

Impact of Chronic Use of Whey Protein Isolate in Two Doses Using an Experimental Model

Quezia D. Jones S. Vasconcelos ¹ , Ana Paula N. Nunes Alves ¹, Ana Cristina H. de Souza ² , Maria Elisabete A. de Moraes ¹ , Gislei F. Aragão ^{1,2,*} 

¹ Faculty of Medicine, Drug Research and Development Center, Federal University of Ceara, Coronel Nunes de Melo street, 1000, 60430-275, Fortaleza, Ceará, Brazil

² Health Science Center, State University of Ceara, Dr. Silas Mugunba avenue, 1700, 60430-275, Fortaleza, Ceará, Brazil

* Correspondence: gislei.frota@uece.br (G.F.A.);

Scopus Author ID 8686354400

Received: 10.12.2021; Accepted: 5.01.2022; Published: 24.03.2022

Abstract: There is limited evidence in the scientific literature regarding the chronic use of whey protein and its potentially adverse effects or toxicity. We evaluated the impact of chronic use of whey protein in two doses using an experimental model. Adult male Wistar rats (n = 30) were supplemented with two different doses: 3125 mg/kg and 6250 mg/kg of whey protein isolate for 90 days. Biochemical, hematological, tissue, morphological, and behavioral analysis were evaluated. The whey-treated groups significantly increased their weight compared to the control (p < 0.05). Biochemical and hematological parameters were unchanged, except for glucose, higher in the 6250 mg/kg group (p < 0.05). The behavioral analysis showed no relevant difference (p > 0.05). The organs analysis showed an absolute increase in liver, right kidney, and heart weights in the whey-treated group compared to the control (p < 0.05). This study suggests that whey protein isolate has no toxicity when supplemented at such doses for 90 days. These doses also appeared to have the same effect regarding weight gain and absolute organs weight. This research corroborates with current literature; however, further work is needed to safely investigate the chronic use of whey supplements at a higher dose.

Keywords: rat; nutritional supplement; whey protein isolate; toxicity evaluation; chronic use.

© 2022 by the authors. This article is an open-access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (<https://creativecommons.org/licenses/by/4.0/>).

1. Introduction

Whey protein (WP) is a commonly ingested nutritional supplement amongst athletes and consumers of sports nutrition products [1]. Its composition, with increased levels of essential amino acids, especially branched-chain amino acids (BCAAs) and bioactive products, is associated with different beneficial outcomes for human health [2]. The whey portion of milk contains α -lactalbumin, β -lactoglobulin, glycomacropeptide, immunoglobulins, protease peptone, and serum albumin. Altogether they make up 85% of the whey protein [3]. Advances in processing technologies have enabled the purification and separation of whey proteins, which are sold as a concentrate (WPC) or isolate (WPI) containing 35–80% and > 90% proteins, respectively. When WPC or WPI are treated with acids, enzymes, or heat, the intact form of protein breaks down into peptides and amino acids, leading to the formation of whey protein hydrolysate (WPH) [4].

WP is purported to provide antimicrobial activity, immune modulation, improve muscle strength and body composition, and prevent cardiovascular diseases and osteoporosis [2, 5]. Some authors showed that diets which are high in protein at the expense of carbohydrates are

beneficial in weight and body fat loss in human subjects [5, 6]. WP has also been associated with the improvement of cognitive functions, such as memory and learning. Studies revealed that vitamin B12 and α -lactalbumin, present in WPI, both contribute to cognitive function in the elderly [7] and cognitive performance in stress-vulnerable subjects [8], respectively. However, there is a lack of rigorous studies regarding WP consumption at a higher dose with long-term use and its impact on cognitive decline, memory, and stress-related issues [9]. A chronic use, associated with a high dose, possibly led by unappropriated guidance and misinformation, ultimately, could promote complications and alterations on the body [10].

Thus, we hypothesized that chronic use of WP above a certain dose could potentially lead to adverse effects or even toxic outcomes to different organs of the body, such as the kidney and liver. There is little understanding of WP's possible consequences or toxicity, specifically regarding biochemical, histological, tissue, and behavior alterations. In line with the above discussion, we propose to conduct an analysis following appropriate and specific guidelines regarding the protocol for chronic toxicity to evaluate WPI supplementation effects on different parameters in two distinct doses.

2. Materials and Methods

Whey protein isolate (WPI), Isofort[®] (Vitafor, São Paulo, Brazil) - vanilla flavor, was purchased from a fitness store. The nutritional facts (per 100g) of Isofort[®] are 2.600g of total protein, 130g of total carbohydrate, 0g of total fat, 4.200 mg of sodium, and 14.300mg of calcium. One serving size has 114 kcal (30g). Composition: whey protein isolate, natural and artificial flavors, xanthan gum, and sucralose. The supplement provides 6g of BCAAs per serving (30g).

2.1. Animals and experimental design.

The design for this study was adapted from the guidelines of the nonclinical toxicology and pharmacological safety studies assessment for the development of drugs by ANVISA (National Health Surveillance Agency, Brazil) [11], and OECD (The Organization for Economic Co-operation and Development) test guidelines for chemicals number 408 (chronic toxicity) [12].

A total of 30 male Wistar rats (*Rattus norvegicus* Albinus), weighing 170±25g, provided by the Federal University of Ceara (UFC) central breeding facility were used. The subjects were housed in polypropylene cages (3 rats/cage) and in a ventilated room of normal light-dark cycle (12h light/dark) and control temperature (25±2°C). Food (Nuvilab rodent food, Quimtia[®], Paraná, Brazil) and water were provided ad libitum.

The project followed the international ethics standard for animal experiments [13] and was approved by the Ethics Committee on Animal Research (CEPA) of UFC (PROTOCOL n° 82/2017). All efforts were made to minimize animal suffering.

Rats were kept under the above-mentioned conditions for 5 days before the beginning of the experiment for acclimatization. Then, they were separated into three different groups: (1) control (C, saline-treated group; n = 10), and WP-treated groups, (2) manufacturer-recommended dose (RD, 3125 mg/kg body mass, n = 10) and (3) high dose (HD, 6250 mg/kg body mass, n = 10) (Figure 1). The animals were treated orally every day for 90 days (volume of 10 mL/kg body weight). WP was dissolved in normal tap water. Their body weight was recorded before the first WP administration and weekly until the last day of treatment.

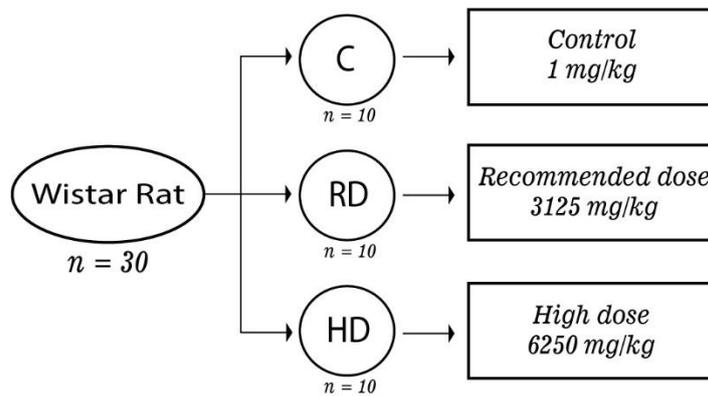


Figure 1. Experimental design. Chronic 90-day treatment of whey protein isolate in two different doses. C, control, RD, recommended dose (3125 mg/kg), and HD, high dose (6250 mg/kg).

