Oregon Health & Science University
School of Medicine

Scholarly Projects Final Report

Title *(Must match poster title; include key words in the title to improve electronic search capabilities.)*

Dietary magnesium manipulation to elucidate distal nephron physiology in a mouse model

Student Investigator's Name

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Date of Submission *(mm/dd/yyyy)*

3/16/2022

Graduation Year

2022

Project Course *(Indicate whether the project was conducted in the Scholarly Projects Curriculum; Physician Scientist Experience; Combined Degree Program [MD/MPH, MD/PhD]; or other course.)*

Scholarly Projects Curriculum

Co-Investigators *(Names, departments; institution if not OHSU)*

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Scholarly Project Final Report

Concentration Lead's Name

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Project/Research Question

In a magnesium-deficient state, does an amiloride bolus cause an increase in magnesium absorption in the distal convoluted tubule when compared to a magnesium-replete state?

Type of Project *(Best description of your project; e.g., research study, quality improvement project, engineering project, etc.)*

Research study

Key words *(4-10 words describing key aspects of your project)*

Magnesium, sodium, potassium, kidney, nephron, NCC, ROMK, ENaC, amiloride

Meeting Presentations
If your project was presented at a meeting besides the OHSU Capstone, please provide the meeting(s) name, location, date, and presentation format below (poster vs. podium presentation or other).

N/A

Publications *(Abstract, article, other)*
If your project was published, please provide reference(s) below in JAMA style.

N/A

Submission to Archive
Final reports will be archived in a central library to benefit other students and colleagues. Describe any restrictions below (e.g., hold until publication of article on a specific date).

N/A

Next Steps
What are possible next steps that would build upon the results of this project? Could any data or tools resulting from the project have the potential to be used to answer new research questions by future medical students?

Future research into the distal nephron may include the use of low Na and low Mg dietary states, paired with amiloride treatment, to evaluate the interplay between NCC, ENaC, and ROMK activities.

Please follow the link below and complete the archival process for your Project in addition to submitting your final report.

https://ohsu.ca1.qualtrics.com/jfe/form/SV_3ls2z8V0goKiHZP

Student's Signature/Date *(Electronic signatures on this form are acceptable.)*
This report describes work that I conducted in the Scholarly Projects Curriculum or alternative academic program at the OHSU School of Medicine. By typing my signature below, I attest to its authenticity and originality and agree to submit it to the Archive.

X _____
Student's full name

Mentor's Approval *(Signature/date)*

X _____
Mentor Name

Introduction *(≥250 words)*

Magnesium is one of the most abundant cations in the body and is heavily involved in a host of cellular processes. Magnesium deficiency is a significant clinical concern, particularly in patients in intensive care treatment,[1] postsurgical care[2], and with chronic kidney disease.[3] Handling of magnesium in the distal nephron is tightly regulated and intertwined with the regulation of sodium and potassium. In this study, we used a mouse model to investigate the effect of hypomagnesemia on sodium and potassium handling in the distal convoluted tubule (DCT) and cortical collecting duct (CCD). Improved understanding of these complex mechanisms can help improve electrolyte management in patient care settings.

On its own, hypomagnesemia has been associated with chronic conditions such as altered glucose handling, arteriosclerosis, osteoporosis, and asthma.[4] Type 2 diabetes mellitus (T2DM) increases GFR and renal tubular flow, thereby enhancing magnesium excretion by the kidney,[5] and potentially causing or worsening hypomagnesemia. Low levels of magnesium precipitate both potassium and calcium loss.[1,6] Hypomagnesemia with hypokalemia predisposes patients to arrhythmias and ectopic ventricular activity, and low magnesium stores with or without concurrent hypocalcemia can lead to neuromuscular derangements.[1]

Magnesium's impact on other electrolytes stems from its renal handling. The sodium-chloride-cotransporter (NCC), epithelial sodium channel (ENaC), and inward-rectifying potassium channel (ROMK) are three basolateral ion channels in the distal nephron that are significantly impacted by magnesium levels.

