Effects of strength training and whey proteins supplementation on the creatinine and urea parameters of rats

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ABSTRACT

Introduction: High protein diets increase the concentration of urea and acids in protein metabolism. Therefore, the use of high doses whey proteins needs to be checked for their effects on kidney function.

Aims: To evaluate the effect of whey proteins consumption of 2 g kg$^{-1}$ d$^{-1}$ and 4 g kg$^{-1}$ d$^{-1}$ on the biochemical marker creatinine and urea after 12 weeks of resistance training in male Wistar rats.

Methods: The sample consisted of 52 male Wistar rats, distributed in 6 groups. The protocol lasted 12 weeks of resistance training with daily supplementation of whey proteins, in three sessions per day. The determination of the biochemical concentration consisted of reading the absorbance and specific equation for the parameter creatinine and urea.

Results: The proportion of creatinine was significantly higher only in the control group compared to the supplemented groups and the trained control group. The higher 24-hour creatinine excretion (mg/kg) in the resistance and supplemented training group compared to the control training shows a possible effect of resistance training on the increase in muscle mass associated with a dose 4 g kg$^{-1}$ d$^{-1}$.

Conclusion: The treatment of male Wistar rats supplemented with whey proteins at doses 2 g kg$^{-1}$ d$^{-1}$ and 4 g kg$^{-1}$ d$^{-1}$ for 12 weeks did not result in impaired renal function.

Keywords: whey proteins; resistance training; creatinine; urea; rats, Wister.
**Introduction**

Whey protein, commercially known as whey proteins, is a by-product of cheese production and a rich source of exogenous amino acids and biologically active proteins, in addition to having nutritional aspects widely studied over the last decades [1]. It is known that \( \alpha \)-lactalbumin and \( \beta \)-lactoglobulin are the main proteins of whey, forming up to 80% of the protein mass, in addition to containing smaller proteins with lactoferrin and lactoperoxidase [2,3].

Research has shown the nutritional qualities of whey proteins which, due to their varied composition, have become commonly used by athletes with the aim of increasing muscle mass [1,4]. Thus, the consumption of whey proteins with a concentration of 80% or even greater than 90% has become increasingly constant in the population [5].

However, the excess of whey proteins in the diet can adversely affect the activity of the organs that participate in its metabolism [6]. One of these organs are the kidneys. Therefore, the indiscriminate use of protein and amino acid-based supplements has aroused interest in assessing possible deleterious health effects associated with the ingestion of excessive doses, especially on renal function [7].

Due to the high rates of prevalence and incidence, chronic kidney disease is a relevant public health problem, affecting thousands of people in Brazil and worldwide [8]. Thus, the use of biomarkers allows an analyze whether there is a lesion and at what stage it is, such as urea and creatinine, which are metabolites used as renal markers [9]. Therefore, when evaluating patients with abrupt drops in the glomerular filtration rate, the relationship between urea and creatinine can be useful and may be altered in different pathological states [10].

Therefore, the concentrations of these markers provide important information about renal function, elevated serum creatinine and urea values may be indicative of renal injury [11]. Thus, the effects of diets with high doses of whey proteins need to be verified against pathological changes that may cause interference with renal function [12,13], due to the increase in glomerular filtration rate and renal acid load [14-15].

The aim of the present study was to evaluate the effect of the consumption of 2 g kg\(^{-1}\) d\(^{-1}\) and 4 g kg\(^{-1}\) d\(^{-1}\) of whey proteins on biochemical markers creatinine and urea after 12 weeks of resistance training in sedentary rats in comparison to rats submitted to resistance training.

**Methods**

**Ethical considerations**

The biological tests were in accordance with the recommendations of the Brazilian Society of Science in Laboratory Animals [16]. The research project was appro-
ved by the Ethics Committee on the Use of Animals (CEUA), of the Universidade Federal do Maranhão, under the registration number: 23115.01804 / 2017-91.