Doses of WP were calculated based on human standards. To translate those doses to animals the HED (human equivalent dose), proposed by the Food and Drug Administration [14], was used. The RD group followed the usage instructions written on the label of the supplement (30g/60kg or 500mg/kg). For the HD group, we searched online fitness communities and estimated the higher dose to be twice the RD (60g/60 kg or 1000mg/kg).

To convert human dose in mg/kg to animal dose in mg/kg we used the following formula (conversion based on body surface area): $HED (mg/kg) = Animal\ dose (mg/kg) \times (Animal\ Km / Human\ Km)$, whereas rat (adult) Km is 6, and human (60kg) Km is 37. Therefore, the animal dose (mg/kg) for the RD group was: $500 = animal\ dose \times (6/37)$, animal dose (mg/kg) equals 3125mg/kg. The animal dose (mg/kg) for the HD group was: $1000 = animal\ dose \times (6/37)$, animal dose (mg/kg) equals 6250mg/kg.

2.2. Blood collection, serum, and tissue preparation.

At the end of the experiment, the animals were sacrificed with a single intraperitoneal injection of a ketamine and xylazine cocktail in a ratio of 9 mg/mL:0.9 mg/mL, followed by exsanguination. Animals have fasted for 4 hours; however, free access to water was maintained. The blood samples were collected via heart puncture, and part of it was collected in tubes containing ethylene diamine tetraacetic acid (EDTA) and used for the evaluation of the hematological parameters (Table 1). The SDH-3 automatic analyzer (Labtest®, São Paulo, Brazil) was used for the hematological analysis. The other part was collected without anticoagulant substances and then centrifuged (3000 rpm for 15 minutes) to obtain serum samples. The serum was stored at -20°C for subsequent biochemical analysis (Table 2). The biochemical diagnostic kits were from Labtest®, São Paulo, Brazil.

The animal's liver, kidney, spleen, heart, and brain were carefully extracted and weighed. Then, they were immersed in a 10% buffered formaldehyde solution for further analyses. The animal's tissues were embedded in a wax block. Paraffin sections were cut with the rotary microtome and placed on proper glass slides. The cuts were made with the aid of a cryostat system (Carl Zeiss model HYRAX C 25 UV model, Carl Zeiss, Oberkocen, Germany) with 20-mm thickness and then stained with hematoxylin and eosin.

2.3. Behavioral testing.

The behavioral testing of both the control and experimental group was performed by the following tests: open field, Y-maze, and passive avoidance. The tests were performed before treatment and on days 89th and 90th of treatment.

2.3.1. Open field.

A 1.2m² open field made of acrylic, transparent walls (50cm high), and a black floor divided into 9 squares of the equal area was used. The animals were placed in the middle and allowed to explore the maze for 6 minutes, with 1 minute for adaptation. Their behaviors were visually scored: line crossing with the 4 paws (ALE), rearing (R), grooming (G), number of fecal boli (NFB), and time spent on the center square (CS) [15].

2.3.2. Y-maze.

This test was shaped to evaluate working memory. Each rat could freely move through the maze for 8 min. The series of arm entries (AE) was visually recorded. Alternations (ALT) were defined as entrances in all three arms on consecutive occasions. The percentage (%) of alternation was calculated as the total of alternations / (total arm entries – 2) [16].

2.3.3. Passive avoidance.

Each rat was placed in space 1 (elevated platform) of the passive avoidance (PA) apparatus. If it stepped on space 2 (grid floor), with the 4 paws, a shock (50 Hz, 0.2 mA, 1s) was given. This continues for a maximum of 120s (habituation trial). 24h later, the animal was placed on the PA apparatus again (test trial).

On both days, habituation and test, the time spent on the step (TS) and the time spent on the grid floor (TG) was visually recorded. The number of times the animal stepped down was measured as the error times (ET). If an animal does not step down within 300s, the trial would be terminated [17].

2.4. Statistical analysis.

All data were analyzed using ANOVA followed by a Tukey's test with GraphPad PRISM version 6.0 for Windows. Results were reported as means \pm standard error of the mean (SEM) (n = 8 for HD group, n = 10 for RD and C groups, for all analysis). The evaluation of the weight gain was analyzed by repeated-measures ANOVA. Differences were considered significant when $p < 0.05$.

3. Results

All the animals survived the treatment, except subjects number 3 and 6 from HD group (6250 mg/kg). Possibly, there was a perforation of the esophagus, trachea, or lungs while administrating WP supplement via oral gavage. During the experiment, the animals did not show any signs of toxicity regarding WP administration.

Figure 2(A) shows that there was no difference between groups before the beginning of the treatment period. Figure 2(B) shows that the RD (316.0 ± 3.408) and HD (311.6 ± 3.790) groups had an increased weight gain from week 1 to week 15 compared with the control. There was no significant difference between RD and HD groups.

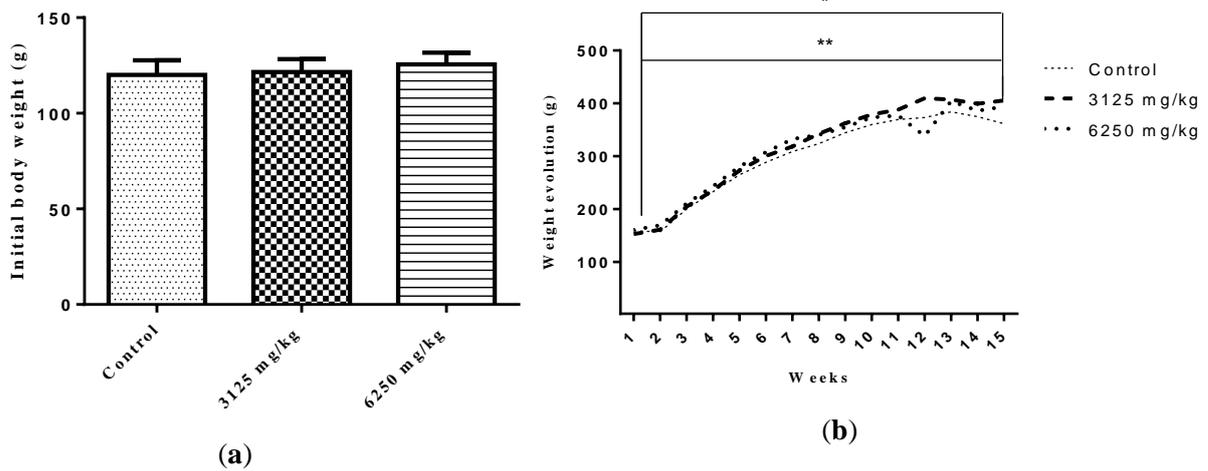


Figure 2. (a) Initial body weight of animals before treatment. No significant difference in the animals' weight before they protein isolate (WPI) treatment. (b) Comparison weight gain between groups. WPI-treated groups significantly increased weight compared to control. *Statistical differences between 6250 mg/kg and control ($p < 0.05$). **Statistical differences between 3125 mg/kg and control ($p < 0.01$). One-way ANOVA followed by Tukeys' multiple comparisons test. Values expressed as mean ($n = 8-10$).