In a low magnesium state, the expression of NCC is decreased.[7] This leads to increased distal sodium delivery, causing ENaC levels to increase in order to preserve sodium chloride reabsorption.[8] ENaC and ROMK activity are paired, such that an increase in ENaC expression and activity leads to an increase in ROMK activity and thereby elevated potassium excretion.[8] This is further supported by the well-known potassium-sparing effect of the drug amiloride, which acts to block ENaC and therefore dissipate the electrochemical gradient for potassium excretion.[9,10] This is one mechanism by which magnesium depletion can precipitate potassium loss.

Magnesium further regulates potassium levels via its interaction with ROMK. In a magnesium-replete state, ROMK is inhibited via magnesium's intracellular blockade of the channel. This limits the outward flux of potassium, leading to retention of the electrolyte.[6] In hypomagnesemia, this inhibition disappears, causing an increase in potassium flow through ROMK.[6] This disinhibition of the channel allows increased potassium excretion and for serum levels to fall. With magnesium repletion, potassium levels stabilize.[6] This is important clinically when considering electrolyte derangement in acute patient care settings.

As discussed above, a low magnesium state disinhibits ROMK in the CCD, allowing for free efflux of potassium into the lumen of the nephron. However, the CCD also contains ENaC, which regulates the influx of sodium from the lumen. Sodium levels also impact the action of ROMK,[11] raising the question of how ENaC action, paired with magnesium levels, impact the function of ROMK. Upon considering the additional layer of NCC activity, these interactions increase in complexity. A low magnesium state is thought to reduce NCC activity,[7] therefore increasing sodium delivery to the CCD. This may be thought

to increase ENaC activity, however, current data on ENaC activity and sodium delivery is unclear.[12] Additionally, the disinhibition of ROMK by a low magnesium state adds more complexity to this situation. Further research on how low magnesium impacts ENaC, ROMK, and both sodium and potassium excretion is warranted.

Previous work by our lab has shown that magnesium deprivation in a mouse model decreases NCC expression when compared with a magnesium-replete diet.[7] However, it was not clear whether NCC was decreased due to injury to the nephron or from a physiologic adaptation to lower serum magnesium. The following experiments attempt to answer this question, while also further delineating the relationship between ENaC and ROMK activity in hypomagnesemia.

Methods *(≥250 words)*

Amiloride Response Test

Fourteen total male mice were used for this experiment, randomized into two treatment groups. This experiment was not blinded.

Seven mice were fed a low magnesium diet, and seven a regular diet with normal magnesium levels, each for 3 days. An injection of 0.09% sterile saline vehicle at a dose of 100 µg per 25 g weight was given to all mice, after which they were placed in metabolic cages to collect urine. Urine was collected for 6 hours, to extrapolate to 24 hours. Amiloride was then injected to all mice at a dose of 40 µg 25 g−1 body weight, and urine collected in metabolic cages for 6 hours. The urine was analyzed via flame photometer for sodium and potassium levels. Cleaning procedures were used for metabolic cages and urine collection vials to control for contaminants.

Levels of urinary sodium and potassium were evaluated in comparison to one another between saline and amiloride injections with Tukey's multiple comparison test. Additionally, the sodium to potassium ratio was calculated for both dietary and intervention groups and standard deviation between calculated using Microsoft Excel.

Magnesium reversal experiment

Twelve total mice were used for this experiment, randomized into three treatment groups. This experiment was not blinded.

Four mice were fed a low magnesium (LM) diet for six days, four a regular magnesium (NL) diet for six days, and four given a low magnesium diet for three days and a regular magnesium diet for three days (LM/NL). At the end of six days, each mouse was anesthetized and bilateral kidneys and cardiac blood were collected. Blood was measured for magnesium content using a magnesium assay and evaluated using standard deviation and Tukey's multiple comparison test. Fresh kidneys were transferred immediately to a sealed container and placed in liquid nitrogen. The frozen kidneys were then ground and centrifuged for eventual use in western blot analysis to evaluate levels of total NCC and phosphorylated NCC. Each western blot result was converted to percentage form based on a 100% (dark color), 75% (medium gray), and less than 50% (light gray). Standard deviation of western blot results was calculated for each dietary condition using Microsoft Excel.

