Assessing blood serum copper levels in hair sheep grazing alfalfa pasture before & after supplementation

Alexandra Glenn, Animal Science/Pre-vet
Mentor: Dr. L. Allen Pettey
Kellogg Honors College Capstone Project

Abstract

Copper toxicity is potentially very dangerous for sheep as they can tolerate very low levels of dietary copper compared with other animals. It is widely believed that when sheep are stressed, copper stores from the liver are released into the blood and cause severe tissue damage, leading to death, but data to show this is very limited. The objective of this study was to see if blood serum copper levels would change after supplementation and after a mildly stressful event. Twenty-five hair sheep were used which had been grazing alfalfa pasture for over 75 days prior to the first blood collection. The stressor event was the act of hoof trimming, which involves flipping the animal onto their back and restraining them for 2 minutes. Blood was collected four times: before and after supplementation with minerals; and before and after the stress event. Blood samples were processed, and the serum was extracted and stored at -70ºC. To measure copper levels, we used a copper assay kit and spectrophotometer. Results show that the copper assay kit we used was able to detect serum copper in sheep. Serum copper levels decreased ($P<0.05$) in lambs after they were stressed, which did not support our hypothesis. This may be due to the fact that hoof trimming may not have been a true stressor for this group of lambs. This study shows that serum copper levels do fluctuate and can be used for further research in assessing copper status in sheep.

Introduction

Sheep are the highly susceptible to Copper toxicity but still require it as an essential mineral in their diet. There are two types of Copper toxicity: acute and chronic; acute occurring after eating a large amount of copper in a feed, chronic occurring after ingesting larger amounts of copper in the diet over a period of time [4]. The copper is then bound in the liver where it continues to build up as sheep cannot excrete copper as efficiently as other species. While stored in the liver, the copper bound in the lysosomes pose no threat to the sheep, but when put under stress (shearing, transport, or extreme weather), these copper stores can be released into the blood stream, causing severe tissue damage throughout the body [2]. When this happens, there are no clinical signs: just dead sheep. There is little one can do to determine copper toxicity if it's acute, but when it's chronic, one of the most trusted ways to determine copper toxicity is by doing a liver biopsy. However, liver biopsies are time consuming and expensive, and are not a practical approach to managing copper toxicity in the field. Very little research has been done to assess how fast copper is being released from the liver or how much stress it will take to elicit an elevated copper response that can be detected in blood serum.

The goal of this study was to try and assess the effects of stress on sheep by using a copper assay kit to measure serum copper in sheep before and after mineral supplementation.

Materials and Methods

Study Design:

- 25 Hair sheep (Dorper-Katahdin cross breed)
  - Blood collected from jugular vein 4 times (T1-T4) using serum separators (Vacu-tainer) and a 20 gauge needle
  - T1 [March 3] = (-) stress/ (-) supplement
  - T2 [March 5]= (+) stress/ (-) supplement
  - T3 [April 4]= (-) stress/ (+) supplement
  - T4 [April 7]= (+) stress/ (+) supplement
- Serum separator was labeled with ear tag number and put on a test tube rack in a cooler
- Stressor: the act of hoof trimming
  - Flipped on their backs and restrained for 2 minutes each
  - After stressing, sheep were allowed to rest for 2 hr before collecting blood
- When not on mineral supplement (T1 & T2) sheep were on a strict alfalfa diet and kept at Spadra Farm
  - T1 and T2 were collected 1 day apart
- Sheep were moved back to the Sheep unit at Cal Poly Pomona to be fed mineral supplement (T3 & T4)
  - T3 and T4 were collected 2 days apart
  - Initial mineral supplement amount given: 50 lbs
  - Were on mineral supplement for 1 month after collecting on T1 and T2

Blood Processing:

- After blood was collected, it was taken to the Equine Research Center (ERC) to be spun down and stored
  - Spun down using Sorvall Rt 6000 refrigerated centrifuge
  - Samples were spun for 5 minutes at 1500 rpm
  - Serum was extracted (see figure 3) and transferred to cryo vial tubes
  - Samples were stored at -70ºC until ready to run copper assay

Serum Copper Assay:

- Spectrophotometer used: Shimazdu UV 160v (uUVvisible recording
  - Located in Bldg 2, room 101 (Nutrition Lab)
  - Read absorbance at 359 nm
- Kit used: QuantiChrom™ Copper Assay Kit (DICU-250)
  - quantitative colorimetric copper determination at 359nm
  - Kit included TCA, Standard, Reagent B & C
  - Mixtures made:
    - Working Standard (WS) = 20µl std + 80µl dH2O
    - Working Reagent (WR) = [10µl B + 300µl C] * # tubes needed
- Final mixtures in cuvette: (according to kit instructions)
  - Blank: 70µl TCA + 300µl Working Reagent (WR)
  - Standard: 70µl TCA + 200µl WS + 300µl WR
  - Samples procedure:
    - Mix 70µl TCA + 200 µl Sample in Eppendorf tube
    - Spin for 2 min at 14,000 rpm if ppt forms
    - Extract 200µl from Eppendorf tube to cuvette
    - **Final mix**: 200µl Sample + 300µl WR
- **Calcuation**: [(ODsample-ODblank)/(ODstd-ODblank)] *300µl/dL
  - Conversion: 100µl/dL = 1 ppm

Figure 1: The hair sheep used in the study. The blue markings designate if they had been sampled

