also significantly decreased the MDA level down to 47.64% compared to the depressed group level.

Discussion:
The present data revealed that the CUS model resulted in depression-like symptoms illustrated by the reduction in the ambulation, rearing and grooming frequencies and the increase in the latency time in the open field test. These results are in agreement with Dang et al., (2009) where a decrease in locomotion was observed in the open field test after the application of an unpredictable chronic mild stress (UCMS) on mice. In addition, the CMS regimen decreased the grooming behavior in mice (Detanico et al., 2009; Yalcin et al., 2005). It was observed in our study that the normal immobility durations in the forced swimming test and the tail suspension test were very low compared to the normal ranges usually obtained in these tests in other published papers but our results were reproducible in other experiments done in our laboratory (Data not shown) and we could not explain these results except by the reasons of strain differences, breeding and housing. This was previously explained in the review article by Harro (2004) who reported that differences between laboratories including the differences and the way of breading, housing and environmental conditions can change the ranges of results obtained in a test from one laboratory to another. The exposure to CUS increased the duration of immobility in both; the forced swimming test and the tail suspension test, indicating the induction of depression. These observations are consistent with the results of Elizalde et al., (2008); Ma et al., (2011).

Treatment with fluoxetine normalized the latency time, ambulation and grooming frequencies and significantly increased the rearing frequency compared to the stressed group. Moreover, the immobility duration was normalized in both the forced swimming test and the tail suspension test. These findings are in agreement with those of Dhir and Kulkarni (2008); Jesse et al., (2010); Rygula et al., (2006).

In the present study, whey protein isolate (300 mg/kg) normalized all the four parameters in the open field test. These results may be attributed to the high tryptophan content in whey protein. Previously the antidepressant-like effect of tryptophan was studied in mice using the forced-swimming test, open-field test and activity cage. A single-dose of tryptophan resulted in an antidepressant-like effect that was dose dependent up to 125 mg/kg. These results were found to be due to the conversion of tryptophan to serotonin (5-HT) (Wong and Ong 2001).

The decrease in brain neurotransmitters; serotonin, norepinephrine and dopamine has been implicated in the incidence of depression (Doris et al., 1999). New theories suggest that the three neurotransmitters are...
interrelated in the treatment of depression (Daws 2009). Converging evidence has indicated that hyper glutamate activity and GABA dysfunction may play important roles in the neurobiology and treatment of depression and other mood disorders (Yang and Shen 2005). Deficient glutamate function has been linked to depressive-like behavior (Elizalde et al., 2010; Tordera et al., 2011). Increase in the glutamate release in certain brain areas may be promising novel therapeutics for mood disorders (Machado-Vieira et al., 2009). On the other hand; mice with disrupted GABA<sub>B</sub> receptors display an antidepressant-like phenotype, with decreased immobility in the forced swim test, suggesting that the blockage of GABA<sub>B</sub> receptors may serve as a novel strategy for the treatment of depression (Mombereau et al., 2004). In the present investigation, the physical stress induced by the CUS model significantly reduced the brain neurotransmitters; serotonin, norepinephrine, dopamine and glutamate while the level of GABA was elevated. These results agree with Vancassel et al., (2008) who reported that the application of CMS for eight weeks on mice resulted in decreased NE and 5-HT brain levels. Moreover; using a depression model of chronic forced swimming stress (CFSS) in rats; relative to control, the CFSS rats had significantly reduced glutamate and glutamine levels in the prefrontal cortex and reduced glutamate and glutamine/GABA ratio in the hippocampus (Li et al., 2008).

Fluoxetine normalized the levels of serotonin, norepinephrine and dopamine. It highly elevated the level of glutamate and significantly decreased the level of GABA compared to the depressed control. These results are in agreement with Bymaster et al., (2002); Kobayashi et al., (2008).

Whey protein isolate (300 mg/kg) significantly increased the serotonin level compared to the depressed control, normalized the GABA level and highly elevated the norepinephrine, dopamine and glutamate levels compared to the normal group and the results were comparable to those of fluoxetine. These results are in agreement with previous studies. In rats, alpha-lactalbumin (one of the most important constituents of whey) enhanced serotonin release and induced anxiolytic and rewarding effects, suggesting that it has beneficial effects on mood (Orosco et al., 2004). Rats feeding on alpha-lactalbumin rich diet showed elevated brain tryptophan and serotonin levels both after acute and chronic administration (Choi et al., 2009; Choi et al., 2011). In another study; twenty-nine highly stress-vulnerable subjects and 29 relatively stress-invulnerable subjects participated in a double-blind, placebo-controlled study. Subjects were exposed to experimental stress after the intake of a diet enriched with either alpha-lactalbumin or sodium-caseinate. Diet-induced change in the plasma Trp-LNAA ratio was measured. Changes in mood were assessed before and after the stressor. Consumption of a dietary protein enriched in tryptophan increased the plasma Trp-LNAA ratio in stress-vulnerable subjects and improved coping ability probably through alterations in brain serotonin (Markus et al., 2000). Moreover, whey protein is rich in tryptophan, glutamine, phenylalanine and tyrosine (Gu et al., 2001; Marshall, 2004). Glutamine plays a major role in the synthesis of the neurotransmitter glutamate in specialized excitatory neurons (Behar and Rothman 2001). Phenylalanine and tyrosine on the other hand, are the precursors of both norepinephrine and dopamine (Gordon et al., 2008). On the other hand, it was reported that serotonin inhibited GABA release and potentiated glutamate release through the action on 5-HT heteroreceptors expressed on gabaminergic and glutaminergic neurons (Harsing 2006).

In the present investigation, there was a marked increase in brain malondialdehyde (MDA) level and decrease in reduced glutathione (GSH) in stressed mice indicating that oxidative stress and depression appear to be synchronized. These findings are in agreement with Kumar et al., (2011) who reported that mice subjected to a CMS paradigm daily for a period of 21 days showed increased lipid peroxidation level and decreased glutathione level.

Fluoxetine normalized the GSH and the MDA levels indicating an antioxidant effect. Similar results were previously reported by Liu et al., (2009); Lobato et al., (2010).

GSH level was significantly increased by whey protein isolate (300 mg/kg) compared to the stressed group. Meanwhile, MDA level was significantly decreased compared to the normal control. These results are expected as previously it was reported that bovine whey protein isolate is rich in cystine (the disulfide form of the amino acid cysteine) and glycine which are the rate-limiting amino acids for the synthesis of glutathione (GSH). Consumption of these products was found to be a useful strategy to elevate the intracellular GSH level (Kent et al., 2003; Marshall 2004). On the other hand, it was previously reported that administration of whey protein (100 mg/kg) for four weeks in mice resulted in a significant decrease in the MDA level and a significant increase in the GSH level in heart tissue and reversed iron induced cardiomyopathy (Bartfay et al., 2003).

Conclusion:

In conclusion, WPI at a dose level of 300mg/kg was found to alleviate the CUS-induced depression-like symptoms in mice. The anti-depressive action may be due to increasing monoamines and glutamate brain levels, inhibition of GABA release and enhancement of antioxidant status in brain tissue. Further investigation on the potential use of WPI in clinical depression is recommended.
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Conflict of Interest:
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