**Sample**

52 males Rattus Novergicus Wistar Albinus were used, with an initial age of 60 days and a body mass of approximately 250 g to 350 g. The rats were allocated in 6 groups, being: supplemented with 2 g kg⁻¹ d⁻¹ (W2) (n = 10) supplemented with 4 g kg⁻¹ d⁻¹ (W4) (n = 7), resistance training and supplemented with 2 g kg⁻¹ day⁻¹ (TW2) (n = 9), resistance training and supplemented with 4 g kg⁻¹ d⁻¹ (TW4) (n = 6), control (TC) (n = 10) and control (C) (n = 10) training.

The rats remained under hygienic conditions in collective cages, kept in an air-conditioned room with temperature control between 24ºC to 28ºC, and under an alternating light/dark cycle of 12 hours. They were fed ad libitum with water and standard balanced feed for rodents (Nuvilab CR-1®).

**Statistical analysis**

Initially, normality was assessed using the Shapiro-Wilk test, and found to be normal, the ANOVA Two-Way test was used to compare the measurement variables. Tukey’s post-hoc was used to determine the statistical differences between all analyzes with a significance level of p<0.05, using the statistical software GraphPad Prism8.1.0.

**Resistance training**

In week 0, before the beginning of the experimental protocol, adaptation to training was carried out. The maximum loaded weight test (PMC), which consisted of up to 9 climbs with an interval of 120 seconds between attempts, was applied 48 hours after the last training familiarization session.

Adopting 75% of the rat’s total body mass as the initial load for the first climb and the 30g increment being added to each attempt. The maximum load adopted was stipulated according to the load of the last complete climb over the entire length of the scale.

The test was considered valid only when the maximum load was identified between 4 and 9 climbs. Otherwise, the test was repeated after 48 hours [17].

The application of the PCM test was carried out every two weeks, during the 12 weeks of training in the TC, TW2 and TW4 groups, in order to identify the maximum load to carry out the maximum strength adaptations over time and the prescription of load intensity for resistance training.

Resistance training had a frequency of 3 weekly sessions, not consecutive. The protocol used was in accordance with the standardization [18], which consists of 4 climbs on the stairs per training session with increasing intensity of 50%, 75%, 90% and 100% of the Maximum Loaded Weight determined in test [17], featuring intense resistance training.
**Supplementation with whey proteins**

The standard solution was calculated based on the amount of protein (22 g) per portion (25 g) of the supplement (H.I Whey: Essential Nutrition®) according to the composition description, using a precision scale for measuring the solute.

The doses administered were 2 g kg⁻¹ d⁻¹ and 4 g kg⁻¹ d⁻¹ of whey proteins, distributed to the rats, by group, as described in the resistance training.

Doses were administered via gavage of a standard solution of whey proteins dissolved in water with a common concentration of 0.323 g/ml of the Supplement (HI Whey: Essential Nutrition®), which corresponds to 0.284 g/ml of Whey proteins, with weekly readjustment based on the total body mass of the rat.

The treatment was carried out for 12 weeks, with three sessions of gavage per day with an interval of 60 minutes. Each gavage was determined according to the rat’s total body mass, with 2 ml for every 100 g of rat’s body weight, with a total volume of 5 ml per gavage session being standardized as stipulated for administration of aqueous solutions [19]. The control (C) and control training (TC) groups were treated with water with the same volume of gavage (5 mL).

Twenty-four hours after the final experimental procedures and with 12 hours of food deprivation, the rats were euthanized with intraperitoneal injection of ketamine and xylazine at 70 mg/kg and 10 mg/kg respectively [20]. These euthanasia criteria were chosen for not causing pain to animals, thus contemplating the Norms of the National Council for the Control of Animal Experimentation [21].

**Biological material**

At the end of the 12-week experiment, the rats were placed in individual metabolic cages for 24 hours, previously sanitized and the urine was collected, in an environment with a light/dark cycle, with free access to food and water [22].

To determine the creatinine concentration, the urine was initially diluted in distilled water with a proportion of 1:25 mL and then the procedure for deproteinization of the urine sample in picric acid was carried out, being stirred and centrifuged at 3000 rpm for 10 minutes. Subsequently, the supernatant was used for analysis to determine the creatinine concentration by two-point kinetics according to the reaction with sodium hydroxide. Two readings were taken at 510 nm absorbance at 30 and 90 seconds, which were used in a specific equation to determine the concentration. The result obtained was multiplied by 25 (Labtest® - Creatinine K Ref. 96).