Figure 2: Blood was collected from each sheep via jugular venipuncture

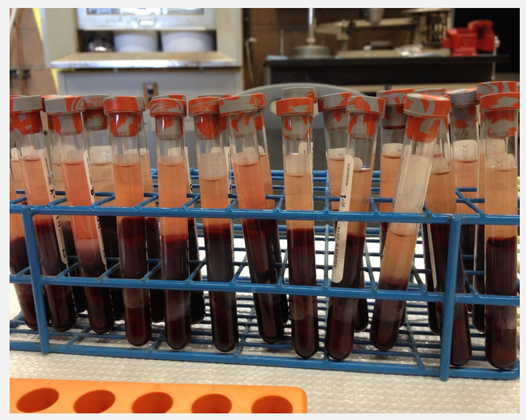


Figure 3: After blood was collected, it was spun down. The top, clear half (serum) was used for the copper assay

Results

Study Design

- 25 sheep were sampled
- All sheep were stressed for 2 minutes
- Mineral supplement ingested: 29 lbs

Blood Collection:

- On T1, there was some hemolysis in the serum separators because we didn't have a test tube rack
- T2-T4, there was less hemolysis because we acquired a test tube rack

Blood Assay:

- Paired T-test was done on T1&T2, T3&T4
- Between T1 and T2, there was a significant decrease in serum copper levels after stress (Graph 1)
- Between T3 and T4, 5 out of 21 sheep included in the results showed a decrease in copper levels, while the rest showed an increase in copper levels after stress (Graph 2)
  - 4 sheep were excluded from these results because their ppm values were negative

Discussion

Why did the copper levels decrease between T1 & T2?

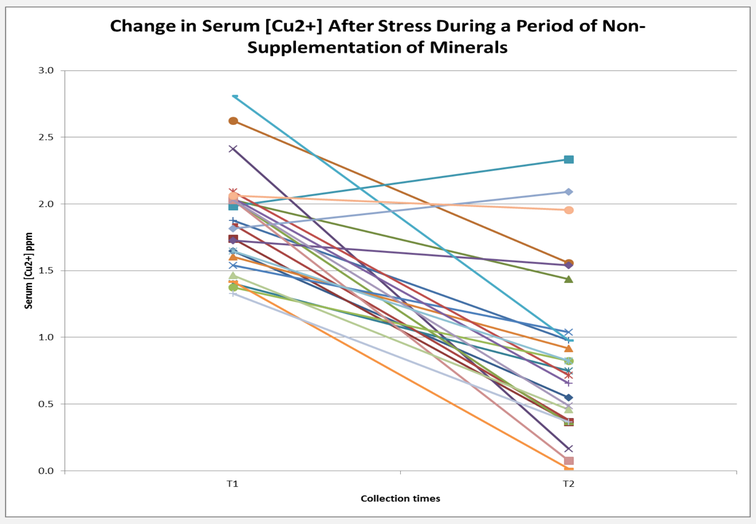
- Stressor was not stressful enough for the animal
- Sheep had been stressed by an even bigger stressor, which made our stressor not as effective
  - the weekend prior to T1 (March 1-2), there was a large storm (by California standards) which probably stressed the sheep
  - This could explain why our sheep's serum copper levels were higher before we stressed them

Why did the copper levels increase between T3 & T4 if our stressor wasn't the sole cause the increased copper serum levels?

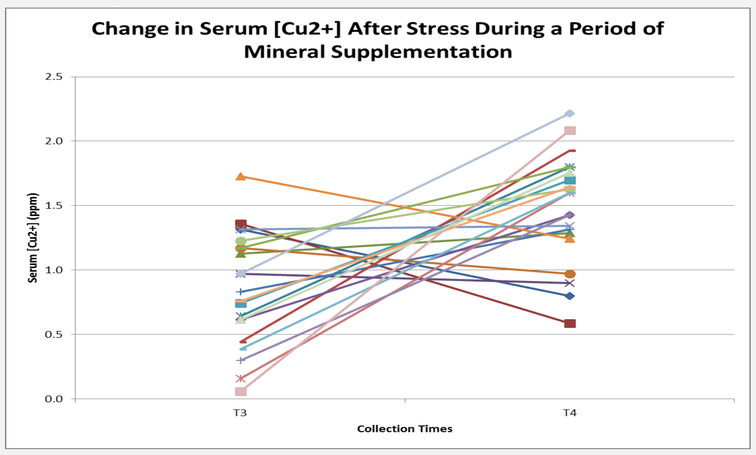
- The average temperature for the month of March was 70ºF-a comfortable temperature
- April 4 (and the 5 days before it) ranged from 65-70ºF
  - April 4 temp: 64ºF
  - April 7 temp: 92ºF
  - Between T3 and T4, the temperature increased drastically by nearly 30 degrees

Other possible reasons why we saw these effects in the data:

- We might have collected too soon or too late after stressing the animal
- User error
  - The copper assay kit was new to us and we were working with a limited supply
  - Samples could have been run in duplicate or triplicate to reduce lab error
- The alfalfa the sheep were on prior to sampling may not have been as devoid of copper as we had thought, which also could have altered our results during T1 and T2
  - The hay that they were fed at the sheep unit may have had less copper than the alfalfa they were on at Spadra Farms



Graph 1: This graph shows what happened between the first two collection times. The general trend of this graph is that the copper concentration decreased (paired t-test; $P< 0.01$) when sampled after the stress event. (T1= no stress, T2= stress)



Graph 2: This graph shows what happened between the 3rd and 4th collection times. The general trend of this graph is that the copper concentration increased (paired t-test; $P< 0.01$) when sampled after the stress event. (T3= no stress, T4= stress)

Conclusions

- Even though we saw an increase in serum copper levels between T3&T4, we can't attribute it all to the stress event of trimming hooves due to the decrease in serum copper from T1 to T2
- Weather events, which were out of our control, may have had an effect on our serum copper levels
- This work showed that measurable differences in serum copper can be detected in hair sheep

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