An additional experiment was conducted to ensure adequate kidney homogenization methods A group of four mice was fed a normal sodium/low potassium (NS/LK) diet (n=2) or normal (NL) diet (n=2) for 3 days, after which the mice were anesthetized and kidneys extracted. Kidneys were homogenized via the same process as described above and analyzed for NCC and phosphorylated NCC via western blot. These results were compared to well-established results from prior studies.[13,14]

Results *(≥500 words)*

Amiloride response test:

No data points were excluded from experimental analysis. In mice fed a normal diet (n=7), both sodium and potassium excretion were not significantly different between vehicle and amiloride injections. Similarly, in mice fed a low magnesium diet (n=7), there were no significant differences in either sodium or potassium excretion between vehicle and amiloride injections.

The sodium/potassium (Na/K) ratio in urine was calculated for both vehicle and amiloride treatments, in both mice fed both normal and low magnesium diets. This is demonstrated visually in Figure 1. Between the normal and low magnesium dietary groups injected with vehicle, there was little difference in the Na/K ratio (P=0.1) (Figure 1). However, between groups injected with amiloride, the was a significant difference between the Na/K ratio (Figure 1). The mice fed a normal diet displayed a greater Na/K ratio than those fed the low magnesium diet (P<0.0001). The Na/K difference was also significant between the normal diet-fed mice injected vehicle versus those injected with amiloride (P<0.0001).

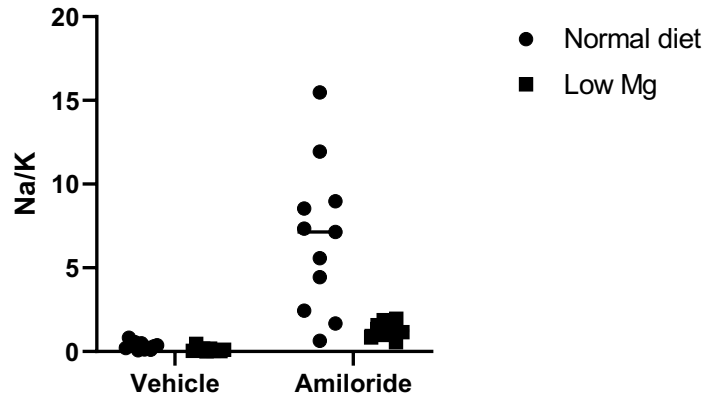

Figure 1: Sodium/potassium ratio between vehicle and amiloride injections in mice fed normal diet or low magnesium diet.

Magnesium reversal experiment

No data points were excluded from experimental analysis. Plasma magnesium levels were found to be significantly lower in the LM diet group (n=4) when compared to the NL diet (n=4) (P <0.0001). The LM/NL diet (n=4) raised plasma magnesium to a level comparable with that of the NL diet, as evidenced by the non-significant P-values between groups. The individual results with standard deviation bars are demonstrated visually in Figure 2.

Total NCC (tNCC) levels are demonstrated in percentage format in Figure 3, an adaptation of the western blot results shown in Figure 11. The LM diet lowered tNCC levels when compared with the NL diet (P=0.01). The tNCC levels with a LM diet were also significantly lower than the LM/NL diet (P=0.01). The NL and LM/NL diets had similar levels of tNCC, as evidenced by a non-significant P value.

Phosphorylated NCC (pNCC) levels were less consistent than those of tNCC, and quantification of the protein with statistical analysis was difficult to perform. Therefore, these results are not discussed in this report.

The techniques used to obtain the tNCC western blot results were evaluated with a second experiment feeding mice a normal sodium/low potassium (NS/LK) diet or normal (NL) diet. The NS/LK diet showed elevated levels of pNCC compared to the NL diet. Additionally, the NS/LK diet showed moderately elevated levels of tNCC compared to the NL diet.

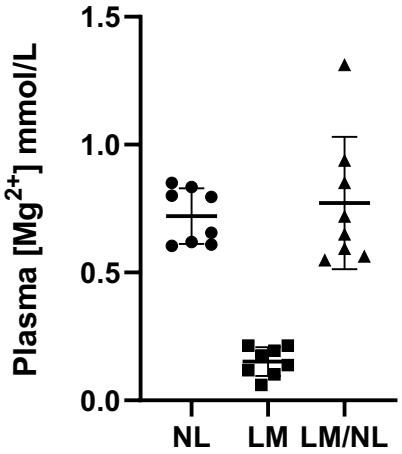

Figure 2: Mg reversal, plasma Mg levels in each dietary group with SD noted. Dietary groups: NL (normal magnesium), LM (low magnesium), LM/NL (low magnesium to normal magnesium)