All hematological parameters, including complete blood count parameters, were significantly unchanged in all groups treated with WP compared to the control group ($p > 0.05$). Biochemical parameters were not statistically changed compared to the control group ($p > 0.05$), except for glucose. There was a significant ($p < 0.05$) increase in glucose in the HD group compared to the control. All these results are presented in Tables 1 and 2.

Table 1. Hematological parameters presented as mean \pm SEM.

Parameter / groups	Control	Recommended dose	High dose
WBC ($10^9/l$)	6,119 \pm 0,9122	7,247 \pm 1,001	7,783 \pm 0,9784
LYM ($10^9/l$)	5,750 \pm 1,035	6,866 \pm 1,135	7,181 \pm 1,110
MID ($10^9/l$)	0,4878 \pm 0,5390	0,7250 \pm 0,5911	1,437 \pm 0,5781
GRA ($10^9/l$)	1,064 \pm 0,5079	1,152 \pm 0,5079	1,269 \pm 0,5079
LY (%)	73,86 \pm 3,630	77,00 \pm 3,981	73,41 \pm 3,893
GR (%)	18,66 \pm 3,090	16,06 \pm 3,389	19,74 \pm 3,314
RBC ($10^{12}/l$)	8,056 \pm 0,2678	7,909 \pm 0,2938	8,169 \pm 0,2873
HGB (g/dl)	13,36 \pm 0,2594	13,31 \pm 0,2845	13,70 \pm 0,2782
HCT (%)	38,57 \pm 1,371	38,13 \pm 1,504	39,39 \pm 1,471
HCT/HGB (%)	2,885 \pm 0,06829	2,866 \pm 0,07490	2,871 \pm 0,07325
MCV (fl)	48,22 \pm 0,6909	48,20 \pm 0,7578	46,86 \pm 0,7411
MCH (pg)	16,67 \pm 0,3831	16,84 \pm 0,4202	16,81 \pm 0,4109
MCHc (g/dl)	34,73 \pm 0,5359	34,94 \pm 0,5878	35,81 \pm 0,5748
RDWc (%)	14,79 \pm 0,1713	14,51 \pm 0,1879	14,50 \pm 0,1838
PLT ($10^9/l$)	696,2 \pm 134,5	860,2 \pm 147,5	887,6 \pm 144,2
PCT (%)	0,3967 \pm 0,05606	0,4790 \pm 0,06149	0,5186 \pm 0,06013
MPV (fl)	5,700 \pm 0,1901	5,770 \pm 0,2085	5,871 \pm 0,2038
PDWc (%)	33,54 \pm 0,6500	33,66 \pm 0,7129	33,70 \pm 0,6971

Values expressed as mean \pm SEM ($n = 8-10$). No significant difference was found (comparison with control). One-way analysis of statistical variance test ($p < 0.05$). *Abbreviations:* white blood cells (WBC), lymphocytes (LYM), monocytes, eosinophils, basophils, and immature cells (MID), granulocytes (GRA), percentage of lymphocytes (%LY), percentage of MID (%MI), percentage of granulocytes (%GR), erythrocyte count (RBC), hemoglobin (HGB), hematocrit (HCT), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), the concentration of mean corpuscular hemoglobin (MCHc), red blood cell distribution width (RDWc), platelets (PLT), procalcitonin (PCT), mean platelet volume (MPV), and platelet distribution width (PDWc).

Table 2. Biochemical parameters presented as mean \pm SEM.

Parameter / groups	Control	Recommended dose	High dose
ALT (U/L)	66,00 \pm 17,46	64,40 \pm 18,52	81,13 \pm 18,52
AST (U/L)	99,10 \pm 21,58	76,40 \pm 22,89	95,75 \pm 22,89
ALP (U/L)	113,5 \pm 19,72	118,0 \pm 20,92	148,6 \pm 20,92

Parameter / groups	Control	Recommended dose	High dose
GLUC (mg/dL)	170,3 ± 40,33	180,1 ± 42,78	280,1 ± 42,78*
DBILI (mg/dL)	0,1010 ± 0,01120	0,1000 ± 0,01188	0,1063 ± 0,01188
TBILI (mg/dL)	0,0940 ± 0,02252	0,0770 ± 0,02389	0,0625 ± 0,02389
TP (U/L)	4,087 ± 0,6747	4,365 ± 0,7157	4,799 ± 0,7157
ALB (g/dL)	3,350 ± 0,5057	3,431 ± 0,5364	3,774 ± 0,5364
CREA (mg/dL)	0,3890 ± 0,07804	0,4210 ± 0,08278	0,4725 ± 0,08278
UREA (mg/dL)	15,10 ± 4,490	16,90 ± 4,762	24,50 ± 4,762

Values expressed as mean ± SEM (n = 8-10). *Significant difference when compared with the control (p < 0.05). One-way analysis of statistical variance test. *Abbreviations:* alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), glucose (GLUC), total bilirubin (TBILI), direct bilirubin (DBILI), total proteins (TP), albumin (ALB), creatinine (CREA).

Regarding the histological analysis, the spleen showed a normal hematopoietic function. However, there was a periarteriolar lymphocytes proliferation with blood-filled sinusoids on the red pulp in all groups. Hepatic samples from the HD group were found with focal rupture of sinusoidal walls, promoting hemorrhage in the liver of most of the animals. A similar result was also found in RD and control groups.

