THE EFFECTS OF CREATINE MONOHYDRATE ON RENAL FUNCTION IN WISTAR RATS

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Dr. Laura Twersky

Mom and Dad

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Without whom, none of this would have been possible.
Abstract:

Creatine is a supplement taken to aid athletes and fitness aficionados alike with their workout. Previous studies have been done to prove the effects creatine has on a workout as well as studies on rats to investigate the benefits of creatine on different aspects of health, mainly the nervous system; however there is minimal data available about the risks. Wistar rats are excellent subjects for human related studies due to the mammalian traits shared between them. Protein over-consumption affects the kidneys and bone via calcium loss; this is a concern with creatine since it is a protein. The rats in the control group did not consume creatine while the rats in the experimental group consumed 1g. kg-1. per day for four weeks. Their urine was collected tested for calcium and protein weekly. Increased concentrations were indicative of renal damage. At the end of the four weeks, the rats were euthanized and the kidneys of each were examined in order to best determine the extent of predicted damage to the kidneys. In order to ensure that creatine use was not being overestimated, a research survey was conducted on thirty six members of the Saint Peter’s University Biology Department of varying ages yielding results that 22.2% of participants use/ have used creatine and 62.5% of those participants used the supplement for 2 months or more.
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Introduction

Creatine is a widely used, non-FDA approved protein supplement used by athletes and bodybuilders alike. The benefits of creatine have been evaluated; however the risks have not been evaluated as extensively. Protein is incredibly important to mammalian physiology given its many functions in the immune system, in the composition of enzymes and hormones, as well its functions in cell structure and cell repair. The function protein plays in cell repair is especially important to note in this study.

Some of these amino acids can be produced by the body, others must be consumed and broken down in order to aid in muscle repair. Protein supplementation originated from the need for muscle cells to repair themselves after strenuous activity. Muscle cells utilize amino acids to manufacture muscle proteins which is why the consumption of protein, particularly in the form of a shake, after a workout has become so common. The influx of protein allows for the body to create the proteins it needs in order to repair the muscle cell after a workout. This being said, it must be noted that recommended protein intake varies for individuals by gender, age and training goals.

As a general rule, the average male and female will do well with 60 grams of protein a day, composing 15% of the diet (President and Fellows of Harvard College, 2006). For women ages 19-70+ the necessary daily value of protein intake is 46 grams; while for men of the same age range, the recommended value is 56 grams (Center for Disease Control and Prevention 2012). Research on this topic varies in results but there is an equation that is widely accepted called the Estimated Average Requirement (EAR) which was compared to the Recommended
Daily Allowance (RDA) in 2008. The study found that the Estimated Average Requirement equation to be: 0.8 g protein \( \cdot \) kg body wt\(^{-1} \cdot \) d\(^{-1} \) after studying the gram of protein constant as 0.50g, 0.75g and 1.00g (Campbell et al 2008).

Athletes are exceptions to these rules since their muscles work so much harder and are put under much more stress than the average person. For endurance athletes the EAR equation is used and the maximal requirement is 1.6g protein \( \cdot \) kg body wt\(^{-1} \cdot \) d\(^{-1} \) (Tarnopolsky, 2004). For strength and power trained athletes the same equation is applied and the recommended “safe” requirement is 1.33g protein \( \cdot \) kg body wt\(^{-1} \cdot \) d\(^{-1} \) (Phillips, 2004).

According to the Harvard Medical School Family Health Guide, “Excess dietary protein increases calcium loss in the urine, perhaps raising the risk for osteoporosis (more a worry for women) and kidney stones (a particular worry for men).” (President and Fellows of Harvard College, 2006).” An issue regarding protein consumption has the ability to affect every organ in the human body. Hormones regulate bodily processes and are the stimulus for organ function; not enough protein will cause severe hormone imbalances while too much protein will cause others.