To determine urinary urea, an enzymatic system was used by two-point kinetics, with the principle of urea hydrolyzing by urease. At first the urine was diluted in distilled water with a proportion of 1:50 mL and two readings were taken in absorbance of 340 nm in the times of 30 and 90 seconds, which were used in a specific equation to determine the concentration. The result obtained was multiplied by 50 (Labtest® - Creatinine K Ref. 96).

After euthanasia, blood was collected by beheading in guillotine. For the analysis of serum creatinine, the procedure for deproteinization of the serum sample
in picric acid was carried out, being agitated and centrifuged at 3000 rpm for 10 minutes. Then, the liquid supernatant was used for analysis to determine the creatinine concentration by two-point kinetics according to the reaction with sodium hydroxide. Two absorbance readings at 510 nm were taken at 30 and 90 seconds, which were used in a specific equation to determine the concentration (Labtest® - Creatinine K Ref. 96).

To determine the concentration of urea in the serum, an enzymatic system by two-point kinetics was used, with the principle of urea hydrolyzing by urease, with two readings at 340 nm absorbance at 30 and 90 seconds, which were used in equation concentration determination (Labtest® - Creatinine K Ref. 96).

**Results**

<table>
<thead>
<tr>
<th></th>
<th>C (n=10)</th>
<th>W2 (n=10)</th>
<th>W4 (n=7)</th>
<th>TC (n=10)</th>
<th>TW2 (n=9)</th>
<th>TW4 (n=6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Creatinine (mg/dl)</td>
<td>102.02 ± 22.29</td>
<td>132.81 ± 141.84</td>
<td>105.25 ± 6.41</td>
<td>83.12 ± 6.35</td>
<td>187.08* ± 30.33</td>
<td>206.98* ± 14.11</td>
</tr>
<tr>
<td>Creatinina de 24h (mg/24h)</td>
<td>14.18 ± 3.69</td>
<td>13.75 ± 1.32</td>
<td>12.23 ± 1.30</td>
<td>8.70 ± 0.89</td>
<td>12.67 ± 1.29</td>
<td>15.48 ± 0.87</td>
</tr>
<tr>
<td>Urea (mg/dl)</td>
<td>2704.96 ± 842.42</td>
<td>5768.06 ± 1078.52</td>
<td>3774.79 ± 322.31</td>
<td>4524.81 ± 334.54</td>
<td>8809.41* ± 1744.99</td>
<td>8620.60* ± 1171.09</td>
</tr>
<tr>
<td>Urea de 24h (mg/24h)</td>
<td>343.44 ± 122.42</td>
<td>583.15 ± 66.73</td>
<td>376.28 ± 25.14</td>
<td>480.34 ± 60.58</td>
<td>608.56 ± 122.04</td>
<td>723.90 ± 130.57</td>
</tr>
</tbody>
</table>

C = Sedentary control not supplemented; W2 = Sedentary supplemented with 2 g kg⁻¹ d⁻¹; W4 = Sedentary supplemented with 4 g kg⁻¹ d⁻¹; TC = Trained not supplemented; TW2 = Trained supplemented with 2 g kg⁻¹ d⁻¹; TW4 = Trained supplemented with 4 g/kg/day. Two-Way post hoc Tukey ANOVA (intergroups): Symbols in the horizontal indicate higher mean (p < 0.05) = *vs. C; # vs. TC; α vs. W4. Equations: a = [Creatinine (mg/dl) x Volume (ml/24h) / 100]; b = [Urea (mg/dl) x Volume (mL/24h)/100]

As shown in table I, in the creatinine marker (mg/dl), there was a higher concentration in the urine of rats in groups TW2 and TW4 in relation to the control group (p = 0.0196) and (p = 0.072,) respectively. Similarly, the TW2 and TW4 groups also showed higher values of creatinine concentration in the urine than the TC group (p = 0.0018) and (p = 0.0008) respectively.