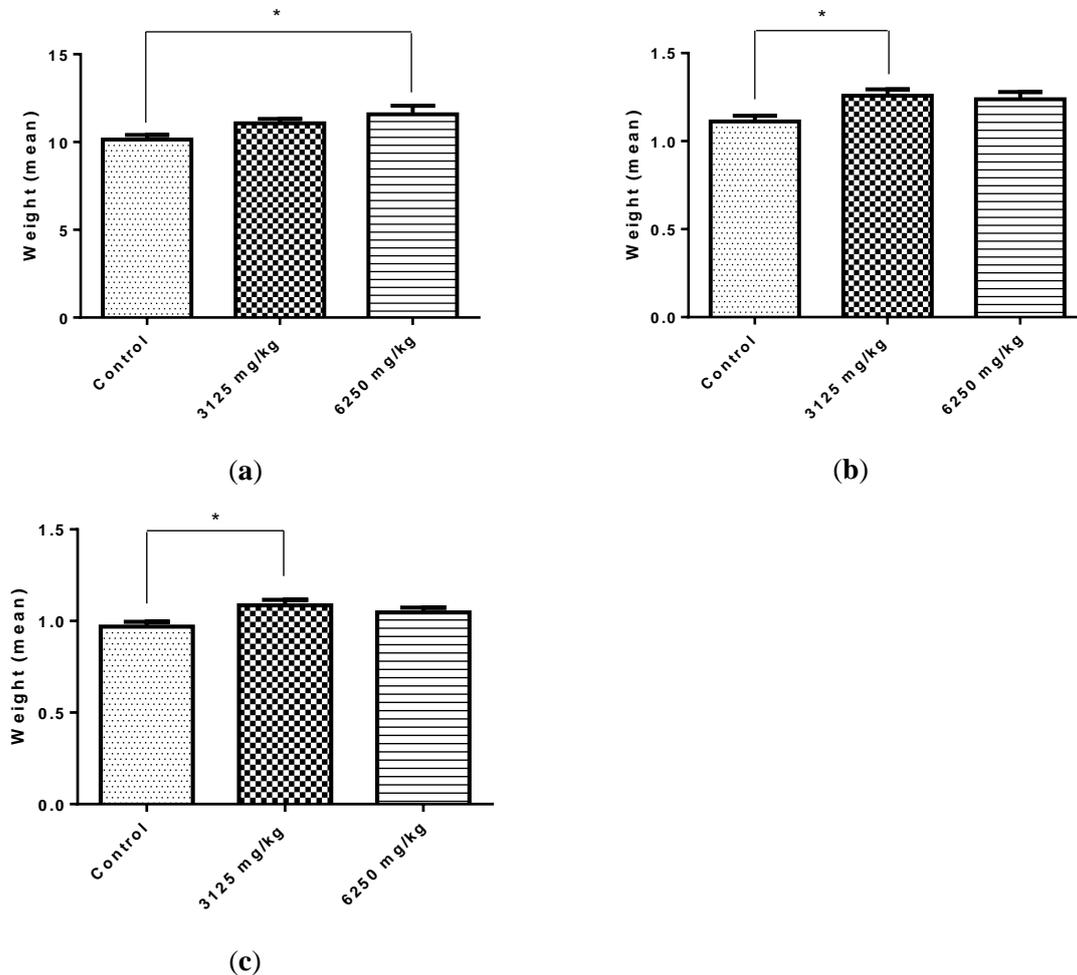


Figure 3. (a). Liver. (b). Right kidney. (c). Heart. The absolute weight of organs (g) of animals treated with whey protein isolate in two different doses, recommended dose (3125 mg/kg) and high dose (6250 mg/kg). Values expressed as mean ± SEM (n = 8-10). *Statistical differences between groups (p < 0.05).

The kidney of the control group showed a dilation in the lumens of both the proximal and distal convoluted tubules. Animals belonging to RD and HD groups maintained normal kidney morphological characteristics. There were no morphological changes observed in any animals' brain and heart tissue, although one subject of the HD group had a small focal

hemorrhage with hemosiderin pigments in the ventricular myocardium. These results were not statistically significant (different) between groups ($p > 0.05$).

In relation to the absolute weight of organs (g), the right kidney, liver, and heart of the animals belonging to WPI-supplemented groups were higher compared with control. Heart and right kidney absolute weights were significantly higher in the RD group. The liver weightiness was higher in the HD group ($p < 0.05$). See figure 3. Brain, spleen, and left kidney showed no significant difference between groups ($p > 0.05$). Additionally, no relevant difference was found in liver and kidney absolute weights between RD and HD groups ($p > 0.05$).

The frequency of rearing and grooming in all groups did not significantly change, comparing before and after the treatment nor compared between groups ($p > 0.05$). The same occurred regarding the time of exploring the middle square ($p > 0.05$). However, both line crossing and the number of fecal boli changed in recommended dose group (3125 mg/kg). There was a significant ($p < 0.05$) decrease in line crossing (mean difference 19.10 ± 6.11), compared to their baseline (Figure 4A). And a substantial increase ($p < 0.05$) in the number of fecal boli (mean difference -3.1 ± 0.72), compared to the baseline (Figure 4B). In addition, the number of fecal boli in the control group was higher compared to the baseline.

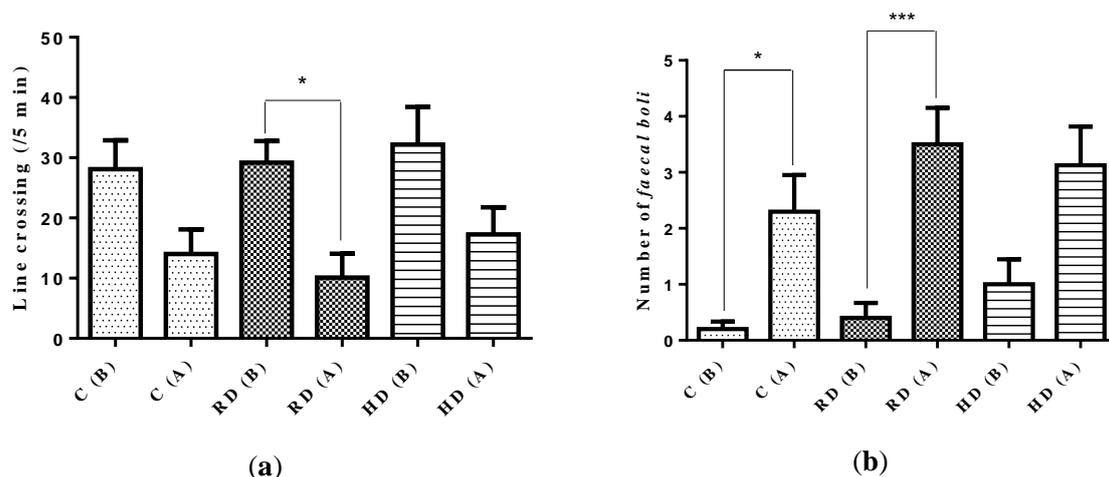


Figure 4. (a). Line crossing of animals before and after supplementation with whey protein isolate in two different doses. (b). Number of fecal boli of animals before and after supplementation with whey protein isolate in two different doses. C, control, RD, recommended dose (3125 mg/kg), HD, high dose (6250 mg/kg). B, before treatment; A, after treatment. Values expressed as mean \pm SEM ($n = 8-10$). *Statistical differences between groups ($p < 0.05$).

There was no significant difference in the analysis of AE, % of AE, ALT, and % of ALT between groups before or after treatment ($p > 0,05$). There was no significant difference in the TS, TG, and ET among groups compared to baseline, in both habituation and test trials, before and after the treatment with WP ($p > 0,05$).

4. Discussion

The current study proposed to evaluate possible toxicity or a significant adverse effect of chronic (90 days) WPI supplementation in two different doses (recommended, 3125 mg/kg, and high, 6250 mg/kg) in males Wistar rats. The main findings revealed that WPI did not promote toxicity nor significant behavioral changes in rats. However, some relevant differences were found. The overall body weight gain of WPI-supplemented groups was higher than in control ($p < 0.05$). In addition, RD and HD groups showed no significant difference regarding

weight gain. Thus, WPI supplementation without exercise may improve whole-body weight gain regardless of the dose [18].

Studies suggest that improvements in muscle strength are minimized when the total protein intake reaches a minimum of ≥ 1.6 g/kg (young adults) [19, 20]. Without physical activity, a dose of approximately 0.24 g/kg body mass appears to be sufficient for stimulating a maximal postprandial response of muscle protein synthesis (MPS) [20]. Certain healthy individuals with a larger lean mass or body mass may benefit from a larger postexercise protein dose. However, doses higher than 40g are related to more harm than good [10, 20].