It is recommended that a gallon of water be consumed per day for those individuals taking creatine due to the excess stress it puts on the filtration system in the kidneys. Consumption of protein in large amounts also tends to dehydrate the subject. (Wang et al., 2009). The dehydration creatine causes in muscle cells is evidenced by a study performed by Young et al. (2004) where creatine was used with the intent of dehydrating the muscle cells of chicken breast.
As mentioned previously, the benefits of creatine have been investigated extensively. Studies have been done on its effects on the nervous system as it is seen to have protective effect when it comes to spinal cord injury in rats (Hausmann et al., 2002), neonatal rats with transient-cerebral hypoxia-ischemia (Adcock et al., 2001), reduction of three-nitropropionic acid induced cognitive and motor abnormalities in rats (Shear et al., 2000) and its protective effects when it comes to damage by global cerebral ischemia through intracerebroventricular administration (Lensmen et al., 2006) as well as preventing the corticosteroid-induced attenuation of growth in the muscle cells of rats (Roy et al., 2002) and the effect of creatine on homocysteine levels and lipid peroxidation which affect the formation of free radicals (Deminice et al., 2009).

It’s function in the physical training arena have been evaluated as well. Belgian researchers found that untrained subjects taking creatine while following a 10-week weight-training program increased their one-rep max on the squat by 25% more than those taking a placebo while following the same program (Vandenberghhe et al., 1997). Researchers have also reported that out of 16 studies investigating the effects of creatine on one-rep max strength, the average increase in strength was about 10% more in those taking creatine as compared to those taking a placebo (Rawson and Volek, 2003).

Other studies of creatine have been on the disadvantages of its consumption. Studies have evaluated the negative effects of creatine on glucose uptake in rats (Young and Young, 2002) and the negative effects of creatine on glomerular filtration rate and renal plasma flow (Ferreira and De Toledo Bergamaschi, 2005). Some studies have been performed on humans however they typically examine durations between approximately one month to just under a year; although in this literary review, no studies were done in a six week period. (Shao and Hathcock,
2006). There has also been one study evaluating the effects of creatine monohydrate on hepatic and renal function in rats (Souza et al., 2009). Blood was collected from the rats and evaluated for calcium and protein levels. The rats were euthanized after the experiment ran to completion and the liver and kidneys of each rat were examined.

**Research Survey**

To ensure the relevancy of this research, prior to conducting an experiment on rats, a survey was conducted of biology majors at Saint Peter’s University. The survey was anonymous and consisted of eleven carefully selected questions to discover gender, level of physical activity, supplementation and further reduced to supplementation with creatine. The surveys were distributed in four classes—General Biology, Development, Research, and Advanced Anatomy and Physiology. Students were given the option to participate and at the conclusion of the class, the surveys were submitted.

There were thirty seven participants that chose to submit their surveys. Of these thirty seven, the of people who exercise and supplement was found and then reduced to those who exercise and take or have taken creatine. These surveys were isolated from the rest for further analysis of patterns such as gender, level of physical activity and duration of creatine use. See Appendix for Research Survey.
Materials and Methods

Materials

- 24 rats purchased from Charles River
- 8 cages
- 8 water bottles
- 8 food dishes
- pure creatine monohydrate purchased from bulksupplements.com
- heat lamp
- test tubes
- test tube racks
- spectrophotometer
- cuvettes
- pipettes
- burettes
- trichloroacetic acid
- centrifuge
- sodium hydroxide
- filter paper
- 20% copper sulfate
- distilled water
- dilute HCl solution
- CO₂ chamber for euthanasia
- CO₂ gas
Experimental Setup

Using the model set in place by Souza et al. (2009), the amount of creatine given to the experimental group of rats in this study will be 1g. kg-1. per day for four weeks. Rather than examining the blood, urine will be tested for protein and calcium using the proper chemicals and a spectrophotometer. Each rat will be kept in a separate cage to ensure that the food consumption and urine production are done solely by each rat.

Animal Maintenance

Rats were purchased from Charles River Laboratories. In order to minimize stress to the rats, the rats were given a four week adjustment period during which they could adjust to their new food and conditions. During this time a four week long trial was done prior to experimentation to investigate the amount of food and water each individual rat consumed. Using this data, I could track differences between the rats before and after their dosage routines began.-- the control group of rats were be given the non-altered version of the green pellets while the experimental group was weighed prior to the beginning of experimentation and each subsequent week so a combination of green pellet and a creatine solution could be custom made for each individual rat. In order to ensure that the texture of the creatine solution added to the food does not affect experimental results, the food pellets were crushed, supplemented with a solution of creatine and water, mixed thoroughly, dried on a low temperature hot plate and reshaped into the pellets to then be fed to the rats See Appendix for a picture of the set up. As previously stated, the rats were weighed at the end of every week and the creatine supplementation to their food was adjusted accordingly See Appendix for a picture of the rat
scale and how it was set up. Using the precedent set by Souza et al. (2009) the rats had access to water *ad libitum* with 12 hours of light and dark each day.
Urinalysis