There was no difference (p > 0.05) between creatinine concentrations for groups supplemented with 2 g kg⁻¹ d⁻¹. However, for the dose of 4 g kg⁻¹ d⁻¹ there was a difference between the groups, with greater excretion of creatinine for the group submitted to training. Groups TW2 and TW4 did not show differences between themselves (p > 0.05) in urinary creatinine. Groups W2 and W4 also showed no difference between them (p > 0.05).

In the results of the urea marker (mg/dl), the TW2 and TW4 groups showed a higher concentration in the urine compared to the control group (p = 0.0018) and (p = 0.0169) respectively. Similarly, TW2 had a higher concentration of urea compared to the TC group (p = 0.447).
The control group and the supplemented groups did not show any difference between themselves, in the same way, the trained and supplemented groups did not present differences between them in urinary urea. When observed, however, creatinine and urea normalized by the 24 h urine volume (mg/24h), all differences in creatinine and urea in mg/dl were normalized.

Table II - Concentration of serum creatinine and urea biomarkers presented as mean and standard error of mean

<table>
<thead>
<tr>
<th></th>
<th>C (n=10)</th>
<th>W2 (n=10)</th>
<th>W4 (n=7)</th>
<th>TC (n=10)</th>
<th>TW2 (n=9)</th>
<th>TW4 (n=6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum creatinine (mg/dl)</td>
<td>0.35 ± 0.8</td>
<td>0.30 ± 0.04</td>
<td>0.48 ± 0.07</td>
<td>0.35 ± 0.02</td>
<td>0.50 ± 0.05</td>
<td>0.51 ± 0.08</td>
</tr>
<tr>
<td>Urea sérica (mg/dl)</td>
<td>50.44* ± 7.19</td>
<td>37.74 ± 1.73</td>
<td>27.98 ± 1.01</td>
<td>37.19 ± 1.36</td>
<td>34.09 ± 2.02</td>
<td>34.70 ± 1.20</td>
</tr>
</tbody>
</table>

C = Sedentary control not supplemented; W2 = Sedentary supplemented with 2 g kg⁻¹ d⁻¹; W4 = Sedentary supplemented with 4 g kg⁻¹ d⁻¹; TC = Trained not supplemented; TW2 = Trained supplemented with 2 g kg⁻¹ d⁻¹; TW4 = Trained supplemented with 4 g kg⁻¹ d⁻¹. Two-Way post hoc Tukey ANOVA (intergroups): Symbols in the horizontal indicate higher mean (p < 0.05) = * vs. W2; # vs. W4; α vs. TW2.

Equations: a = [Creatinine (mg/dl) x Volume (ml/24h)/100]; b = [Urea (mg/dl) x Volume (ml/24h)/100]

According to table II, there was no significant difference (p > 0.05) in the serum creatinine concentration (mg/dl) between the groups. In the serum urea marker, the control group had a higher mean compared to W2, W4 and TW2 (p = 0.0190), (p = 0.0018) and (p = 0.0301) respectively. There was no difference between the TC group and the other groups (p > 0.05). Likewise, groups TW2 and TW4 showed no differences between them (p > 0.05).

Table III - Renal function estimation equations, with results presented as mean and standard error

<table>
<thead>
<tr>
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<th>W4 (n=7)</th>
<th>TC (n=10)</th>
<th>TW2 (n=9)</th>
<th>TW4 (n=6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Creatinine in 24h (mg/kg)</td>
<td>31.14 ± 7.74</td>
<td>38.31 ± 2.32</td>
<td>24.24 ± 2.59</td>
<td>18.05 ± 1.60</td>
<td>28.51 ± 2.55</td>
<td>37.89* ± 1.57</td>
</tr>
<tr>
<td>Creatinine Clearance (ml/min)</td>
<td>4.47 ± 2.20</td>
<td>3.57 ± 0.54</td>
<td>2.34 ± 0.49</td>
<td>1.77 ± 0.19</td>
<td>1.87 ± 0.27</td>
<td>2.61 ± 0.70</td>
</tr>
<tr>
<td>Urea/Creatinine Ratio (mg/g)</td>
<td>236.40* ± 52.75</td>
<td>121.63 ± 11.05</td>
<td>76.88 ± 15.97</td>
<td>110.06 ± 7.67</td>
<td>72.58 ± 7.12</td>
<td>84.93 ± 23.38</td>
</tr>
</tbody>
</table>