These findings must be linked to the "muscle full" hypothesis. After a dose of 25-30g of WP, the supplement has no additional benefit regarding MPS or anabolic response [19, 21]. One of how muscle hypertrophy occurs is through stimulation and signal of the mammalian target of rapamycin (mTOR) [21]. mTOR exerts a critical role in mediating the sign necessary for mRNA translation initiation, an important regulatory site in cellular protein synthesis [19, 22]. mTOR activation postexercise without protein is minimal, and it is the amino acids (AA), specifically BCAAs, overload that promotes this stimulation. However, this signaling and cell response tend to plateau after a certain dose, whereas the excess of AA does not promote more MPS [21]. In addition, timing and dosage are factors that should be taken into consideration when exploring the mechanisms of WP reducing weight gain. It is likely that uncontrolled factors, such as protocol of ingestion, dose, eating patterns, and macronutrient distribution, could also interfere with body weight gain [23, 24].

In this study, a high dose of WPI augmented the level of glucose compared to RD and control groups ($p < 0.05$). Other parameters remained unchanged between groups. Oppositely, in one study, WPI decreased glucose levels in both healthy Wistar and Zucker diabetic fatty (ZDF) rats [25]. Similar results were also found with WP concentrate (WPC) [26]. WP bioactivity peptides may assist in the modulation of energy metabolism by promoting higher glucose uptake in the cells and subsequent higher glycogen synthesis [27]. However, there is still no consensus in the literature about the quantity and quality of protein capable of reducing or maintaining blood-glucose concentrations at the desired range without causing adverse effects [28]. WP consumption could result in postprandial hyperinsulinemia by an increase in plasma BCAA (leucine, isoleucine, valine) levels [29]. BCAAs contribute to the production of glucose in the liver (gluconeogenesis) through the alanine-glucose cycle, connected with the maintenance of glycemic homeostasis [30]. Thus, this BCAA influx may lead to a metabolic imbalance further, especially if not associated with exercise [30, 31]. In this study, the absence of physical activity associated with a high-protein diet could have increased glucose levels in the serum of the HD animals. However, authors are still not sure if high-protein diets, specifically supplemented with WP, are beneficial or not, whereas dose and time could play an important role in glycemic response and regulation [32, 33].

The hematological parameters showed no significant difference between groups. Studies still disagree regarding the use of WP supplementation and improvement on blood parameters [34-36]. Karandish *et al.* (2008) [34] showed that after one month of WP supplementation (6.6 g/day) in health exercise training individuals, no significant changes were found in red blood cell counts, hemoglobin, and hematocrit or other hematological parameters. The opposite result was found by Ronghui [35]. Whey Protein is a high-quality protein that contains significant amounts of all the essential AA needed compared to other dietary protein sources, possibly improving immune response and antioxidant capacity [37, 38]. However, high-protein intake, specifically BCAAs, can have a role in the pathogenesis of viral infections

[39, 40]. Butorov [39] found that increased BCAA intake elevated the risk of HIV replication and subsequent acceleration of immunosuppression and progression of the disease. Interestingly, in another study, WP from mother's milk showed a protective role against SARS-CoV-2 infection *in vitro*, and WP from other species have also shown similar results [40]. Both human and milk WP present similar compositions with the presence of antiviral peptides [41]. It seems that WP's effects on the immune system are dose-dependent, as well as the specific amount of AA that is being used [38-41]. However, more studies are required to corroborate these findings [39-40].

The right kidney, heart, and liver had an increase in their absolute weight in WPI-supplemented groups compared to control. Studies have been inconclusive about the deleterious or adaptation effects of organs caused by high-protein intake [42, 43]. Aparicio *et al.* (2011) [43] work revealed that high consumption of WP hydrolysate (WPH), without exercise, increased the absolute liver and kidney weights, corroborating with the findings of this study. Contrarily, Oryan *et al.* (2011) [44] found a protective effect of WPI in reducing liver toxicity in rats, with decreased ALT and AST levels, and in its absolute weight, as well as an improvement of its morphological aspects.

Some papers suggest that physical activity may influence how much damage is done to the liver and the kidney associated with a high-protein diet [45, 46]. In this study, not surprisingly, the livers absolute weight increased significantly in the HD group compared to the control ($p < 0.05$). Additionally, the recommended dose promoted the same effect on the right kidney and the heart ($p < 0.05$). Consumption of high-protein diets is postulated to negatively influence liver and renal health [45, 47], and exercise may have a protective role against these alterations [43, 46]. Therefore, only a high intake of AA when not associated with physical activity may have a deleterious effect on health [10, 48].

In our findings, the histopathological analysis demonstrated that the spleen, liver, and kidneys had some degree of tissue damage. However, such results were found in all groups. It is possible that the vascular congestion and hemorrhage observed in the liver, heart, and kidneys are not related to the toxicity of the WP but due to animal euthanasia. Animals were euthanized by a single intraperitoneal injection of a ketamine and xylazine cocktail. Ketamine/xylazine is a commonly used combination for anesthesia and euthanasia in mice and rats and is often followed by a secondary method of euthanasia, such as exsanguination, thoracotomy, or cervical dislocation [49]. Miller *et al.*'s (2015) [50] study showed that the use of the ketamine and xylazine cocktail promoted tissue damage on the lungs of rats. Thus, we cannot associate such findings with WP supplementation because the tissue damage was also found on the animals belonging to the control group.

Observing stress-related anxiety in rodents typically relies on species-specific behaviors such as an increased risk assessment, the reduction of exploration, seeking shelter, escaping, burying or defecating [51]. The open-field test is able to evaluate the exploratory activity and locomotor effects in rats [15]. In our results, animals belonging to all groups tended to reduce their locomotion activity (showed by decreased ALE) as well as an increase in NFB compared to their baseline. Thus, we believe that these effects are related to the chronic (90-day) gavage administration and manipulation of animals and not because of the whey protein supplementation itself.

Oral gavage is a widely used method for administering substances to animals in pharmacological and toxicological studies [51]. This method allows researchers precise control over the test dose and timing of compound administration [52]. However, the restraint required

by this method could be stressful for rodents, possibly causing an increase in heart rate, body temperature, and blood glucocorticoid levels [53]. The stress promoted by gavage seems to be associated with a vehicle- and dose volume-dependent approach [54].

No significant differences were found regarding learning, spatial and long-term memory. Ohsawa *et al.* (2015) [55] supplemented rats with Calpis sour milk whey (fermented milk), in a single dose of 2000 mg/kg, and the animals were also treated with scopolamine to evaluate immediate spatial and working memory. Results showed improved memory impairment induced by scopolamine during the spontaneous alternation behavior test (Y-maze). Garg *et al.* (2018) [56] supplemented WPC (300 mg/kg for 28 days) in male Wistar rats to evaluate its neuroprotective effect. Results showed that WPC upregulated the expression of marker genes associated with neurodegeneration and downregulated the expression of inflammatory markers in the brain. Contrarily, Żebrowska *et al.*'s (2019) [57] study revealed that a high-protein diet induces oxidative stress in the rat cerebral cortex and hypothalamus. Generally, studies are still inconclusive regarding high amounts of WP with memory and anxiety improvements [55-57].