Urine from each rat will be collected using clear plastic wrap attached to the bottom of each rat’s cage (Kurien and Scofield, 1999). Urine will be tested for protein and calcium every week for both experimental and control groups using a spectrophotometric analysis. Protein content in urine will be detected using the biuret method (Hiller et al., 1948) while calcium content in the urine will be detected using the lanthanum method of spectrophotometric analysis (Trudeau and Freier, 1967). After the 4 weeks of experimentation, a graph will be created using the spectrophotometric data of each rat.

Calcium Concentration Urinalysis:

Each rat’s urine will be tested for calcium. In a procedure outlined by Trudeau and Freier, the urine sample must first be deproteinized using trichloroacetic acid. (Trudeau and Freier, 1967). In order to perform the biuret method of spectrophotometric analysis, the urine of each rat must be filtered prior to treatment with chemicals in order to eliminate any precipitates that would affect an error in spectrophotometric analysis. The recommendation of urine to test is between 0.5mL and 5.0mL, therefore 1.0mL of urine will be put into a test tube along with an equal volume of a 10% solution trichloroacetic acid which is then shaken to mix and allowed to rest for 10 minutes. After this resting period, the test tube is then centrifuged for 10 minutes at 2500 rpm. The supernatant solution will be decanted leaving a protein precipitate and the test tube is inverted over filter paper for 2 minutes to drain. After draining the precipitate, 2 mL of a 3% solution of sodium hydroxide will be added and then shaken gently taking great care to
ensure no foam develops. This solution is then allowed to stand. A clear gel at the bottom of the test tube is indicative of protein that has not dissolved completely and may be overlooked; however if the test tube is swirled and thin transparent strands rise from the the bottom the protein is not dissolved completely-- the solution is complete when there are no more transparent strands rising from the bottom of the test tube after shaking the solution. More of the 3% sodium hydroxide solution is added until the volume of the test tube equals 10mL. 1 mL of this solution is then added to a cuvette and the remaining liquid is saved for a later portion of experimentation. 1mL of sodium hydroxide is added to another cuvette to serve as a blank. Using the sodium hydroxide solution to blank the spectrophotometer at 560μ. A reading will then be taken of the cuvette containing the protein solution and recorded.

**Protein Concentration Urinalysis**

1mL of the remaining protein solution from the test tube will then be put into another test tube and 0.25mL of 20% copper sulfate is added from the 5 mL marked burette which is then stoppered and shaken immediately and vigorously approximately 15 times. 1 mL of sodium hydroxide should be put into another test tube to undergo the same procedure. If shaking is not immediate, clumps of copper hydroxide may form on the sides of the test tube and the procedure must be repeated. The tubes are allowed to stand for 10 minutes (the blanks with solely sodium hydroxide and copper sulfate should stand for no more than 10) for the biuret color to develop and are then centrifuged for 4 minutes at 2500 rpm. 1 mL of the solution is then put into a cuvette for the spectrophotometer to read at a wavelength of 560μ and then recorded. Test tubes and cuvettes should then be cleaned using a dilute HCl solution to remove precipitates, then rinsed with tap water 5 times and finally distilled water 3-4 times (Hiller et. al 1948). After
the 8 weeks of experimentation, a graph will be created using the spectrophotometer readings of each rat’s urine.
Results

Research Survey Results

Supplementation

Of the thirty six sampled, 27% used a supplement. Of those supplementing, there was an equal gender distribution, 90% chose to supplement with protein and 10% chose to use other supplements. Of the 90% protein supplementers, 40% were all athletes and were all female the other 50% consisted of all male non-athletes.