C = Sedentary control not supplemented; W2 = Sedentary supplemented with 2 g kg⁻¹ d⁻¹; W4 = Sedentary supplemented with 4 g kg⁻¹ d⁻¹; TC = Trained not supplemented; TW2 = Trained supplemented with 2 g kg⁻¹ d⁻¹; TW4 = Trained supplemented with 4 g/kg/day. Two-Way post hoc Tukey ANOVA (intergroups): Symbols in the horizontal indicate higher mean (p < 0.05) = * vs. W2; α vs. W4; # vs. TC; β vs. TW2; Ω vs. TW4. Equations: a = [Creatinine 24 h (mg/24h) / Body Mass (kg)]; b = [Urinary Creatinine (mg/dl) x Volume (ml/24h) / Serum Creatinine (mg/dl)]; c = [Serum Urea (mg/dl)/Serum Creatinine (mg / dl)]

CAs shown in table III, the 24-hour creatinine in mg/kg showed a significant difference between the TW4 and TC groups, with TW4 higher than the TC (p = 0.0378). Groups TW2 and TW4 showed no difference between them (p > 0.05) in relation to 24-hour creatinine relative to body mass. The control group and the TC group showed
no difference between them (p > 0.05). And, there was no significant difference in the creatinine clearance concentration (ml/min) between the groups (p > 0.05). When the urea / creatinine ratio (mg/g) is observed, it is noted that in the control group there is a higher concentration compared to the groups W2 (p = 0.0013), W4 (p = 0.0015), TC (p = 0.0002), TW2 (p = 0.0008) and TW4 (p = 0.0100). In this way, all groups showed a difference in relation to the control. However, there was no difference between them (p > 0.05).

Discussion

In the present study, it was exposed the changes in the creatinine and urea markers for the doses of 2 g kg⁻¹ d⁻¹ and 4 g kg⁻¹ d⁻¹. Previous studies have also used doses of whey proteins in an animal model, mostly presenting lower doses than those presented. Haraguchi et al. [23] investigated the influence of whey proteins on liver enzymes, lipid profile and bone formation in hypercholesterolemic Fisher rats. The serum creatinine concentration in the groups with whey protein and whey protein hypercholesterolemic diets did not show any significant difference, agreeing with our findings. Regarding the serum urea concentration, the results were similar between the groups, differently from our results, since the non-supplemented sedentary control group had a higher mean concentration compared to W2, W4 and TW2. However, in our study, we did not use the hypercholesterolemic diet, which may have generated a protective effect against impaired renal function, as suggested by the authors [23].

The results found by Athira et al. [24] when analyzing the potential for improving the hydrolysate of whey proteins (WPH) against oxidative stress induced by paracetamol in 24 mice showed similar to ours, since the administration of WPH (4 mg/kg) significantly decreased the concentration of creatinine serum. The reduction in serum creatinine values after intraperitoneal and oral treatment with WPH established the antioxidant effect in vivo. Therefore, it was concluded that WPH develops a protective effect against impaired renal function induced by paracetamol [24].

Chen et al. [25], when examining the improvement in exercise performance and biochemical profiles in mice supplemented with whey proteins (dose of 4.1 g kg⁻¹ d⁻¹), did not identify significant differences in serum creatinine between the supplemented and trained supplemented sedentary groups. However, it is noteworthy that this study lasted 6 weeks, being, therefore, a shorter period than the one adopted in our study. Lollo et al. [26] investigated the effects of whey proteins (17% protein) and casein plus leucine in trained Wistar rats, however, no clear changes in serum creatinine values were detected, with no changes in this marker, similar to the findings of the present study.