Regarding the strengths of this present work, we believe that no other study aimed to evaluate the effects of chronic whey protein supplementation, focusing on its use and physiological outcomes, comparing different doses. Thus, we were able to evaluate WP toxicity as a whole, following appropriate guidelines. However, we also had some limitations. The whole energy and water intake of the animals were not measured, making it difficult to calculate the influence of their diet on the outcomes found. Although, they consumed a normal pellet diet provided by Quimtia[®] and the only difference was the WP supplementation.

5. Conclusions

A chronic supplementation (90-day) of WPI in two different doses resulted in no significant change regarding biochemical, hematological, morphological, and behavioral parameters, as well as toxicity. However, regardless of the dose used, WPI promoted weight gain, increased the absolute weight of the liver, the right kidney, and the heart, and, in a higher dose, raised the levels of serum glucose. We believe that our study brought relevant results that corroborate current literature and new insights about WPI supplementation. We also highlighted that more studies should be made. Further research should focus on metabolic changes associated with a higher intake of WP, proposing a safe dosage of consumption to improve the individual's health, reducing damage, specifically on the liver and the kidney.

Funding

This research was funded by a grant from the Cearense Foundation of Scientific and Technological Support – FUNCAP (Ceara, Brazil).

Acknowledgments

We thank the Group of Studies in Neuroinflammation and Neurotoxicology (GENIT), of the State University of Ceara for the support in this work. Additionally, we thank Débora R. A. Cavalcanti and Francisco Evanir Gonçalves de Lima for contributing, respectively, with the histopathological and hematological/biochemical analysis and Yasmin I. S. Oliveira for contributing to the animals' manipulation and treatment.

Conflicts of Interest

The authors declare no conflict of interest.

References

1. Master, P.B.Z.; Macedo, R.C.O. Effects of dietary supplementation in sport and exercise: a review of evidence on milk proteins and amino acids. *Crit Rev Food Sci Nutr* **2021**, *61*, 1225–1239, <https://doi.org/10.1080/10408398.2020.1756216>.
2. Vasconcelos, Q.D.J.S.; Bachur, T.P.R.; Aragao, G.F. Whey protein: composition, use and benefits - a narrative review. *EJPESS* **2018**, *4*, 173–183, <https://doi.org/10.5281/zenodo.1161636>.
3. Piri Damaghi, M.; Mirzababaei, A.; Moradi, S.; Daneshzad, E.; Tavakoli, A.; Clark, C. C. T.; Mirzaei, K. Comparison of the effect of soya protein and whey protein on body composition: a meta-analysis of randomised clinical trials. *Br J Nutr* **2021**, 1–11, <https://doi.org/10.1017/S0007114521001550>.
4. Minj, S.; Anand, S. Whey Proteins and its derivatives: bioactivity, functionality, and current applications. *Dairy* **2020**, *1*, 233–258, <https://doi.org/10.3390/dairy1030016>.
5. Simonson, M.; Boirie, Y.; Guillet, C. Protein, amino acids and obesity treatment. *Rev Endocr Metab Disord* **2020**, *21*, 341–353, <https://doi.org/10.1007/s11154-020-09574-5>.
6. Frestedt, J.L.; Zenk, J.L.; Kuskowski, M.A.; Ward, L.S.; Bastian, E.D. A whey-protein supplement increases fat loss and spares lean muscle in obese subjects: a randomized human clinical study. *Nutr Metab (Lond)* **2008**, *27*, 8–10, <https://doi.org/10.1186/1743-7075-5-8>.
7. Zajac, I.T.; Herreen, D.; Bastiaans, K.; Dhillon, V.S.; Fenech., M. The effect of whey and soy protein isolates on cognitive function in older Australians with low vitamin B12: a randomised controlled crossover trial. *Nutrients* **2018**, *11*, 19–23, <https://doi.org/10.3390/nu11010019>.
8. Markus, C.R.; Olivier, B.; de Haan, E.H. Whey protein rich in alpha-lactalbumin increases the ratio of plasma tryptophan to the sum of the other large neutral amino acids and improves cognitive performance in stress-vulnerable subjects. *Am J Clin Nutr* **2002**, *75*, 1051–1056, <https://doi.org/10.1093/ajcn/75.6.1051>.
9. Glenn, J.M.; Madero, E.N.; Bott, N.T. Dietary protein and amino acid intake: links to the maintenance of cognitive health. *Nutrients* **2019**, *11*, 1315–1359, <https://doi.org/10.3390/nu11061315>.
10. Vasconcelos, Q.D.J.S.; Bachur, T.P.R.; Aragão, G.F. Whey protein supplementation and its potentially adverse effects on health: a systematic review. *Appl Physiol Nutr Metab* **2020**, *46*, 27–33, <https://doi.org/10.1139/apnm-2020-0370>.
11. Brasil. National Health Surveillance Agency (ANVISA). Law N° 26, of 13 of may of 2014. Dispõe sobre a Vigilância Sanitária a que ficam sujeitos os medicamentos, as drogas, os insumos farmacêuticos e correlatos, cosméticos, saneantes e outros produtos, e da outras providencias. Diário Oficial da União, 2004, p. 12647. Available online: <https://www.in.gov.br/en/web/dou/-/resolucao-n-679-de-21-de-novembro-de-2019-241336577> (Accessed on 06 december **2021**).
12. OECD. Repeated dose 90-day oral toxicity study in rodents, test guideline no. 408. OECD guidelines for the testing of chemicals, OECD. Paris; **1998**, <https://doi.org/10.1787/9789264070707-en>.
13. Clark, J.D.; Gebhart, G.F.; Gonder, J.C.; Keeling, M.E.; Kohn, D.F. Special report: the 1996 guide for the care and use of laboratory animals. *ILAR J* **1997**, *38*, 41–48, <https://doi.org/10.1093/ilar.38.1.41>.
14. FDA (Food and Drug Administration). Center for Biologics Evaluation and Research. Estimating the safe starting dose in clinical trials for therapeutics in adult healthy volunteers, U.S. Food and Drug Administration; 2002. Available online: <https://www.fda.gov/media/72309/download>. (Accessed on 02 march **2022**).
15. Roth, K.A.; Katz, R.J. Stress, behavioral arousal, and open field activity--a reexamination of emotionality in the rat. *Neurosci Biobehav Rev* **1979**, *3*, 247–263, [https://doi.org/10.1016/0149-7634\(79\)90012-5](https://doi.org/10.1016/0149-7634(79)90012-5).
16. Sarter, M.; Bodewitz, G.; Stephens, D.N. Attenuation of scopolamine-induced impairment of spontaneous alternation behaviour by antagonist but not inverse agonist and agonist β -carbolines. *Psychopharmacology (Berl)* **1988**, *94*, 491–495, <https://doi.org/10.1007/BF00212843>.
17. Branchi, I.; Ricceri, L. Active and passive avoidance. In Behavioral Genetics of the Mouse (Cambridge Handbooks in Behavioral Genetics, 1st ed.; Crusio W., Sluyter F., Gerlai R., Pietropaolo S.; Cambridge: Cambridge University Press, United Kingdom, 2013; Volume 1, pp. 291–298, <https://doi.org/10.1017/CBO9781139541022.029>.