Total Supplementers: 27.8%

- Male Supplementers: 50%    Female Supplementers: 50%
- Protein Supplementers: 90%  Non-Protein Supplementers: 10%
- Athletic Protein Supplementers: 40%  Non-Athletic Protein Supplementers: 50%
  - all female athletes        - all male subjects

Creatine User Data

Of the thirty six sampled, 22.2% used creatine, of the Creatine Users, 75% were male, 25% were female, 25% were athletes consisting of an equal gender distribution in this category and of the 75% non-athlete users all were male.

Total Creatine Users: 22.2%

- Male Users: 75%    Female Users: 25%
- Athlete Users: 25%  Non-Athlete Users: 75%
  - equal gender distribution  - 100% male users

Exercise Patterns of Creatine Users

Of the 22.2% of Creatine Users, 75% exercise identify themselves as exercising frequently. Of this 75%, 33.3% are females of equal distribution between athlete and non-athlete, 66.7% are males and all non-athletes. 33.3% of the frequent exercisers do so for 45 minutes a day, 16.7% do so for 60 minutes a day and 50% do so for more than 60 minutes a day. Of the 22.2% of Creatine users, 25% identify themselves as always exercising, all of these were male and did so for more than 60 minutes a day; this was distributed equally between both athletes and non-athletes.

Exercise of Creatine Users:

- Frequently: 75%
  - Female: 33.3%  Male: 66.7%
    - equal athletes/non athletes  - all non-athletes
  - 45 minutes a day: 33.3%  60 minutes a day: 16.7%  60+ minutes a day: 50%

- Always: 25%
  - Female: 0%  Male: 100%
    - 45 minutes a day: 0%  60 minutes a day: 0%  60+ minutes a day: 100%
    - equal athletes and non athletes
Reasons

Of the 22.2% of Creatine Users surveyed, 100% of both the male and female participants identify a reason for their exercise being their health, 87.5% of the total identify body image and Athletics as reasons with 100% of the females identifying both and 83.3% of the males doing so, 75% of all Creatine Users identify stress relief as a reason with 100% of females citing this and 66.7% of males. Participants were also given a fill-in for this question if they felt there was another reason for their exercise. 16.7% of males cited “Aesthetics” and another 16.7% cited feeling “good”.

Reasons:

- Health: 100% Female: 100% Male: 100%
- Body Image: 87.5% Female: 100% Male: 83.3%
- Athletics: 87.5% Female: 100% Male: 83.3%
- Stress Relief: 75% Female: 100% Male: 66.7%
- Other- Aesthetics: 16.7% (100% male) Feels Good: 16.7% (100% male)

Creatine Usage

Of the 22.2% of Creatine Users surveyed, 37.5% only used it once with 66.7% of those being female and 33.3% of those being male, 62.5% used creatine for two months or longer, all of which were males, another 12.5% supplemented with creatine for a year or longer, all subjects
being male again. Of the 22.2% of creatine users surveyed 87.5% had used the supplement in the past whereas 12.5% were currently using creatine.

**Creatine Usage:**

- Once: 37.5%  
  Female: 66.7%  
  Male: 33.3%
- Two+ months: 62.5%  
  Female: 0%  
  Male: 100%
- Over 1 year: 12.5%  
  Female: 0%  
  Male: 100%
- Past Usage: 87.5%
- Current Usage: 12.5%

**Creatine and Supplementation**

Of the thirty six participants, 26.8% supplemented and 22.2% used creatine. 62.5% of those two groups have used both creatine and protein to supplement at different points in their lives. Of ten supplementers, there are 8 creatine users so the correlation between supplementation and creatine use is 80%. 40% of participants are supplementing with creatine and protein simultaneously with 25% being females and 75% being males.

**Creatine and Supplementation:**

- Total Supplementers: 26.8%
- Total Creatine Users: 22.2%
- Creatine Users that also supplemented with protein: 62.5%
- Creatine supplementation correlation: 80%
- Participants supplementing with creatine and protein simultaneously: 40%
  - Female: 25%  - Male: 75%
Urinalysis

Protein Urinalysis:

The protein concentrations in the experimental groups urine increased over time while the protein concentration in the control group stayed relatively stable. From the data collected and illustrated in Figure 1, it can be seen that the concentration seems to spike at week three and dip slightly at week four.