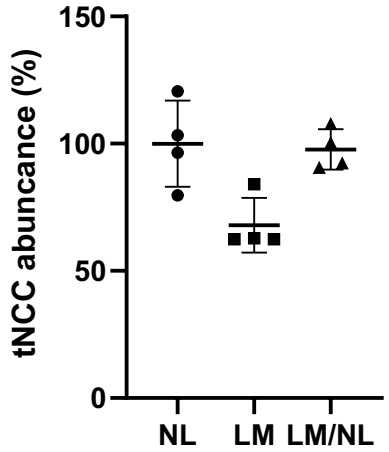

Figure 3: Total NCC (tNCC) abundance (%) in each dietary condition with SD noted. Dietary groups: NL (normal magnesium), LM (low magnesium), LM/NL (low magnesium to normal magnesium)

Discussion *(≥500 words)*

Amiloride response test:

Increased sodium excretion is a natural response to amiloride treatment, as amiloride is a well-known inhibitor of ENaC. This is demonstrated in the mice fed a normal diet under the amiloride condition, with a significantly increased sodium to potassium ratio (Figure 1). Therefore, these results indicate that there is a mechanism in the low magnesium state that causes increased potassium loss compared to the normal diet, thereby causing a sodium to potassium ratio that appears similar to the vehicle treatment (Figure 1). It is possible that in the low magnesium state, the combination of NCC inhibition and ROMK disinhibition leads to increased potassium excretion at the CCD, and elevated levels of urinary potassium. This is not seen in the urine results from the normal diet mice, as NCC action is preserved and ROMK inhibited, leading to decreased potassium loss. It is unclear why these proposed changes in NCC and ROMK activity with varying dietary magnesium levels and amiloride treatment are not reflected in the gross sodium and potassium excretion results. Further studies, with greater sample sizes, may help to further elucidate these actions.

Additional areas of research include the impact on potassium balance of ENaC modulation in a model of enhanced ROMK action. The above experiment hints at the complexity of interactions between ENaC inhibition or action, as regulated by amiloride, when paired with the impact of magnesium on ROMK and NCC. A low sodium/low magnesium experiment is warranted to evaluate the interplay of sodium and magnesium on NCC, ENaC, and ROMK activity. The addition of an amiloride challenge to this experiment could help to further elucidate regulation of ENaC and ROMK. These are important questions to answer, as hypomagnesemia is a frequent clinical problem, particularly in ICU patients,[1] and amiloride is a common potassium-sparing diuretic.[15]

Magnesium reversal experiment

Plasma magnesium levels dropped as expected in mice fed a LM diet, as compared to the mice fed a NL diet. Plasma magnesium levels were able to recover with the return to normal dietary magnesium levels, demonstrated in Figure 2. Therefore, we can conclude that magnesium absorption is normal in these mice, as plasma magnesium levels do not persist at a low level in the LM/NL dietary condition.

The LM diet was found to decrease levels of tNCC, however, adding magnesium back into the diet raised tNCC expression to magnesium-replete levels (Figure 3). The finding of decreased levels of tNCC in the LM diet group is supported by prior work, as a study by Ferdaus et al. found that a low magnesium state decreased expression of NCC.[20] The increase of tNCC with magnesium repletion, however, has not been shown previously. This result suggests that there is no permanent tubular damage affecting NCC, rather, the change in expression is a physiologic change mediated by NCC's complex regulation pathway, likely involving NEDD4-2, as discussed above.

The second experiment we performed to validate methodology showed that a low potassium diet was correlated with elevated levels of both pNCC and tNCC, findings that are consistent with previous reports.[13,14] Therefore, we concluded that our techniques for kidney homogenization and Western blot are adequate for reliable data collection.

Conclusions *(2-3 summary sentences)*

In summary, the amiloride response experiment helps support the conclusion that a low magnesium state causes increased urinary potassium excretion, likely through a combination of NCC inhibition and ROMK disinhibition. The magnesium reversal experiment supports the conclusion that no permanent tubular damage occurs to the distal nephron affecting NCC expression. Further studies are necessary to

better understand the complex relationships between magnesium, potassium, and sodium management by NCC, ROMK, and ENaC in the distal nephron.

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