18. Duarte, N.M.; Cruz, A.L.; Silva, D.C.; Cruz, G.M. Intake of whey isolate supplement and muscle mass gains in young healthy adults when combined with resistance training: a blinded randomized clinical trial (pilot study). *J Sports Med Phys Fitness*, **2020**, *60*, 75–84, <https://doi.org/10.23736/S0022-4707.19.09741-X>.
19. Reidy, P.T.; Fry, C.S.; Igbini, S.; Deer, R.R.; Jennings, K.; Cope, M.B.; Mukherjee, R.; Volpi, E.; Rasmussen, B.B. Protein supplementation does not affect myogenic adaptations to resistance training. *Med Sci Sports Exerc* **2017**, *49*, 1197–1208, <https://doi.org/10.1249/MSS.0000000000001224>.
20. Gwin, J.A.; Church, D.D.; Wolfe, R.R.; Ferrando, A.A.; Pasiakos, S.M. Muscle protein synthesis and whole-body protein turnover responses to ingesting essential amino acids, intact protein, and protein-containing mixed meals with considerations for energy deficit. *Nutrients* **2020**, *15*, 2457, <https://doi.org/10.3390/nu12082457>.
21. Atherton, P.J.; Etheridge, T.; Watt, P.W.; Wilkinson, D.; Selby, A.; Rankin, D.; Smith, K.; Rennie, M.J. Muscle full effect after oral protein: time-dependent concordance and discordance between human muscle protein synthesis and mTORC1 signaling. *Am J Clin Nutr* **2010**, *92*, 1080–1088, <https://doi.org/10.3945/ajcn.2010.29819>.
22. Olaniyan, E.T.; O'Halloran, F.; McCarthy, A.L. Dietary protein considerations for muscle protein synthesis and muscle mass preservation in older adults. *Nutr Res Rev* **2021**, *34*, 147–157, <https://doi.org/10.1017/S0954422420000219>.
23. Churchward-Venne, T.A.; Pinckaers, P.J.M.; Smeets, J.S.J.; Betz, M.W.; Senden, J.M.; Goossens, J.P.B.; Gijzen, A.P.; Rollo, I.; Verdijk, L.B.; van Loon, L.J.C. Dose-response effects of dietary protein on muscle protein synthesis during recovery from endurance exercise in young men: a double-blind randomized trial. *Am J Clin Nutr* **2020**, *112*, 303–317, <https://doi.org/10.1093/ajcn/nqaa073>.
24. Hector, A.J.; Phillips, S.M. Protein recommendations for weight loss in elite athletes: a focus on body composition and performance. *Int J Sport Nutr Exerc Metab* **2018**, *28*, 170–177, <https://doi.org/10.1123/ijsem.2017-0273>.
25. Gregersen, S.; Bystrup, S.; Overgaard, A.; Jeppesen, P.B.; Sønderstgaard Thorup, A.C.; Jensen, E.; Hermansen, K. Effects of whey proteins on glucose metabolism in normal Wistar rats and Zucker diabetic fatty (ZDF) rats. *Rev Diabet Stud* **2013**, *10*, 252–269, <https://doi.org/10.1900/RDS.2013.10.252>.
26. Franzen, J.M.; Graciano Vaz, J.; Zancanaro, V.; Mariano de Bitencourt, R. Baixa dose de Whey Protein reduz glicose, triglicérides e controla o peso corporal em ratos Wistar. *RBONE* **2016**, *10*, 133–144. Available online: <http://www.rbone.com.br/index.php/rbone/article/view/425>.
27. Weigle, D.S.; Breen, P.A.; Mattys, C.C.; Callahan, H.S.; Meeuws, K.E.; Burden, V.R.; Purnell, J.Q. A high-protein diet induces sustained reductions in appetite, ad libitum caloric intake, and body weight despite compensatory changes in diurnal plasma leptin and ghrelin concentrations. *Am J Clin Nutr* **2005**, *82*, 41–48, <https://doi.org/10.1093/ajcn.82.1.41>.
28. Rietman, A.; Schwarz, J.; Tomé, D.; Kok, F.J.; Mensink, M. High dietary protein intake, reducing or eliciting insulin resistance? *Eur J Clin Nutr* **2014**, *68*, 973–979, <https://doi.org/10.1038/ejcn.2014.123>.
29. Newgard, C.B.; An, J.; Bain, J.R.; Muehlbauer, M.J.; Stevens, R.D.; Lien, L.F.; Haqq, A.M.; Shah, S.H.; Arlotto, M.; Slentz, C.A.; Rochon, J.; Gallup, D.; Ilkayeva, O.; Wenner, B.R.; Yancy, W.S. Jr.; Eisenson, H.; Musante, G.; Surwit, R.S.; Millington, D.S.; Butler, M.D.; Svetkey, L.P. A branched-chain amino acid-related metabolic signature that differentiates obese and lean humans and contributes to insulin resistance. *Cell Metab* **2009**, *9*, 565–566, <https://doi.org/10.1016/j.cmet.2009.02.002>.
30. Mann, G.; Mora, S.; Madu, G.; Adegoke, O. Branched-chain amino acids: catabolism in skeletal muscle and implications for muscle and whole-body metabolism. *Front Physiol* **2021**, *12*, 702826, <https://doi.org/10.3389/fphys.2021.702826>.
31. Lynch, C.J. and Adams, S.H. Branched-chain amino acids in metabolic signalling and insulin resistance. *Nat Rev Endocrinol* **2014**, *10*, 723–736, <https://doi.org/10.1038/nrendo.2014.171>.
32. Akhavan, T.; Luhovyy, B.L.; Brown, P.H.; Cho, C.E.; Anderson, G.H. Effect of premeal consumption of whey protein and its hydrolysate on food intake and post meal glycemia and insulin responses in young adults. *Am J Clin Nutr* **2010**, *91*, 966–975, <https://doi.org/10.3945/ajcn.2009.28406>.
33. Elovarris, R.A.; Hutchison, A.T.; Lange, K.; Horowitz, M.; Feinle-Bisset, C.; Luscombe-Marsh, N.D. Plasma free amino acid responses to whey protein and their relationships with gastric emptying, blood glucose- and appetite-regulatory hormones and energy intake in lean healthy men. *Nutrients* **2019**, *11*, 2465–2469, <https://doi.org/10.3390/nu11102465>.