Figure 1:
**Calcium Urinalysis:**

The calcium concentrations in the experimental group consistently rose as the weeks progressed while the control group calcium concentrations spiked at week one and dipped to week three until a smaller spike at week four. It is also important to note that it was one rat in the control group with abnormally high levels in both situations. Tests were run on the samples of this rat multiple times in order to discern if there was any errors made in testing; however, this was not the case as the values were the same with each re-test.

**Figure 2:**

![Calcium Absorbance by Week](image)
Discussion

Research Survey:

This research survey was designed to justify the use of laboratory animals in the study of creatine's effects on the mammalian physiology of the kidney and its function. A survey was conducted in the Biology department of a Division 1 University to give an idea as to the use of creatine in the general college age population-- particularly in such a setting where there are students competing in athletics at a very high level. Were the study to be conducted in the gyms on campus, the prevalence of creatine and overall supplementation usage would be expected to be even higher than those results found in this survey. Of the thirty six participants surveyed only six were athletes-- one male and five females, all of whom did not supplement.

There was a pattern in the Creatine Users surveyed-- all users identified themselves as working out either frequently or always and for 45 minutes a day or more. All cited more than one reason for their exercise with a unanimous decision for all being health. If these people are citing the main reason for their exercise as their health, they should know what effects the supplement that they are choosing to use has the ability to do to their bodies.

Many people assume that creatine is safe in large part to its prevalence. There have been no warnings associated with it for the masses other than workers in health food stores recommending the purchaser drink a lot of water.
Urinalysis:

As expected, the concentrations in the experimental groups had increased significantly over the course of the four weeks. Calcium concentrations in the experimental group grew steadily as seen in Figure 2 while the protein concentrations seemed to level off between weeks three and four in Figure 1. The protein concentrations in the urine of the control’s remained steady but had a slight spike at week three, where the protein concentration spikes slightly for the experimental group as well. A longer study would allow for us to see if the protein concentrations remained at a steady level, decreased or continued to rise after the four week period.

From the data collected and illustrated in Figure 1, it can be seen that the concentration seems to spike at week three and dip slightly at week four. This could mean one of three things—that the concentration will remain steady around 0.07 and 0.08, that the concentration of protein in the urine may decrease, or perhaps the concentration will continue to rise after the period of four weeks. If the concentration levels off it means that the rat’s bodies have found a way to metabolize and utilize the protein in the bloodstream up to a certain point but the remainder is being excreted through the urine at a steady rate. A further decrease would indicate the the bodies of the rats have found a way to utilize the extra protein in their diets and it would decrease the likelihood of kidney problems and calcium loss. A further increase in the concentration of protein in the urine would indicate that the body has not found a way to metabolize the majority of the protein which could lead to further kidney problems and calcium loss.
From the data collected and seen in Figure 2, it can be seen that the control group had a spike in the beginning and another at the end. It is also important to note that it was one rat with abnormally high levels in both weeks one and four. Tests were run on the samples of this rat multiple times in order to discern if there was any errors made in testing; however, this was not the case as the values were the same with each re-test. Suppositions as to why this particular rat had these levels range from dehydration to milk-alkali syndrome which would cause excess calcium in the urine. If there had not been any spikes, the levels of the calcium concentration in the urine would have been very level—staying around an absorbance reading of 0.005 to 0.01. The important thing to note about the graph was the steady increase in the calcium concentrations of the controls. The concentrations showed a steady increase in the graphs which with a study of longer duration we would be able to see if the concentrations continue rising or eventually level off at some point.