Franzen et al. [27], in their chronic study with Wistar rats treated with low doses of protein (10% whey in feed), found no significant differences between baseline and end of treatment in any experimental group in relation to the values of urea.
and serum creatinine. Therefore, no changes were observed in the values of these markers, similarly to our findings in the serum creatinine marker and diverging in the serum urea marker. We also emphasize that the dose adopted in this finding was lower than the dose adopted in our study.

Santos et al. [7], when researching the effects of dietary supplementation with a dose of 1.8 g/kg/day whey proteins sedentary Wistar rats, showed that no statistically significant differences were found between the treated groups and the control group for the values serum creatinine and urea, indicating that there was no impairment of renal function. These results corroborate the findings in this study, with no significant changes in serum creatinine values, however it diverges as values in the concentration of serum urea. However, our study had a considerably longer duration and dosage.

Khairallah et al. [28] investigated the effect of diets containing 22.5 (g%) of protein as milk protein isolate (MPI), whey protein isolate (WPI), soy protein isolate (SPI), soy protein concentrate (SPC) and enzyme-treated soy protein (SPE) on muscle function in 50 Sprague-Dawley rats. However, similarly to our study, there were no significant differences in serum creatinine between groups at the end of the study. Thus, there were no changes in the concentrations of this marker. However, while our study lasted 12 weeks, this study administered the diets for 8 weeks [28].

When we analyzed the serum urea data, the results of this study showed a significant difference between the sedentary control group and almost all supplemented groups, with the exception of only TW4, unlike the findings by Aparicio et al. [29] who did not identify a significant difference in serum urea between groups when examining the effects of whey proteins and soy protein intake on plasma renal parameters.

In turn, the results presented by Aparicio et al. [30], when examining the effects of consuming high doses of protein on renal parameters in rats, do not corroborate our findings, since the groups with diets enriched with whey showed higher values of serum urea compared with the groups with normoproteic diet. Accordingly, Nebot et al. [31] who examined the effects of the amount of diet and protein source on the bone status of rats and the interactions that occur between these nutritional factors, found higher values of serum urea in the groups with diets based on whey proteins (45%) in comparison to the normoproteic group. Although the findings differ from the results of this study, both serum urea and serum creatinine values remain within the established reference range [32-34].

In relation to urinary biomarkers, we found in this study that the values of creatinine (mg/dl) differ significantly in the TW2 and TW4 groups with the sedentary control and trained control groups. Similarly, urea values (mg/dl) are significantly different between groups TW2 and TW4 with sedentary control and between TW2 and sedentary control. However, when we verified the results of both 24-hour creatinine and 24-hour urea, no results were found with significant differences between groups.
Finally, when we analyzed the serum urea/creatinine ratio, we found significantly lower values in the supplemented groups compared to the control group. However, again it was not possible to make comparisons of these results with results from the literature, because, unlike this study, in none of the studies that comprise the systematic review calculated these variables, as well as the urinary biomarkers.

In this way, the importance of the findings is highlighted in order to contribute to the search for the presence or absence of a protein threshold that can cause deleterious effects on renal function, which can be indicated by changing the investigated biomarkers.

However, it is important to emphasize that some limitations of the present study must be considered. Thus, additional studies should be carried out over a longer period for this dose, as well as for higher doses in order to verify with precision if there is a threshold in the dose of this substance that causes alterations and/or side effects in the renal function of individuals healthy.

### Conclusion

Considering the results obtained in this study, it can be suggested that the treatment of male Wistar rats with resistance training and supplementation with whey proteins at doses of 2 g kg⁻¹ d⁻¹ and 4 g kg⁻¹ d⁻¹ for 12 weeks did not result in impairment of renal function, as the values of serum biomarkers were within the ranges of reference values. While urinary biomarkers, although showing significant difference between groups, when normalized by the 24h urine volume (mg/24h), all differences in creatinine and urea were normalized in mg/dl.

### Conflict of interest

No conflict of interest with relevant potential.

### Financing source

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### Author’s contributions

- **Conception and design of the research:** Navarro F, Navarro AC, Silva AJS, Vieira EP.
- **Data collection, statistical analysis and writing of the manuscript:** Vieira EP, Silva AJS.
- **Critical review of the manuscript:** Vieira EP, Navarro AC, Navarro F.

### References