34. Eslami, S.; Karandish, M.; Marandi, S.; Zand-Moghaddam, A.. Effects of whey protein supplementation on hematological parameters in healthy young resistances male athletes. *J Appl Scie* **2010**, *10*, 991–995, <https://doi.org/10.3923/jas.2010.991.995>.
35. Ronghui, S. The research on the anti-fatigue effect of whey protein powder in basketball training. *Open Biomed Eng J* **2015**, *9*, 330–334, <https://doi.org/10.2174/1874120701509010330>.
36. Abbas, A.K. and Fathi, Q.M. Effect of whey protein supplement on physiological parameters in building body athletes. *Iraqi J Sci* **2018**, *58*, 2332–2337. Available online: <https://ijs.uobaghdad.edu.iq/index.php/eijs/article/view/102>.
37. Bumrungpert, A.; Pavadhgul, P.; Nunthanawanich, P.; Sirikanchanarod, A.; Adulbhan, A. Whey protein supplementation improves nutritional status, glutathione levels, and immune function in cancer patients: a randomized, double-blind controlled trial. *J Med Food* **2018**, *21*, 612–616, <https://doi.org/10.1089/jmf.2017.4080>.
38. Tai, C.S.; Chen, Y.Y.; Chen, W.L. β -lactoglobulin influences human immunity and promotes cell proliferation. *Biomed Res Int* **2016**, 7123587, <https://doi.org/10.1155/2016/7123587>.
39. Butorov, E.V. Impact of high protein intake on viral load and hematological parameters in HIV-infected patients. *Curr HIV Res* **2017**, *15*, 345–354, <https://doi.org/10.2174/1570162X15666171002121209>.
40. Fan, H.; Hong, B.; Luo, Y.; Peng, Q.; Wang, L.; Jin, X.; Chen, Y.; Hu, Y.; Shi, Y.; Li, T.; Zhuang, H.; Zhou, Y.H.; Tong, Y.; Xiang, K. The effect of whey protein on viral infection and replication of SARS-CoV-2 and pangolin coronavirus *in vitro*. *Sig Transduct Target Ther* **2020**, *5*, 275, <https://doi.org/10.1038/s41392-020-00408-z>.
41. Ha, D.J.; Kim, J.; Kim, S.; Go, G.W.; Whang, K. Y. Dietary whey protein supplementation increases immunoglobulin g production by affecting helper t cell populations after antigen exposure. *Foods* **2021**, *10*, 194, <https://doi.org/10.3390/foods10010194>.
42. Avila, E.T.P.; Rosa, L.T.; Tibana, R.A.; de Almeida, P.C.; Fraga, G.A.; de Souza, S.M.; Corona, L.F.P.; Navalta, J.W.; Rezaei, S.; Ghayomzadeh, M.; Damazo, A.S.; Prestes, J.; Voltarelli, F.A. Effects of high-protein diet containing isolated whey protein in rats submitted to resistance training of aquatic jumps. *Nutrition* **2018**, *53*, 85–94, <https://doi.org/10.1016/j.nut.2018.01.018>.
43. Aparicio, V.A.; Nebot, E.; Kapravelou, G.; Sánchez, C.; Porres, J.M.; López Jurado, M.; Aranda, P. Resistance training reduces the metabolic acidosis and hepatic and renal hypertrophy caused by the consumption of a high protein diet in rats. *Nutr Hosp* **2011**, *26*, 1478–1486, <https://doi.org/10.1590/S0212-16112011000600040>.
44. Oryan, A.; Eftekhari, M.H.; Ershad, M.; Panjehshahin, M.R.; Tabatabaei, H.R. Hepatoprotective effects of whey protein isolate against acute liver toxicity induced by dimethylnitrosamine in rat. *Comp Clin Pathol* **2011**, *20*, 251–257, <https://doi.org/10.1007/s00580-010-0986-5>.
45. de Assis, T.P.; Gonçalves, C.S.; Giannetto, M.L.N.; Carlos, C.P.; Cury, P.M.; Caldas, H.C.; Mendes, G.E.F. Does protein supplementation and exercise interfere with renal function and structure? *Rev Bras Med Esporte* **2020**, *26*, 527–531, <https://doi.org/10.1590/1517-869220202606181869>.
46. Chen, W.C.; Huang, W.C.; Chiu, C.C.; Chang, Y.K.; Huang, C.C. Whey protein improves exercise performance and biochemical profiles in trained mice. *Med Sci Sports Exerc* **2014**, *46*, 1517–1524, <https://doi.org/10.1249/MSS.0000000000000272>.
47. Ko, G.J.; Rhee, C.M.; Kalantar-Zadeh, K.; Joshi, S. The Effects of High-Protein Diets on Kidney Health and Longevity. *JASN* **2020**, *31*, 1667–1679, <https://doi.org/10.1681/ASN.2020010028>.
48. Ali, A.A.; Almukhtar, S.E.; Sharif, D.A.; Saleem, Z.S.M.; Muhealdeen, D.N.; Hughson, M.D. Effects of bodybuilding supplements on the kidney: A population-based incidence study of biopsy pathology and clinical characteristics among middle eastern men. *BMC Nephrol* **2020**, *21*, 164–169, <https://doi.org/10.1186/s12882-020-01834-5>.
49. Heng, K.; Marx, J. O.; Jampachairsi, K.; Huss, M. K.; Pacharinsak, C. Continuous rate infusion of alfaxalone during ketamine-xylazine anesthesia in rats. *JAALAS* **2020**, *59*, 170–175, <https://doi.org/10.30802/AALAS-JAALAS-19-000122>.
50. Miller, D.L.; Dou, C.; Raghavendran, K. Anesthetic techniques influence the induction of pulmonary capillary hemorrhage during diagnostic ultrasound scanning in rats. *J Ultrasound Med* **2015**, *34*, 289–297, <https://doi.org/10.7863/ultra.34.2.289>.
51. Bailey, K.R.; Crawley, J.N. Anxiety-related behaviors in mice. In: *Methods of behavior analysis in neuroscience*, 2nd ed.; Buccafusco, J.J., Boca Raton: CRC Press/Taylor & Francis, United Kingdom, 2009, pp. 10–15. Available online: <https://www.ncbi.nlm.nih.gov/books/NBK5221>.

52. Ferguson, S.A.; Boctor, S.Y. Use of food wafers for multiple daily oral treatments in young rats. *J Am Assoc Lab Anim Sci* **2009**, *96*, 292–295. Available online: <https://www.ncbi.nlm.nih.gov/pmc/articles/PMC2696833>.
53. Kramer, K.; Voss, H.P.; Grimbergen, J.A.; Mills, P.A.; Huetteman, D.; Zwiers, L.; Brockway, B. Telemetric monitoring of blood pressure in freely moving mice: a preliminary study. *Lab Anim* **2000**, *34*, 272–280, <https://doi.org/10.1258/002367700780384663>.
54. Turner, P.V.; Vaughn, E.; Sunohara-Neilson, J.; Ovari, J.; Leri, F. Oral gavage in rats: animal welfare evaluation. *J Am Assoc Lab Anim Sci* **2012**, *51*, 25–30. Available online: <https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3276962>.
55. Ohsawa, K.; Uchida, N.; Ohki, K.; Nakamura, Y.; Yokogoshi, H. Lactobacillus helveticus-fermented milk improves learning and memory in mice. *Nutr Neurosci* **2015**, *18*, 232–240, <https://doi.org/10.1179/1476830514Y.0000000122>.
56. Garg, G.; Singh, S.; Singh, A.K.; Rizvi, S.I. Whey protein concentrate supplementation protects rat brain against aging-induced oxidative stress and neurodegeneration. *Appl Physiol Nutr Metab* **2018**, *43*, 437–444, <https://doi.org/10.1139/apnm-2017-0578>.
57. Żebrowska, E.; Maciejczyk, M.; Żendzian-Piotrowska, M.; Zalewska, A.; Chabowski, A. High protein diet induces oxidative stress in rat cerebral cortex and hypothalamus. *Int J Mol Sci* **2019**, *20*, 1547–1549, <https://doi.org/10.3390/ijms20071547>.