The importance of these findings is how they relate to kidney function. Since the kidneys are the filters of the body, it is important that they are cared for. The main concern of this study was seeing the calcium increase in the urine since it could cause osteoporosis or kidney stones in the long run. Excess calcium being filtered through the kidneys could result in a buildup causing the stones and since the excess protein can cause the calcium loss and thus osteoporosis, it is important to note how the concentrations in protein change in the urine over time. Seeing the increase in the protein concentrations can be correlated to the increase in the calcium concentrations in the urine. Once again, since the kidneys are filtering the blood and urine, anything that the filters can’t handle will bypass the filters and get excreted— in this case it would be too much protein.
There were other interesting things observed over the course of this study that do not necessarily pertain to the specialized aspect of research; however, seemed important enough to note. Prior to the dosing experimentation, the rats were at Saint Peter’s for four weeks. Throughout those four weeks I noted how much each rat was eating and how often I would refill their water bottles. Once experimentation began I noticed that the experimental rats were eating less than the controls, while the experimental rats were also drinking significantly more water. The control rats were still having their water bottles refilled every three days; however, the rats in the experimental group needed their water bottles refilled every two days. The experimental rats were also much more active in their cages once they began the dosage routine— they would climb on the tops of the cages, do flips off the sides and would run in circles in the cages while the control rats remained fairly sedentary. Another observation along this vein was that the male experimental rats, and only the males, became more aggressive. In order to dose the rats I would make a sound for them to come to the front of the cage, then they would get on their hind quarters and I would give them a pellet through the slats in the top of the cage. Prior to experimentation, both control and experimental rats would take the pellet and then remain towards the front of the cage, sometimes meandering towards the back. Once the dosage routine began the male experimental rats would snatch the food from my hand, sometimes biting at the nitrile glove covering it then run to the back of the cage to consume the pellet then run to the front again for more.
Conclusion

Creatine is a supplement taken after a workout, as a protein supplement is, in order to increase the ability of the muscles cells to heal. The popularity of this supplement is growing and not enough is known about the effect it may have on an individual’s health. Every creatine user int the research survey cited health as a reason for their exercise, but what if they are putting something into their bodies that is doing more harm than good? Creatine is not FDA-approved--like many substances and although it is a highly researched supplement, it is not being researched for side effects. Since creatine is a widely used supplement for the athletic and serious exercising communities, not enough is known about other health aspects of its function which is astonishing, particularly when 62.5% of creatine users have done so for two months or more. Although the sample size of this research survey was small, it was meant to represent the average college-age population-- young men and women concerned about their health, their bodies, athletics, and who want to cope with stresses of everyday college life. There were athletes, and average students concerned with their health and well-being in this sample-- it was taken in a classroom not a gym and the numbers may seem low, however, this is the general population of college age students and 22.2% of that using this substance is a large number.

From the data collected we can see that the concentrations of protein and calcium increase in the urine over time-- to what extent we do not yet know. However, from the data collected we can tell that the creatine is putting excess strain on the kidney’s of the rats. To know whether or not the damage goes deeper, we would need a longer study and an autopsy to be completed at the finale of the study.
Other areas of study based upon the data collected and observed would primarily be related to a longer duration of the study, eight weeks up to a year-- the results would no doubt be intriguing. Another area of study based on this would be following the dosing pattern that the container of creatine suggests.

Figure 3:

As seen in Figure 3, this particular brand's bottle suggested a creatine loading phase that consisted of four "rounded teaspoons" a day for a week then maintaining by taking one teaspoon of creatine per day with one of the optimal times to take it being with "your post workout protein shake". The final study I would recommend would be, examining the effects of creatine on testosterone production in the male rats since proteins are the basis of hormones, excess protein can affect hormones and the male experimental rats became more aggressive once their dosage routines began.
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Appendix

Research Survey

1. What is your gender? Male Female

2. Are you on an athletic team at Saint Peter’s? Yes No

3. If yes, please specify ________________________________

4. How often do you exercise?

Never Rarely Sometimes Frequently Always

5. I exercise_________ mins a day.  30  45  60  60+

6. Why do you exercise? (circle all that apply)

Health Body image Athletics Stress Relief

Other: ________________________________

7. Do you take supplements? (i.e. protein shakes, pre-workout, energy pills, etc.) Yes No

8. Do you specifically take supplementary protein? Yes No

9. If yes, do you supplement with creatine? Yes No

10. Have you ever supplemented with creatine? Yes No

11. If yes to 9 or 10, how long have you been/ were you using creatine?

________________________________

Thank you for your time in completing this survey!
Rat Scale:

This is the exact scale used to weigh the rats each week. It was set up on the floor to minimize possible harm to the rats should they have escaped.

Materials for Dosed Pellet Preparation:

This was how the materials were set up for the dosed pellet food preparation each week.
The creatine powder was weighed using this scale and weight boat for every dosed pellet produced.

This is how the dosed pellets were arranged to dry. It was done in this manner to ensure the dosed pellets (custom made for each rat) did not get mixed up.